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Chemical Modification of the *Beta* Adrenergic Receptors Coupled with Adenylate Cyclase by Disulfide Bridge-Reducing Agents

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SUMMARY

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Dithiothreitol, a disulfide bridge-reducing agent, decreased 10-fold the apparent activation constant of (−)-isoproterenol for the adenylate cyclase system in C6 glioma cells, rat liver cells, and avian erythrocytes. In liver plasma membranes, the apparent activation constant of glucagon for the adenylate cyclase was unchanged. In C6 glioma cells, dithiothreitol also decreased by 10-fold the affinity of the *beta* adrenergic receptor for (−)-[^{3}H]dihydroalprenolol without modifying the total number of sites. Thus, with a submaximal concentration of (−)-[^{3}H]dihydroalprenolol (10 nM), the specific binding was reduced by 70–80% in the presence of dithiothreitol (10 mM) or other disulfide bridge-reducing agents: 2-mercaptoethanol (50 mM) and cysteine (20 mM). Addition of dithiothreitol to intact cells or to broken cell preparations had a similar effect. The fall in affinity was due to a 3–4-fold increase in the dissociation rate constant and a 2–3-fold decrease in the association rate constant. The reduced receptor was reoxidized by simple exposure to air. The opening of the disulfide bridge may have changed the receptor spatial configuration, since there were 10-fold and 25-fold losses in affinity for the (−)- and (+)-isoproterenol isomers, respectively.

INTRODUCTION

The specificity of the membrane-bound *beta* adrenergic receptor and its coupling with adenylate cyclase were recently studied in different target cells using labeled *beta* adrenergic blocking agents such as propranolol, hydroxybenzylpindolol, and (−)-[^{3}H]dihydroalprenolol (1, 2). The solubilized *beta* adrenergic receptor was shown to be a protein (3). Dithiothreitol, a disulfide bridge-reducing agent, altered the *beta* adrenergic contractile response in guinea pig atria (4). The purpose of the present work was to investigate the possibility that this alteration corresponds to a chemical modification at the *beta* adrenergic receptor level. In that case, the formation of reactive sulfhydryl groups on the receptor molecule might be a useful tool for characterizing and purifying the receptor, as was shown for the acetylcholine receptor (5). It was also of interest to find out whether DTT[3] would modify the *beta* adrenergic receptor, since several authors routinely used this reagent to protect

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[3] The abbreviation used is: DTT, dithiothreitol.

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sulfhydryl groups belonging to the catalytic component of the *beta* adrenergic receptor-adenylate cyclase systems.

MATERIALS AND METHODS

Clone C6 glioma cells were taken from glial tumors induced by repeated injections of nitrosomethylurea into Wistar rats (6) and cultured as previously described (2). Particulate fractions from C6 glioma cells were prepared according to a previous report (2). Adrenalectomized female albino Wistar rats were used to prepare purified plasma membranes from liver (7). Detailed information on the adenylate cyclase assay and (−)-[^{3}H]dihydroalprenolol binding measurements in C6 glioma cells was reported elsewhere (2). Briefly, particulate fractions (20–40 μg of protein) were incubated at 30° in 50 μl containing 100 mM Tris-HCl (pH 8), 5 mM $MgSO_4$, 1 mM cyclic 3′,5′-AMP, 0.2 mM ATP, 0.2 mg/ml of creatine kinase, 20 mM phosphocreatine, and 1 mM EDTA. For adenylate cyclase assays, tracer amounts of cyclic [^{3}H]AMP and [α-^{32}P]ATP were added after 8 min of incubation, and the reaction was stopped 5 min later. For binding measurements, (−)-[^{3}H]dihydroalprenolol was added at the beginning of the incubation, and the amount of this antagonist bound to the *beta* adrenergic receptor was determined after 10 min. Then 1 ml of cold (4°) washing medium containing 50 mM Tris-HCl (pH 8) and 20 mM $MgCl_2$ was added to each tube. The diluted mixture was filtered through two GF/C glass fiber filters (2). Specific binding was defined as the difference between the amount of (−)-[^{3}H]dihydroalprenolol bound in the absence (total binding) and presence of 10 μM unlabeled alprenolol (nonspecific binding). Values are the means of triplicate determinations. Adenylate cyclase assay conditions for liver plasma membranes have already been described (8).

(−)-[^{3}H]Dihydroalprenolol, cyclic [^{3}H]-AMP, and [α-^{32}P]ATP were purchased from New England Nuclear; (−)-isoproterenol, from Sigma; (+)-isoproterenol, from Sterling-Winthrop; and crystalline porcine glucagon, from Novo Laboratories.

RESULTS

Effects of several disulfide bridge-reducing agents on (−)-[^{3}H]dihydroalprenolol binding. DTT (10 mM), 2-mercaptoethanol (50 mM), and cysteine (20 mM) caused a 70–80% reduction in specific binding of (−)-[^{3}H]dihydroalprenolol (10 nM, a submaximal concentration) to particulate C6 glioma cell fractions (Table 1). The DTT effect was dose-dependent (Fig. 1), maxi-

TABLE 1

Effects of disulfide bridge-reducing agents on specific and nonspecific binding of (−)-[^{3}H]dihydroalprenolol to beta adrenergic receptors

The concentration of (−)-[^{3}H]dihydroalprenolol was 10 nM. Values are the means and standard deviations of three determinations.

Reducing agent	Specific binding	Nonspecific binding
	pmole (−)-[^{3}H]dihydroalprenolol bound/mg protein	
None (control)	0.173 ± 0.012	0.051 ± 0.004
DTT (10 mM)	0.037 ± 0.005	0.045 ± 0.005
2-Mercaptoethanol (50 mM)	0.053 ± 0.003	0.043 ± 0.003
Cysteine (20 mM)	0.045 ± 0.000	0.046 ± 0.007

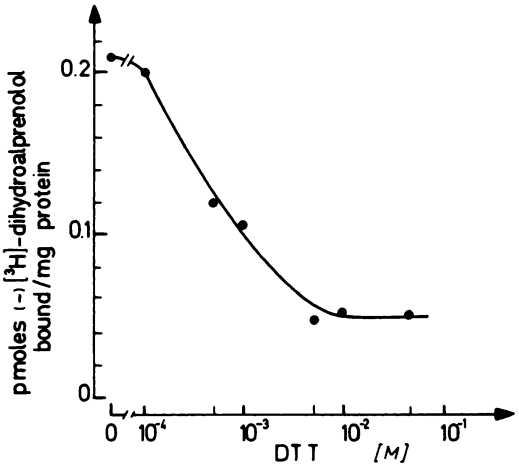

FIG. 1. *Dose-dependent effect of DTT on (−)-[^{3}H]dihydroalprenolol specific binding to beta adrenergic receptors*

Particulate fractions of C6 glioma cells were incubated for 20 min in the presence of 10 nM (−)-[^{3}H]dihydroalprenolol and various DTT concentrations. Nonspecific binding was determined by adding 10 μM (−)-alprenolol and was not affected by DTT.

mal inhibition being obtained with 5 mM DTT when incubation proceeded for 20 min (Fig. 1). Specific (−)-[^{3}H]dihydroalprenolol binding obtained with a submaximal concentration of this ligand never dropped below a certain level even with high DTT concentrations. Two hypotheses could be proposed to explain these results. (a) There are two populations of *beta* adrenergic receptors in particulate fractions of C6 glioma cells, one not binding (−)-[^{3}H]dihydroalprenolol after DTT treatment, and the other (20–30% of the total population) being unaffected. (b) There is a change in the affinity of the *beta* adrenergic receptor after DTT treatment, and the residual specific binding corresponds to the number of sites occupied by 10 nM (−)-[^{3}H]dihydroalprenolol.

Time course of DTT effect on (−)-[^{3}H]dihydroalprenolol binding. The DTT effect was time-dependent (Fig. 2). At the concentration used (0.5 mM) maximal inhibition was obtained after 15 min. The presence of the ligand during the reducing procedure changed only slightly the inhibition time course. Maximal inhibition was similar whether the ligand was added before or after DTT (Fig. 2). Three minutes of incubation with 15 nM (−)-[^{3}H]dihydroalprenolol were sufficient to reach binding equilibrium (2).

Change in beta adrenergic receptor affinity after treatment with DTT. DTT reduced the affinity of the *beta* adrenergic receptor for (−)-[^{3}H]dihydroalprenolol (Fig. 3). In the presence of a maximal DTT concentration (10 mM), the affinity of the *beta* adrenergic receptor was 10 times lower (42 nM) than in the control assay (3.4 nM) (Fig. 3). A submaximal concentration produced intermediate affinity (Fig. 3). No difference in the total number of receptors was observed. A Scatchard plot of the data showed straight lines for the control assay and for a maximal DTT concentration (10 mM), whereas with a submaximal concentration of reducing agent a curve was obtained (Fig. 3). The straight lines indicated a system in which the *beta* adrenergic receptors all had the same affinity. The affinity of the

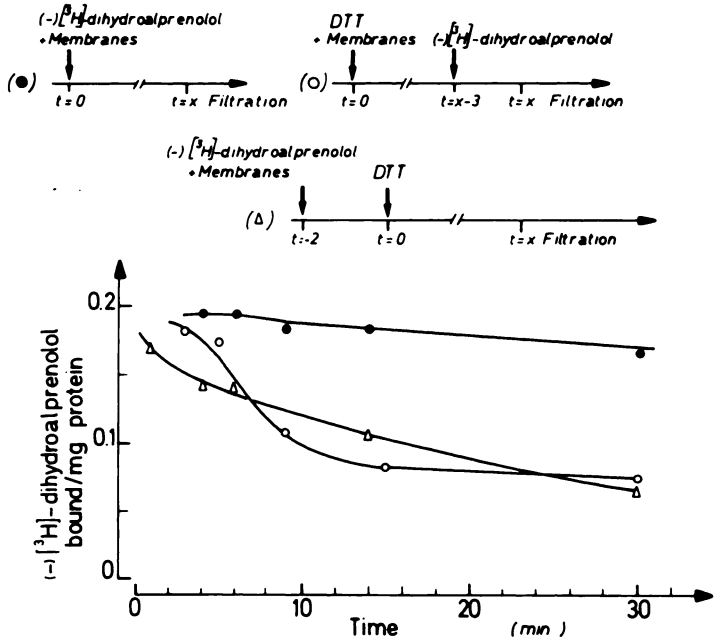

FIG. 2. *Time course of DTT effect on (−)-[^{3}H]dihydroalprenolol binding to beta adrenergic receptor in C6 glioma cells*

(−)-[^{3}H]Dihydroalprenolol (15 nM) was added either 2 min before incubation with DTT was started or 3 min before filtration. The DTT concentration was 0.5 mM.

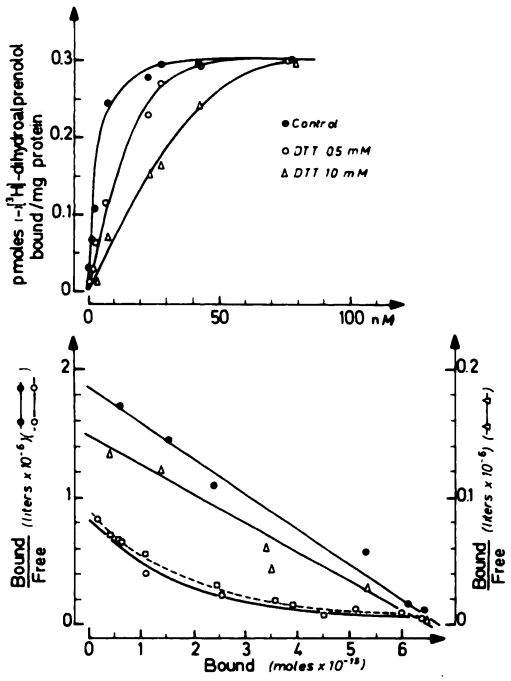

FIG. 3. *DTT-induced change in beta adrenergic receptor affinity*

Beta adrenergic receptor affinities for (−)-[^{3}H]dihydroalprenolol, calculated from Scatchard plots of the dose-binding curves, were 3.4 nM for the control assay (●) and 42 nM in the presence of 10 mM DTT (△). Note that the scale on the ordinate was changed 10-fold for the Scatchard plot of the binding curve obtained with 10 mM DTT. In the presence of 0.5 mM DTT (○), the Scatchard plot was nonlinear and was superimposable on the theoretical Scatchard plot (□) obtained assuming that the particulate fractions contained 40% native and 60% reduced *beta* adrenergic receptors with respective affinities of 3.4 and 42 nM.

control (3.4 nM) should be that of the native *beta* adrenergic receptor, and the affinity of the receptor measured in the presence of 10 mM DTT (42 nM) should be that of the *beta* adrenergic receptor after reduction. Assuming in this experiment that in the presence of 0.5 mM DTT the particulate fraction contained 40% native and 60% reduced receptors, the theoretical Scatchard plot drawn fits our experimental plot (Fig. 3). Thus, it is clear that the residual binding in Fig. 1 corresponds to the amount of sites occupied by 10 nM (−)-[^{3}H]dihydroalprenolol under conditions of completely reduced receptors.

Change in apparent affinity of beta adrenergic-sensitive adenylate cyclase for (−)-isoproterenol induced by DTT. DTT (10 mM) reduced 10-fold (from 0.1 to 1 μM) the apparent affinity of the *beta* adrenergic receptor-adenylate cyclase system for (−)-isoproterenol activation ($K_{a\,app}$ = concentration of agonist giving 50% maximal activation) (Fig. 4). A submaximal DTT

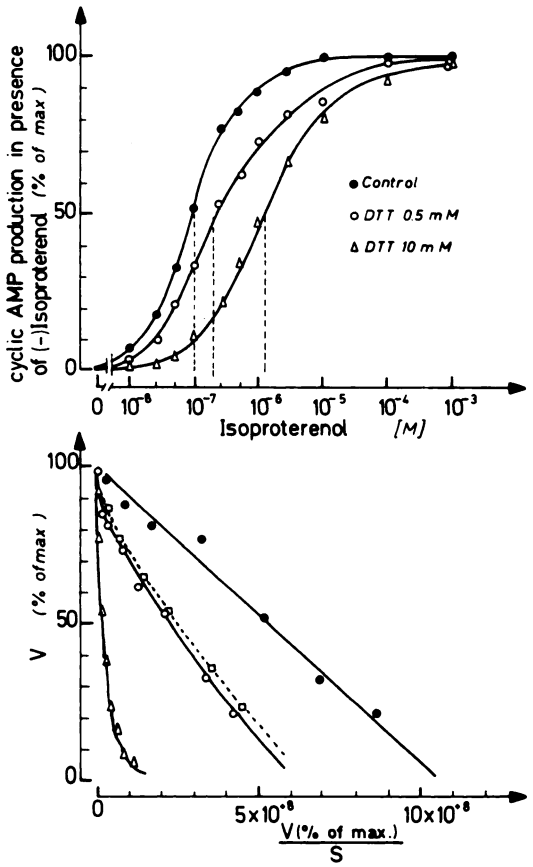

FIG. 4. *Change in apparent adenylate cyclase activation constant ($K_{a\,app}$) for (−)-isoproterenol activation induced by DTT*

The $K_{a\,app}$ calculated from Hofstee plots of the dose-activation curves was equal to 0.095 μM for the control assay and 1 μM in the presence of DTT (10 mM). The Hofstee plot of the dose-activation curve obtained with 0.5 mM DTT was nonlinear and superimposable on the theoretical Hofstee plot (□- - -□) obtained assuming that, in this experiment, the particulate fractions contained 65% native *beta* adrenergic receptors ($K_{a\,app}$ = 0.095 μM) and 35% reduced receptors ($K_{a\,app}$ = 1.25 μM). Basal adenylate cyclase activities (picomoles of cyclic AMP per min/mg of protein) were: control assay, 50.9; 0.5 mM DTT, 37; 10 mM DTT, 53.2; and in the presence of 0.1 mM (−)-isoproterenol, control assay, 600; 0.5 mM DTT, 600; 10 mM DTT, 786.

concentration gave intermediate apparent affinity (Fig. 4). A Hofstee plot of the data gave straight lines for the control and for a maximal DTT concentration (10 mM) (Fig. 4), whereas a nonlinear plot was obtained with a submaximal concentration of reducing agent (0.5 mM) (Fig. 4). This might indicate that under control conditions or in the presence of 10 mM DTT, the *beta* adrenergic receptor-adenylate cyclase system was homogeneous, the form of receptor coupled with the adenylate cyclase being either native ($K_{a\ app}$ = 0.095 μM) or reduced ($K_{a\ app}$ = 1.25 μM). In the presence of a submaximal DTT concentration, a Hofstee plot of the curve of dose vs. activation by (−)-isoproterenol indicated that the particulate fractions contained a mixture of two different *beta* adrenergic receptor-adenylate cyclase systems, one formed with the native receptor and the other with the reduced receptor. In this experiment (Fig. 4), the experimental Hofstee plot corresponds exactly to the theoretical Hofstee plot obtained assuming that 65% of the *beta* adrenergic receptor was native in form ($K_{a\ app}$ = 0.095 μM) and 35% reduced ($K_{a\ app}$ = 1.25 μM). A DTT-induced loss in apparent affinity of the *beta* adrenergic receptor was also observed when adenylate cyclase was activated with (−)-norepinephrine or dopamine (data not shown).

Receptor specificity of DTT effect. DTT decreased the $K_{a\ app}$ for *beta* adrenergic-sensitive adenylate cyclase in liver plasma membranes (Fig. 5). In the same membranes the apparent affinity of the glucagon receptor coupled with adenylate cyclase was unchanged (Fig. 5). DTT also reduced the apparent affinity of the *beta* adrenergic receptor in turkey and duck erythrocyte membranes (data not shown).

Modification by DTT of association and dissociation rate constants for (−)-[^{3}H]dihydroalprenolol binding to beta adrenergic receptor. The kinetics of association and dissociation of (−)-[^{3}H]dihydroalprenolol for native and reduced *beta* adrenergic receptors were determined in the same experiment. The dissociation rate constants (k_{-1}) were 0.17 min^{-1} and 0.65 min^{-1} for the native and reduced forms, respectively (Fig. 6). For the native system it was possible to measure with high accuracy the $t_{1/2}$ value for the associ-

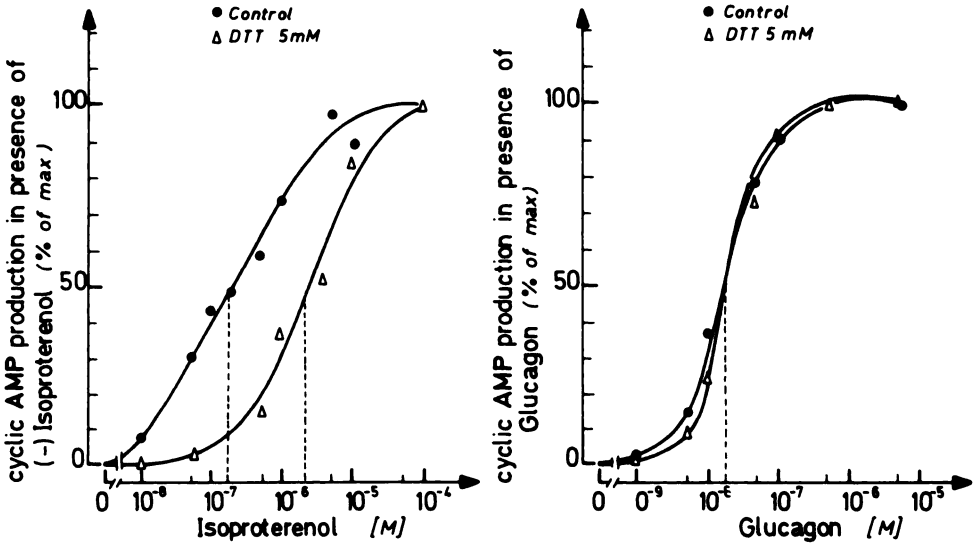

FIG. 5. *Effect of DTT on beta adrenergic and glucagon receptors coupled with adenylate cyclase in liver plasma membrane*

The membranes were incubated for 10 min at 30° with the complete assay mixture except for [α-^{32}P]ATP, in the presence and absence of DTT (5 mM). [α-^{32}P]ATP was then added, and the reaction was allowed to proceed for 10 min. Basal adenylate cyclase activities (picomoles of cyclic AMP per 5 min/mg of protein) were: control, 175; 5 mM DTT, 140; in the presence of 10 μM (−)-isoproterenol and 10 μM GTP, control, 775; 5 mM DTT, 400; and in the presence of 1 μM glucagon, control, 1650; 5 mM DTT, 1875.

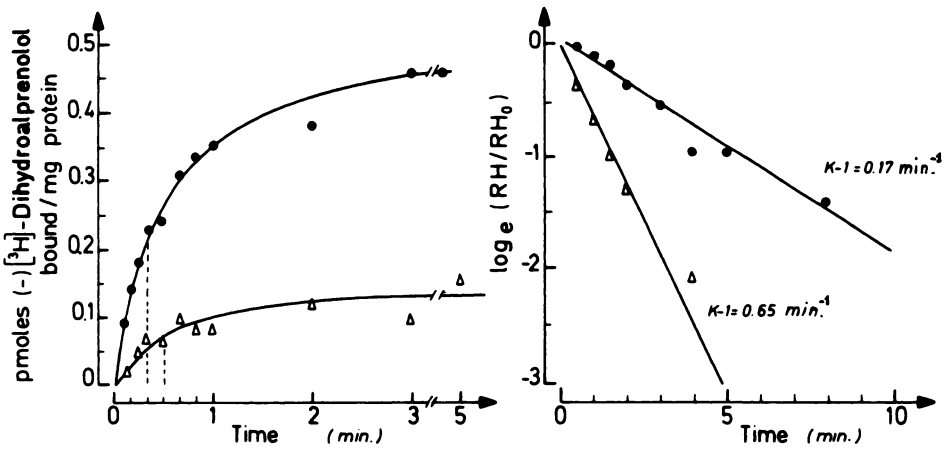

FIG. 6. *Time course for (−)-[³H]dihydroalprenolol association with and dissociation from C6 glioma cell particulate fractions*

Left: association processes. Particulate fractions of C6 glioma cells were incubated at 30° for 15 min with (△) or without (●) DTT, 5 mM, in 45 μl of the usual incubation medium. Then (−)-[³H]dihydroalprenolol (final concentration, 26 nM), with or without (−)-alprenolol (final concentration, 10 μM), was added in 5 μl. The incubation period was determined for each point starting from the addition of radioactive ligand up to the addition of 1 ml of 50 mM Tris-HCl, pH 8, and 20 mM $MgCl_2$. Filtration was immediately performed. The $t_{1/2}$ for association (- - -) is given by the equation (9) $t_{1/2} = \log_e 2/(k_1H + k_{-1})$. The $t_{1/2}$ values were 20 and 30 sec in the absence and presence of DTT, respectively. Since dissociation rate constants (k_{-1}) were 0.17 and 0.65 min^{-1} in the absence and presence of DTT, respectively (see right-hand graph), association rate constants (k_1) of 7.3×10^7 M^{-1} min^{-1} for the native system and 2.8×10^7 M^{-1} min^{-1} for the reduced system could be calculated.

Right: semilogarithmic plots of the dissociation processes. Particulate fractions of C6 glioma cells were incubated for 15 min at 30° in the presence of 26 nM (−)-[³H]dihydroalprenolol with (△) or without (●) 5 mM DTT. Then 5 μl of (−)-alprenolol were added, giving a final concentration of 0.1 mM, in order to follow the dissociation kinetics of the labeled ligand. *RH* is the concentration of the hormone-receptor complex; RH_0 is the concentration of the hormone-receptor complex when the dissociation was started. Each point is the mean of three determinations, which varied less than 10%. Similar results were obtained in two other experiments.

ation process [20 sec with 26 nM (−)-[³H]dihydroalprenolol]. This allowed calculation (see legend to Fig. 6) of an association rate constant of 7.3×10^7 M^{-1} min^{-1}, which correlates well with the association rate constant computed previously in this system (2). Although less accurate (Fig. 6), the $t_{1/2}$ for the association process of (−)-[³H]dihydroalprenolol in the reduced system could be estimated as equal to 30 sec, allowing the calculation of an association rate constant of 2.8×10^7 M^{-1} min^{-1} (see legend to Fig. 6). The determinations of these kinetic constants allowed computation of K_d values of 2.3 and 23 nM for the native and reduced *beta* adrenergic receptors, respectively.

Reoxidation of reduced beta adrenergic receptors. When intact C6 glioma cells were treated with DTT and washed and the particulate fractions were prepared immediately, the *beta* adrenergic receptor coupled with adenylate cyclase was in the reduced form [$K_{a\ app}$ = 1 μM (Fig. 7A)]. This $K_{a\ app}$ for adenylate cyclase activation by (−)-isoproterenol was approximately the same as the $K_{a\ app}$ measured when DTT was added directly to the particulate fractions prepared from either untreated or DTT-treated cells (Fig. 7A). This suggests that the reduction of the *beta* adrenergic receptor by DTT was complete in intact C6 glioma cells. When DTT-treated cells were washed and exposed for 4 hr to air in HAM F-10 medium, the *beta* adrenergic receptor coupled with the adenylate cyclase was reoxidized [$K_{a\ app}$ = 0.15 μM (Fig. 7B)]. This $K_{a\ app}$ was very close to

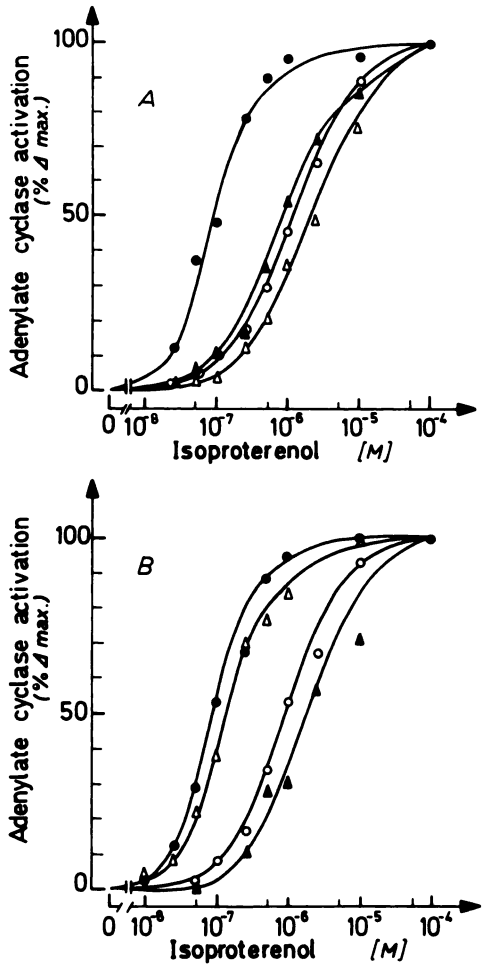

FIG. 7. *Reduction of beta adrenergic receptor on intact cells and its reoxidation by exposure to air*

A. C6 glioma cells were incubated in 0.9% NaCl and 25 mM Tris-HCl, pH 8 for 30 min in the presence (△, ▲) and absence (○, ●) of 5 mM DTT. Cells were washed once in a large volume of 0.9% NaCl, lysed in hypotonic medium (25 mM, Tris-HCl, pH 8, and 5 mM EDTA), homogenized, and centrifuged for 30 min at 5000 × *g*. The pellet was resuspended in the same hypotonic medium, and the adenylate cyclase assay was performed immediately in the presence (○, △) and absence (●, ▲) of 10 mM DTT. Basal adenylate cyclase activities (picomoles of cyclic AMP per 5 min/mg of protein) were 28 (●), 17 (○), 16 (▲), and 16 (△); and in the presence of 100 μM (−)-isoproterenol, 525 (●), 414 (○), 296 (▲), and 238 (△).

B. Four batches of C6 glioma cells were incubated for 30 min in 0.9% NaCl and 25 mM Tris-HCl, pH 8, in the presence (△, ▲) and absence (○, ●) of 5 mM DTT. Cells were washed once in a large volume of 0.9% NaCl. Particulate fractions of two batches of cells (○, ●) were prepared as described in Fig. 7A, and adenylate cyclase activities were determined in the presence (○) and absence (●) of 10 mM DTT. Two other batches of cells (△, ▲) were resuspended in 90 ml of HAM F-10 medium in 500-ml Corning bottles and incubated for 4 hr at 37° with (▲) and without (△) 5 mM DTT. Particulate fractions were prepared and adenylate cyclase activities were determined in the absence of DTT. Basal adenylate cyclase activities (picomoles of cyclic AMP per 5 min/mg of protein) were 27 (●), 28 (○), 35 (△) and 4.4 (▲); and in the presence of 100 μM (−)-isoproterenol, 297 (●), 218 (○), 217 (△), and 27 (▲).

the $K_{a\ app}$ of the native system, which was 0.1 μM in this experiment (Fig. 7B). The reoxidized receptor could be reduced again by adding DTT directly to the incubation medium, the $K_{a\ app}$ obtained being 2 μM (Fig. 7B).

Comparison of stereospecificities between native and reduced receptors coupled with adenylate cyclase. As already seen, the apparent affinities for (−)-isoproterenol of the native and reduced *beta* adrenergic receptors coupled with adenylate cyclase were 0.1 and 1 μM, respectively (six experiments). Measured in the native and reduced systems, respectively, the apparent affinity of (+)-isoproterenol fell from 1 to 24 μM (experiment 1), from 2 to 50 μM (experiment 2), and from 1.1 to 25 μM (experiment 3). Hofstee plots of the dose-activation curves of experiment 3 are presented in Fig. 8. These plots give straight lines, whose slopes give the apparent affinity of agonist for adenylate cyclase activation. The scales of the abscissae were made different in order to obtain the same slope for dose-activation curves obtained with both isomers in the absence of DTT. The Hofstee plots of the dose-activation curves for the two isomers in the presence of DTT are not superimposable, suggesting that reduction of the *beta* adrenergic receptor affects its affinity for (−)- and (+)-isoproterenol differently.

DISCUSSION

DTT and other disulfide bridge-reducing agents diminished the affinity of the *beta* adrenergic receptors coupled with an adenylate cyclase for its specific ligands without modifying the total number of recep-

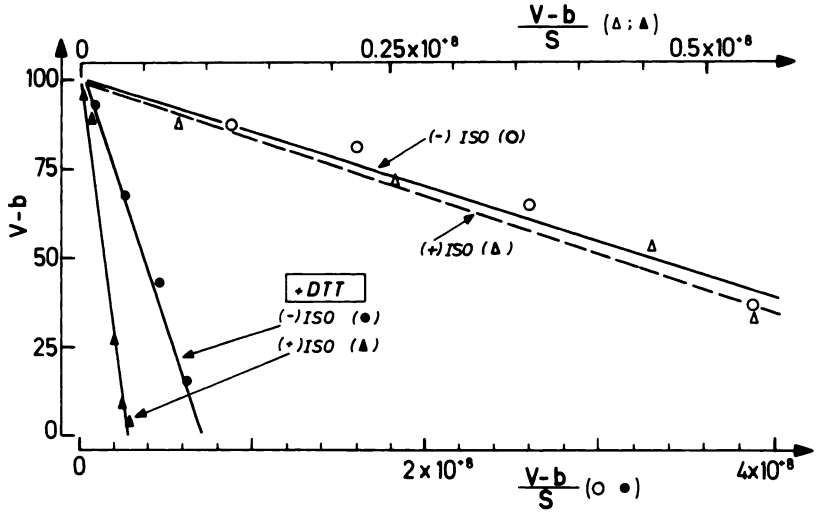

FIG. 8. *Hofstee plots of adenylate cyclase dose-activation curves obtained with stereoisomers of isoproterenol (ISO) in the presence and absence of DTT*

Particulate fractions of C6 glioma cells were incubated in the presence (●, ▲) and absence (○, △) of 10 mM DTT and with different agonist concentrations. Adenylate cyclase activities were measured during 5 min, starting with the addition of [α-^{32}P]ATP 8 min after the beginning of the reaction. V = velocity of the reaction in the presence of agonist, b = velocity of the reaction in the absence of agonist, and S = concentration of agonist. The amounts of cyclic AMP formed in the presence of agonist ($V - b$) were expressed as percentages of the maximal amounts of cyclic AMP formed in the presence of an optimal concentration of agonist, which were 216 [(−)-isoproterenol with (●) and without (○) DTT] and 181 [(−)-isoproterenol with (▲) and without (△) DTT]. Two different scales were chosen for the abscissae to obtain the same slope for both (−)- and (+)-isoproterenol when activities were measured without DTT. This representation indicates clearly that the shift in $K_{a\,app}$ induced by DTT was different for the two isoproterenol isomers. The $K_{a\,app}$ values for (−)-isoproterenol in the presence and absence of DTT were 1.42 and 0.16 μM, respectively; the corresponding values for (+)-isoproterenol were 25 and 1.1 μM. These results are representative of three experiments.

tors present in the particulate fractions of C6 glioma cells (Figs. 3 and 4). In contrast, sulfhydryl-blocking agents did not alter labeled antagonist binding to *beta* adrenergic receptors (10).

We found no modification either of the affinity of the *beta* adrenergic receptor for (−)-[^{3}H]dihydroalprenolol or of the total number of binding sites in the presence of 6 mM *N*-ethylmaleimide for 15 min.[4] The DTT effect was observed when the agent was added to intact cells (Table 2), when it was present during particulate fraction preparation, and when it was added directly to the incubation medium (Figs. 3, 4, and 7). This loss of affinity was determined either by measuring the labeled antagonist K_d for binding sites or by determination of the $K_{a\,app}$ for adenylate cyclase activation by (−)-isoproterenol (Figs. 4 and 7), norepinephrine, or dopamine (data not shown). Both K_d and $K_{a\,app}$ decreased 10-fold when reduction was maximal. Although we focused our interest on the DTT-induced change in the apparent affinity of the *beta* adrenergic receptor coupled with the adenylate cyclase, in some experiments (see Figs. 4, 5, and 7) we also observed slight modifications in the adenylate cyclase activities determined under basal or isoproterenol-stimulated conditions. In the presence of a submaximal DTT concentration, a mixture of the native and reduced receptors was observed (Figs. 3 and 4).

The decrease in affinity was observed for ligands with different chemical structures. Furthermore, the observation that the intensity of the DTT effect was a function of incubation time for receptor

[4] Unpublished observations.

TABLE 2

Compilation of data on effect of DTT on $K_{A\ app}$ for adenylate cyclase activation by (−)-isoproterenol

When present, DTT was added either during the preparation of broken cell systems or during the adenylate cyclase assay.

Beta-sensitive adenylate cyclase system	$K_{a\ app}$		Reference
	−DTT	+DTT	
	μM	μM	
C6 glioma cells	0.02		11
C6 glioma cells	0.05–0.1		2; this report
Liver plasma membranes	0.18		This report
Zadjela hepatoma cells	0.20		12
Frontal cerebral cortex	0.10		13
Cat ventricular heart muscle	0.25		14
Rat ventricular heart muscle	0.63		15
Frog erythrocytes	0.30		16
C6 glioma cells		1	This report
C6 glioma cells		1	17
Turkey erythrocytes		1	18
Frog erythrocytes		1	19
Liver plasma membranes		2.5	This report

plus DTT and not for ligand plus DTT indicates that the chemical modification occurred at the receptor level and not at the ligand level (Fig. 2). The 10-fold decrease in the *beta* adrenergic receptor affinity after reduction resulted both from a 3–4-fold increase in the dissociation rate constant (3.8-fold in the experiment reported in Fig 6; from 0.17 to 0.65 min^{-1}) and from a 2–3-fold decrease in the association rate constant (2.6-fold in the experiment reported in Fig. 6; from 7.3×10^7 to 2.8×10^7 M^{-1} min^{-1}).

The disulfide bridge implicated in receptor affinity could be reoxidized simply by exposing the cells to air (Fig. 7B).

Whereas there was a 10-fold drop in the $K_{a\ app}$ for the isoproterenol (−) isomer, in the case of the (+) isomer there was a 25-fold drop. This suggests that adenylate cyclase activation by the (+) isomer is not due to contamination with the (−) isomer. Furthermore, these results indicated that reduction of the *beta* adrenergic receptor modified its spatial configuration. This could explain the earlier report stating that the apparent molecular size of the *beta* adrenergic receptor solubilized with digitonin was larger when it was chromatographed on Sepharose 6B in the presence of 2-mercaptoethanol (1 mM) (3, 20).

DTT has been used by several authors to protect the reactive sulfhydryl groups of the catalytic component of the *beta* adrenergic receptor–adenylate cyclase system. As suggested by our results, this can lead to artifacts such as the discrepancy often reported between the affinity of the *beta* adrenergic receptor for its ligands when measured *in vivo* and *in vitro* (e.g., ref. 17) and the measurement of low affinity constants. In this connection a nonexhaustive compilation of data from the literature on the various $K_{a\ app}$ values for adenylate cyclase activation by (−)-isoproterenol in 13 experimental systems, depending on the absence or presence of DTT during the preparation or in the incubation of the membranes, is reported in Table 2. The average $K_{a\ app}$ calculated was 1.3 ± 0.7 μM ($N = 5$) with DTT, compared with 0.22 ± 0.19 μM ($N = 8$) without DTT (Table 2). Moreover, the specificity of the reduced *beta* adrenergic receptor might be modified, as has been reported for the acetylcholine receptor (21). Finally, when experimental conditions lead to a mixture of native and reduced receptors, a nonlinear Scatchard plot might be obtained (Fig. 3) and interpreted as indicating the presence of negative cooperativity between sites.

We observed that the nonlinear experimental Scatchard plot for binding in the presence of 0.5 mM DTT could be fitted to a theoretical plot obtained assuming a mixture of 40% native and 60% reduced receptors, while under the same experimental conditions a Hofstee plot of the adenylate cyclase dose-activation curve of (−)-isoproterenol fit a theoretical plot assuming the presence of 65% native and 35% reduced receptors coupled with the adenylate cyclase. This could be due to nonlinear coupling between *beta* adrenergic receptors and adenylate cyclase

activation in C6 glioma cells, described previously (2). Indeed, with a native system, we have shown that 40% receptor occupancy led to 70% adenylate cyclase activation (2).

It is therefore possible to reduce the S—S bridge of the *beta* adrenergic receptor, and this leads to the formation of reactive sulfhydryl groups. Their alkylation by an affinity label derived from a *beta* adrenergic agonist or antagonist could be useful in isolating this receptor and studying its characteristics.

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