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β-Adrenergic Receptors Display Intramolecular Disulfide Bridges *In Situ*: Analysis by Immunoblotting and Functional Reconstitution

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SUMMARY

The molecular nature of mammalian β-adrenergic receptors *in situ* was probed using immunoblotting and functional reconstitution techniques. Membrane proteins of cells replete with β-adrenergic receptors were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose and then probed with anti-receptor antibodies. When cell membranes were first treated with agents that cleave disulfides of proteins, immunoblots of these membranes revealed intense immunoreactive bands with electrophoretic mobility similar to that of protein standards of M_r 65,000–67,000, comigrating with purified, reduced, and alkylated β-adrenergic receptors. However, when cell membranes were prepared under anaerobic conditions, solubilized in the presence of agents that alkylate thiols, and denatured in the absence of added thiols, immunoblotting revealed receptor with M_r 55,000, rather than 65,000. This faster electrophoretic mobility is associated with the presence of intramolecular disulfides in the purified receptor and demonstrates that β-adrenergic receptors possess intramolecular disulfide bridges *in situ*. Purified receptors that demonstrate this faster mobility (M_r 55,000 under non-reducing conditions) were co-reconstituted into phospholipid vesicles with the stimulatory GTP-binding protein G_s and their ability to catalyze the binding of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate to G_s was measured. Agonist (isoproterenol) as well as thiol increased the receptor-promoted activation of G_s. Taken together, these data demonstrate that native β-adrenergic receptors possess one or more intramolecular disulfide bridges *in situ*, reduction of which causes functional activation of the receptor.

Many physiological processes are regulated by catecholamines via their interaction with β-adrenergic receptors (1). The binding of β-adrenergic agonists to these cell surface receptors leads to the stimulation of adenylate cyclase activity and accumulation of intracellular cyclic AMP. The adenylate cyclase system through which stimulatory hormones act is composed of at least three components: the β-adrenergic receptor, a stimulatory, regulatory GTP-binding protein (G_s), and adenylate cyclase itself (2).

β-Adrenergic receptors are sensitive to the effects of thiol compounds, such as dithiothreitol and 2-mercaptoethanol. The ability of β-adrenergic receptors to bind radioligands has been shown to be decreased in membranes exposed to dithiothreitol (3, 4). The analysis of purified mammalian β-adrenergic receptors provided the first direct evidence to support the existence of intramolecular disulfides in these receptors. Chemical reduction of disulfide bridges by thiols has been shown to alter the electrophoretic mobility of purified β-adrenergic receptors. β-Adrenergic receptors isolated from RFCs (β_1-subtype) and from S49 mouse lymphoma cells (β_2-subtype), treated with thiols and then subjected to SDS-PAGE, migrate with apparent M_r = 65,000–67,000. However, we observed that these purified receptors migrated as M_r 55,000 species when subjected to SDS-PAGE under non-reducing conditions (5, 6). The thiol-induced change in molecular weight on SDS-PAGE was not observed as a general phenomenon for other membrane proteins (5), but has been reported for the hepatic glucagon receptor (7), the opiate receptor purified from bovine striatum (8), and receptors for interleukin-2 (9), interleukin-3 (10), and leutinizing hormone-human chorionic gonadotropin (11). A critical question to be answered in this regard is, do intramolecular disulfides exist in these receptors *in situ*?

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ABBREVIATIONS: G_s, the stimulatory regulatory protein of the adenylate cyclase system; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; RFC, rat fat cell; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; IAPB, iodoazidobenzylpindolol.

Pedersen and Ross (12) reported the effects of thiols on purified avian β-adrenergic receptors that had been co-reconstituted into unilamellar phospholipid vesicles with G_s purified from rabbit liver. Reduction with thiols resulted in functional activation of the avian receptor such that it catalyzed nucleotide exchange by G_s more efficiently and was active even in the absence of agonists (12). We (5, 12) and others (13, 14) have proposed a role for intramolecular disulfides and sulfhydryls in the structure and activation of β-adrenergic receptors. More recently this hypothesis has been extended to other G-protein-linked receptors based upon primary sequence information (15). In the present study we utilize immunoblotting techniques and anti-receptor antibodies to probe for the existence of intramolecular disulfides in the β-adrenergic receptors *in situ*, capitalizing upon the molecular weight of these receptors on SDS-PAGE, which has been shown to be sensitive to reduction by thiols (5). Using a defined reconstitution system, we investigate also whether the M_r 55,000 form of the β-adrenergic receptor is the species responsible for mediating activation of G_s by agonist ligands as well as by thiols.

Materials and Methods

Membrane preparations. Isolation of RFCs, as well as preparation of crude and highly purified membranes from these cells, was performed as described (16). The preparation of membranes from S49 wild-type mouse lymphoma cells was described previously (6). In some instances S49 mouse lymphoma cell membranes were prepared under anaerobic conditions (an atmosphere of 100% nitrogen) and in the presence of one of two reagents that alkylate "free" sulfhydryl groups of proteins, either sodium iodoacetate (22 mM) or *N*-ethylmaleimide (50 mM). Protein was determined by the method of Lowry *et al.* (17).

Purification of β-adrenergic receptors. β-Adrenergic receptors were purified from membranes of RFCs (16) and from S49 wild-type mouse lymphoma cells (6) using affinity, ion exchange chromatography and steric exclusion high performance liquid chromatography.

Polyacrylamide gel electrophoresis (PAGE). Samples were prepared for electrophoresis by incubation in 0.05 ml of solubilizing solution containing 0.125 M Tris-Cl (pH 6.8), 4% SDS, 20% sucrose, either without a thiol or with dithiothreitol (10 mM) or 2-mercaptoethanol (20%), as described elsewhere (18). Specific conditions for each experimental protocol are provided in the legends to the figures. Electrophoresis was performed on 10% polyacrylamide gels in the presence of SDS (19). Silver staining of gels was performed as described previously (6).

Antisera and immunoblotting. The preparation and characterization of the rabbit polyclonal antiserum against β-adrenergic receptors (6EN-1) used in these studies have been described in detail elsewhere (18). Aliquots of cell membrane preparations as well as purified β-adrenergic receptors were subjected to SDS-PAGE and the separated proteins were transferred electrophoretically to nitrocellulose by the method of Towbin *et al.* (20), as modified by Erickson *et al.* (21). The nitrocellulose was processed and incubated with anti-receptor antiserum and the immunoreactivity on the blots was made visible using an alkaline phosphatase-linked second (goat anti-rabbit IgG) antibody (see figure legends for final dilution), as previously described (18). Alternatively, a radioiodinated second (goat anti-rabbit IgG) antibody was used in tandem with autoradiography to identify immune complexes on blots.

Reconstitution of receptor-G_s vesicles. β-Adrenergic receptors purified from S49 mouse lymphoma cells and G_s purified from rabbit liver (22) were co-reconstituted into unilamellar vesicles composed of phosphatidylethanolamine, phosphatidylserine, and cholesterol (3:2:1) (23, 24). Functional activation of G_s was measured according to [^{35}S]GTPγS binding at 30° in medium containing 2 mM free Mg^{2+} and 200 nM [^{35}S]GTPγS, exactly as described earlier (12, 25).

Materials. All other materials were obtained from sources previously described (12, 18).

Results and Discussion

When treated with thiols to cleave disulfide bridges, β_2-adrenergic receptors purified from S49 mouse lymphoma cells display M_r = 65,000, according to silver-stained SDS-PAGE gels (Fig. 1, lane *1*). This molecular weight is in good agreement with those of mammalian β-adrenergic receptors isolated from several other sources (16, 26, 27). Recently, antibodies that specifically recognize both β_1- and β_2-adrenergic receptors were prepared in rabbits and characterized against several different β-adrenergic receptors (18). In the present studies we examined the ability of one anti-receptor antiserum raised against guinea pig lung β-adrenergic receptor (6EN-1) to recognize purified as well as membrane-bound receptors of both the β_1- and β_2-subtypes (Figs. 1, lanes 2–5, and 2). Purified β-adrenergic receptors were reduced with thiol, subjected to SDS-PAGE, and transferred to nitrocellulose paper. The blots were then

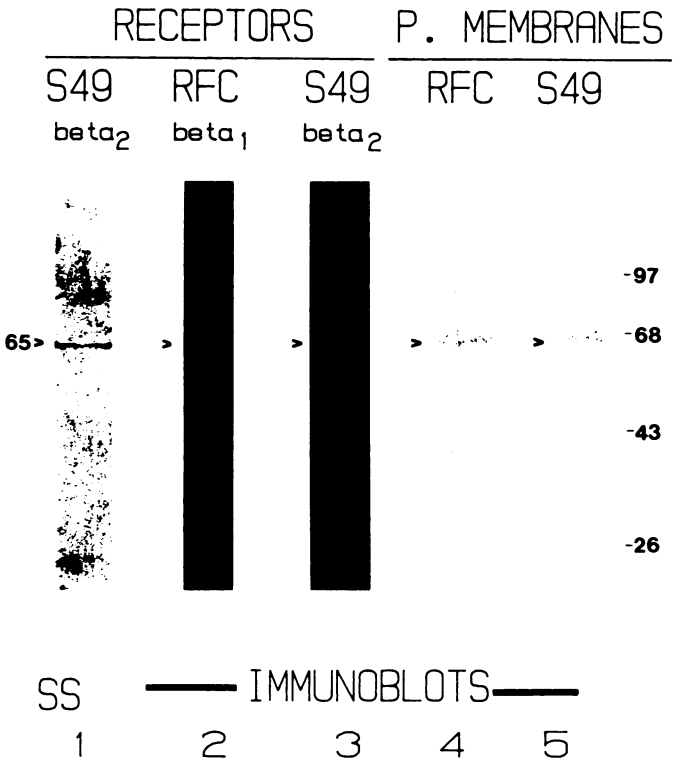

Fig. 1. Analysis of purified and cell membrane-bound β-adrenergic receptors on SDS-PAGE after reduction by thiols. Purified β_2-adrenergic receptors (300 ng per lane) of S49 mouse lymphoma cells were reduced with 2-mercaptoethanol (20%), subjected to SDS-PAGE, and stained with silver (lane *1*). β-Adrenergic receptors purified from RFCs (100 ng per lane) and from S49 mouse lymphoma cells (100 ng per lane) were treated with 10 mM dithiothreitol, subjected to SDS-PAGE, and transferred to nitrocellulose (lanes *2* and *3*, respectively). Highly purified membranes (0.3 mg of protein per lane) prepared from RFCs (lane *4*) and from S49 cells (lane *5*) were treated with 10 mM dithiothreitol in SDS followed by 30 mM *N*-ethylmaleimide. Samples were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blots were incubated with anti-receptor antiserum (1:200 dilution). The immunoreactivity was stained with a goat anti-rabbit IgG second antibody (1:400 dilution) that was radioiodinated and detected by autoradiography or was conjugated to alkaline phosphatase and detected by the appearance of colored product, exactly as described (18). The $M_r \times 10^{-3}$ of protein standards is provided in the margin.

probed with the anti-receptor antibodies. Peptides with M_r = 65,000–67,000 displayed immunoreactive staining by this antiserum at dilutions of 1:100, 1:200, and 1:400 (Fig. 2, lanes *2*, *3*, and *4*, respectively), but not by preimmune serum (1:100; Fig. 2, lane *1*). At 1:200 dilution, specific staining of 25, 50, and 75 ng of receptor was demonstrable (Fig. 2, lanes *5*, *6*, and *7*, respectively). Both β_1-adrenergic receptor purified from RFCs and β_2-receptors purified from S49 mouse lymphoma cells displayed specific immunoreactivity with a molecular weight in agreement with that of receptor made visible by silver staining (Fig. 1, lanes *1–3*).

Blots of plasma membranes subjected to treatment with thiols and then SDS-PAGE were probed next with the anti-receptor antiserum (Fig. 3). A prominent species of immunoreactivity with M_r 65,000–67,000 was specifically stained in immunoblots of S49 mouse lymphoma cell membranes probed with anti-receptor antiserum at 1:50, 1:100, 1:200, and 1:400 dilution (Fig. 3, lanes *2–5*, respectively), but not preimmune serum (1:50; Fig. 3, lane *1*). Immunoreactive staining by anti-

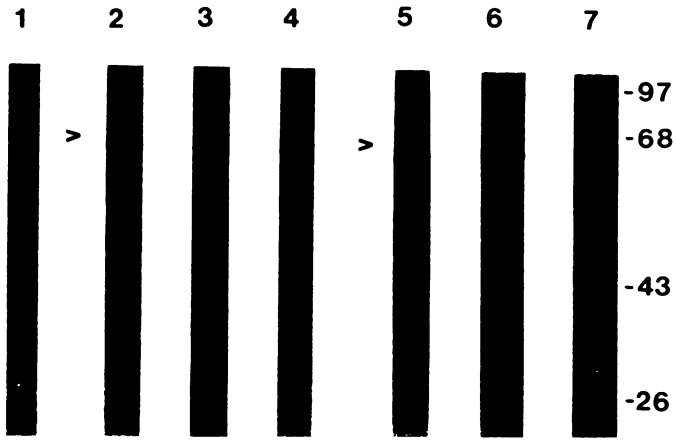

Fig. 2. Analysis of immunoblots of thiol-treated, purified β-adrenergic receptors subjected to SDS-PAGE and probed with anti-receptor antiserum 6EN-1. Purified β_2-adrenergic receptor isolated from S49 mouse lymphoma cells were reduced with 2-mercaptoethanol (20%), subjected to SDS-PAGE, and electroblotted onto nitrocellulose strips. Blots of receptor (50 ng) were probed with anti-receptor antiserum 6EN-1 (lanes *2–4*) or preimmune serum (lane *1*) at final dilutions of 1:100 (lanes *1* and *2*), 1:200 (lane *3*), or 1:400 (lane *4*). Blots of increasing amounts of receptor (lane *5*, 25 ng; lane *6*, 50 ng; lane *7*, 75 ng) were probed with 6EN-1 at 1:200. The immune complexes were detected using a radioiodinated second antibody in tandem with autoradiography.

receptor antiserum could be detected on immunoblots of 0.1 mg of membrane protein (Fig. 3, lane *6*), and staining increased with increasing protein loading (Fig. 3, lanes *6–8*). Membranes isolated from cells having β_1 (RFCs)- or β_2 (S49 mouse lymphoma wild-type cells)-adrenergic receptors also were treated in an identical fashion to chemically cleave disulfides and subjected to SDS-PAGE (Fig. 1, lanes *4* and *5*). Immunoblot analysis using anti-receptor antibodies revealed predominant immunoreactive staining of peptides with M_r = 65,000–67,000 in blots of membranes from both RFCs (Fig. 3, lane *4*) and S49 mouse lymphoma cells (Fig. 3, lane *5*).

The existence of intramolecular disulfide bridges in β-adrenergic receptor has been reported based upon data from structural and functional analyses performed with purified receptors (5, 12). Using cell membranes and purified β-adrenergic receptors subjected to SDS-PAGE under non-reducing conditions in tandem with immunoblotting, the molecular weight of the native receptor was investigated (Fig. 4). Membranes freshly prepared from isolated RFCs and S49 cells were subjected to SDS-PAGE without prior treatment with thiols. Staining of immunoblots of membranes from RFCs (Fig. 4, lane *1*) and S49 mouse lymphoma cells (Fig. 4, lane *2*) revealed immunoreactivity in M_r 55,000 peptides, as compared to peptides with M_r = 65,000 observed after chemical cleavage of disulfides with thiols (see Figs. 1–3). The M_r of purified β_1 (Fig. 4, lane *3*)- as well as β_2 (Fig. 4, lane *4*)-adrenergic receptors subjected to SDS-PAGE under these same conditions was also 55,000, as shown in immunoblots of the gels probed with anti-receptor antibodies (Fig. 4). β_2-Adrenergic receptor (S49 cells) subjected to SDS-PAGE and stained with silver (Fig. 4, lane *5*) also displayed M_r = 55,000, in agreement with the electrophoretic mobilities of the receptors that were solubilized from cell membranes (Fig. 4, lanes *1* and *2*) or purified to essential homogeneity (Fig. 4, lanes *3* and *4*). Minor M_r 55,000 species were observed occasionally on immunoblots of cell membranes that had been treated with thiols (Fig. 3, *small arrowhead*, lanes *2–5*). Incomplete chemical reduction of disulfides or proteolysis are likely explanations for the appearance of these species. The M_r of the membrane-associated receptors subjected to SDS-PAGE in the absence of treatment with thiols (M_r = 55,000, Fig. 4) suggested the presence of intramolecular disulfide bridges in the receptor *in situ*.

The possibility that β-receptors possess disulfide bridges *in situ* was further probed by study of the receptor in intact cell

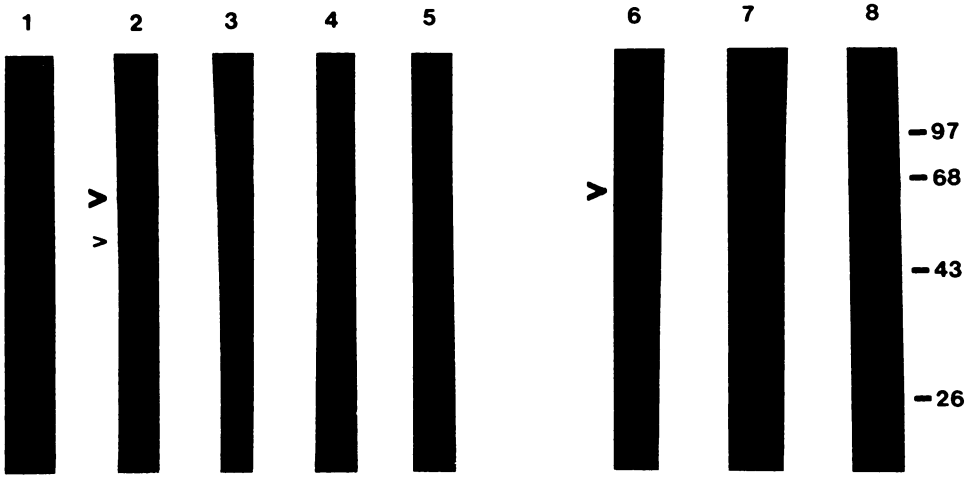

Fig. 3. Analysis of immunoblots of thiol-treated, membrane-bound β-adrenergic receptors subjected to SDS-PAGE and probed with anti-receptor antiserum 6EN-1. Highly purified membranes (0.2 mg, lanes *1–5* and *7*; 0.1, lane *6*; 0.4, lane *8*) were isolated from S49 mouse lymphoma cells, treated with 2-mercaptoethanol (20%), and subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and the blots probed with preimmune (lane *1*) or 6EN-1 (lanes *2–8*) antisera at a final dilution of 1:50 (lanes *1* and *2*), 1:100 (lane *3*), 1:200 (lanes *4*, *6*, *7*, and *8*), or 1:400 (lane *5*). The immune complexes were identified using a radioiodinated second antibody in tandem with autoradiography.

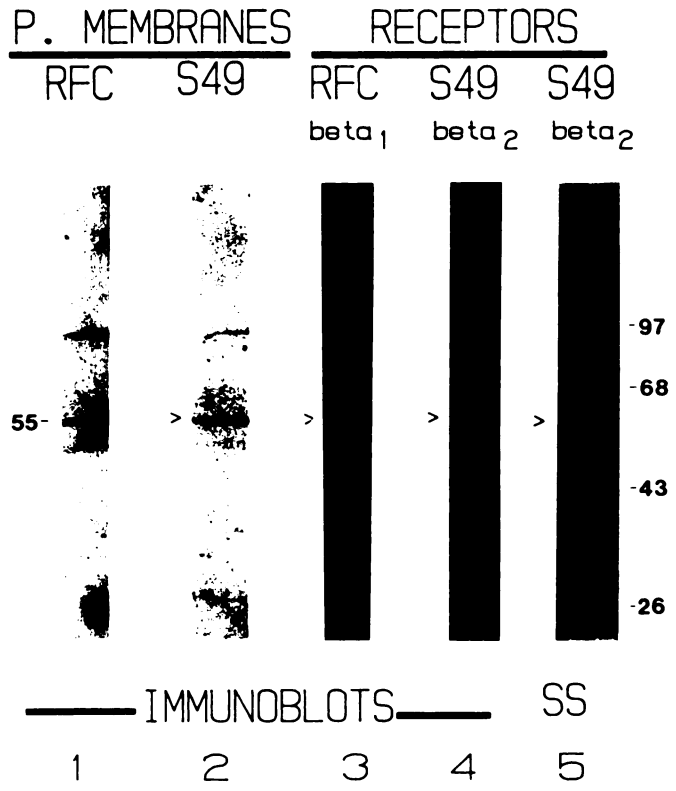

Fig. 4. Analysis of purified and cell membrane-bound β-adrenergic receptors on SDS-PAGE without prior exposure to added thiols. Membranes (0.2 mg of protein per lane) from RFCs (lane *1*) and from S49 cells (lane *2*) were prepared and solubilized in SDS in the absence of added thiol, subjected to SDS-PAGE, and transferred to nitrocellulose. β-Adrenergic receptors (100 ng per lane) purified from RFCs (specific activity > 12 nmol/mg of protein; lane *3*) and from S49 cells (specific activity > 9 nmol/mg of protein; lane *4*) were treated likewise. The nitrocellulose blots were incubated with anti-receptor antiserum (1:200 dilution) as described under Materials and Methods. β_2-Adrenergic receptors (50 ng) purified from S49 cells (lane 5) were also subjected to SDS-PAGE under non-reducing conditions and stained with silver. The separated proteins were transferred to nitrocellulose and probed with anti-receptor antibodies and made visible by staining as described under Materials and Methods. The $M_r \times 10^{-3}$ of marker proteins is provided in the margin.

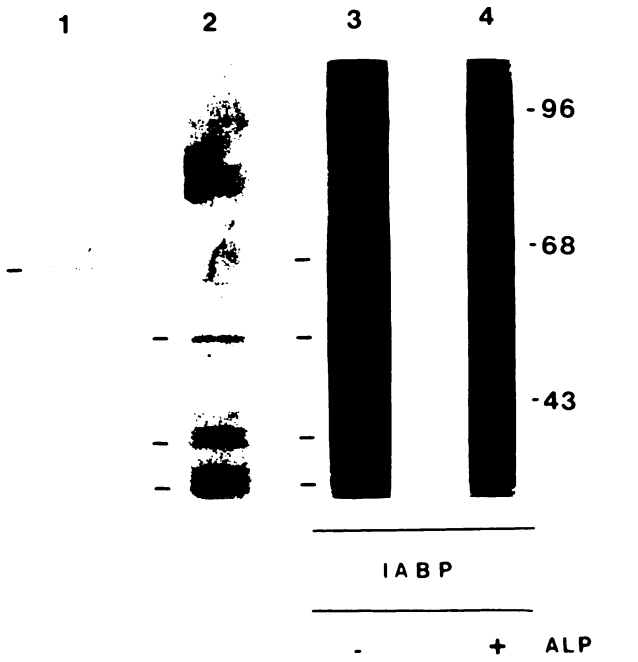

Fig. 5. Immunoblot analysis of the molecular weights of β-adrenergic receptors of membranes prepared from S49 cells under anaerobic conditions and in the presence of *N*-ethylmaleimide. Membranes were prepared from S49 mouse lymphoma cells that were washed and processed up to and including solubilization in SDS in the presence of 50 mM *N*-ethylmaleimide and under an atmosphere of 100% nitrogen. The solubilized membrane proteins were subjected to SDS-PAGE and the separated proteins were transferred to nitrocellulose. Blots from thiol-treated and alkylated S49 cell membranes (0.2 mg, lane *1*) and from the membrane prepared under anaerobic conditions in the presence of 50 mM *N*-ethylmaleimide (0.2 mg, lane 2) were probed with 6EN-1. Samples (0.3 mg) of S49 cell membranes were incubated with [^{125}]IABP alone (lane *3*) or in the presence of 1 μM (−)-alprenolol (*ALP*) for 30 min at 30° in the dark. At the end of the incubation the membranes were photolyzed, washed, solubilized in SDS, reduced with dithiothreitol, and immediately subjected to SDS-PAGE. The gels were stained, destained, dried, and exposed to Kodak XAR-5 film for 24 hr. Note that in addition to the predominant M_r 65,000 and 55,000 peptides specifically labeled by IABP are several smaller fragments which co-migrate with fragments specifically stained by 6EN-1 (compare lanes *2* and *3*).

preparations. The immunostaining of non-reduced, denatured receptors is generally less intense than that commonly observed for receptors and have been first reduced with thiols and then alkylated (compare Figs. 1 and 4). The development times for the visualization of the immunoreactive species on the blots on non-reduced receptor thus were extended. S49 cells were harvested, washed, and maintained under anaerobic conditions and β-adrenergic receptors were examined on immunoblots of membranes prepared from these cells. An M_r of 55,000 was observed for immunostained receptor of membranes prepared from cells under anaerobic conditions (i.e., an atmosphere of 100% nitrogen) in 20 mM Hepes buffer, pH 7.8, containing 2 m*M* $MgCl_2$, 1 mM EDTA, 150 mM NaCl, protease inhibitors, and the presence of either 50 mM *N*-ethylmaleimide (Fig. 5, lane 2) or 22 mM sodium iodoacetate (data not shown), agents that alkylate free sulfhydryl groups of proteins. An immunoblot of reduced and alkylated receptor from these cells, displaying M = 65,000, is shown for comparison (Fig. 5, lane *1*). Several immunoreactive fragments of receptor [M_r < 43,000 were observed in this particular blot (Fig. 5, lane 2), even though the procedures were performed in a cocktail of protease inhibitors (16). The presence of these fragments was found to be quite variable. Minor fragments of similar M_r were observed routinely in S49 membranes that had been incubated with the photoaffinity ligand [^{125}I]IABP and photolyzed (Fig. 5, lane 3). The specific radiolabeling of these fragments by IABP, as well as both the M_r 65,000 and 55,000, species (compare Fig. 5, lanes *3* and *4*), in agreement with the specific staining by anti-receptor antibodies, suggests that these fragments are derived from the β-adrenergic receptor. As previously discussed (5, 15), the photolysis appears to result in a species of receptor (M_r 55,000) that does not migrate as an M_r 65,000 species, if the thiol treatment follows rather than precedes the photolysis with IABP. The fact that the predominant receptor species of cell membranes prepared under these stringent, anaerobic conditions display the faster electrophoretic mobility (M_r 55,000; see lane 2, Fig. 5), associated with the presence of disulfides, provides compelling evidence that β-adrenergic receptors possess intramolecular disulfide bridges *in situ*.

The influence of incubating cell membranes with increasing concentrations of dithiothreitol on the electrophoretic mobility of the β-adrenergic receptor was investigated using immunoblotting and anti-receptor antibodies. Highly purified RFC

membranes were prepared and incubated with various concentrations of dithiothreitol. The membranes were treated with excess *N*-ethylmaleimide and analyzed by SDS-PAGE and immunoblotting (Fig. 6). In the absence of dithiothreitol, predominant immunoreactive staining was observed in an M_r 55,000 species. Several minor, immunoreactive fragments of receptor with (M_r ranging from 43,000 to less than 20,000) generated during the incubation were made visible by extended incubation of blots with phosphatase-substrate necessitated by the lesser immunoreactivity of non-reduced receptor (see above). Treatment with 10 mM dithiothreitol resulted in a shift in the apparent M_r of the major immunoreactive species from 55,000 (Fig. 6, lane *1*) to 67,000 (Fig. 6, lane *5*). Increasing amounts of the M_r 67,000 species were observed as the concentration of added thiol increased from 0.5 to 5.0 mM (Fig. 6, lanes *2–4*). The immunoblots of cell membranes treated with the two lowest concentrations of thiol (0.5 mM, lane 2; 1.0 mM, lane 3 in Fig. 6) reveal multiple receptor species and prompt speculation that more than one disulfide bond is being cleaved by thiol. However, we have been unable to define more precisely the nature of these receptor species. Reduction of purified β-adrenergic receptors by dithiothreitol or 2-mercaptoethanol has been shown previously to result in a concentration-dependent destabilization of the ability of receptor to bind antagonist radioligand (5). The concentrations of dithiothreitol required to destabilize the receptor in that study paralleled those required here to shift the electrophoretic mobility of the receptor on SDS-PAGE. These data suggest that β-adrenergic receptors exist *in situ* with intramolecular disulfide bridges that are at the least essential for the stability of the ligand-binding domain of the molecule.

The possibility that the chemical reduction of the disulfide bridges of the β-adrenergic receptor was a reversible process was examined using receptor isolated from S49 cells (Fig. 7). Receptor was first treated with thiol (2-mercaptoethanol). Aliquots were then subjected to lyophilization followed by resuspension in dilute buffer, a cycle repeated five times. The samples were then subjected to alkylation with excess iodoacetamide and to electrophoresis on an 8–15% acrylamide gel. Comparison of thiol-treated receptor (10%, 5%, and 2% 2-mercaptoethanol; Fig. 7, lanes *2*, *3*, and *4*, respectively) to receptor that was thiol-treated, lyophilized and then washed repeatedly (Fig. 7, lanes 5 and 6) demonstrates that the effect of thiol treatment on receptor molecular weight is reversible. Receptor that was alkylated without prior chemical reduction is shown for comparison (Fig. 7, lane 7). These data also show that the M_r 55,000 species is *not* a proteolytic product of the M_r 65,000 species, but rather a more highly oxidized form of the β-adrenergic receptor.

In previous studies, Pedersen and Ross (12) demonstrated that reduction of intramolecular disulfides in the avian β-adrenergic receptor resulted in an increase in its regulatory activity as well as in a decrease in its stability. Receptors purified from S49 lymphoma cells were co-reconstituted with rabbit hepatic G_s into unilamellar phospholipid vesicles and their ability to promote the activation of G_s was assayed according to [^{35}S]GTPγS binding (25). The data presented in Fig. 8 demonstrate two features of the purified mammalian receptor. The first is that these receptors which display M_r 55,000 on SDS-PAGE under non-reducing conditions are competent with respect to mediating agonist stimulation of G_s activation. Second, and equally important, is that these receptors, like their

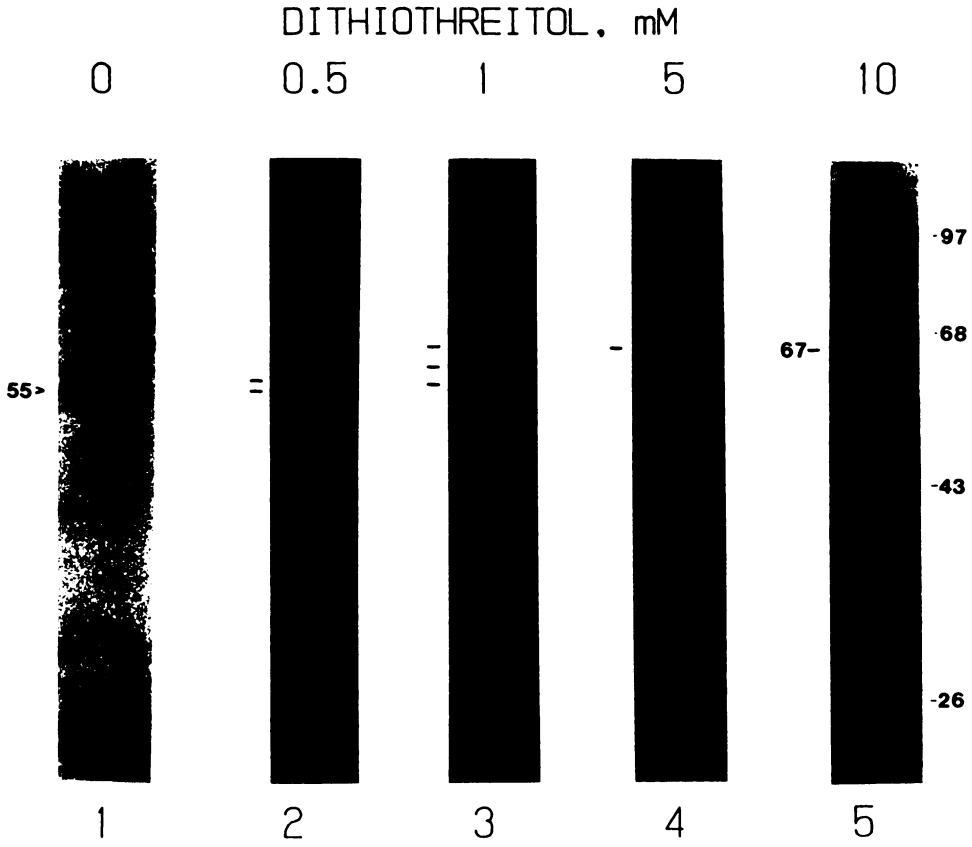

Fig. 6. Immunoblot analysis of the electrophoretic mobility (M_r) of β-adrenergic receptors in cell membranes treated with dithiotreitol. Highly purified membranes prepared from RFCs were isolated and then treated with increasing concentrations of dithiothreitol (0–10 mM) for 20 min at 4° and then treated with a 10 mM excess of *N*-ethylmaleimide (5). The membranes were solubilized in SDS and subjected to SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and β-adrenergic receptors were made visible by immunoreactive staining of the blots with anti-receptor antiserum as described under Materials and Methods. The band of immunostaining with M_r > 100,000 was not routinely observed (see Fig. 1).

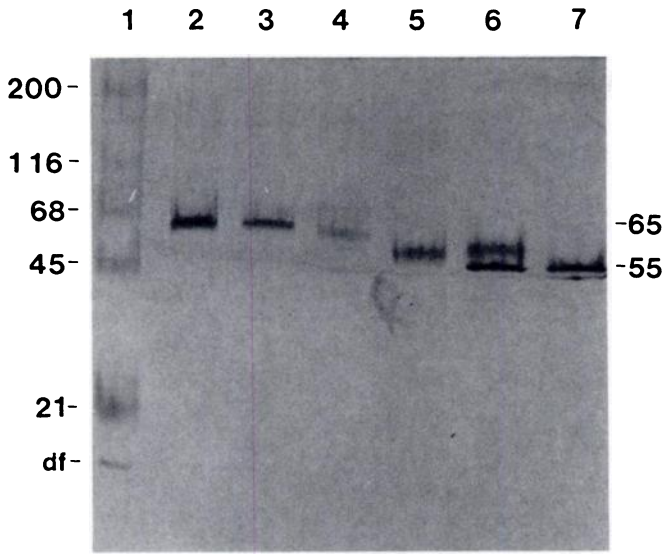

Fig. 7. Chemical reduction of disulfide bridges of β-adrenergic receptors: reversibility. Aliquots of purified β-adrenergic receptors (10 ng) were lyophilized to dryness and reconstituted in Laemmli sample buffer containing the following concentrations of 2-mercaptoethanol: 10% (lane 2), 5% (lanes 3 and 5), and 2% (lanes 4 and 6). Two of the samples (lanes 5 and 6) were lyophilized, resuspended in buffer by vortexing, and allowed to sit on ice for 30 min. The samples were then lyophilized to dryness and the washing was performed four additional times. All samples were then treated with excess iodoacetamide at pH 8.0 and loaded onto an 8–15% acrylamide gradient Phast gel. The gels were subjected to electrophoresis for 30 min and stained automatically with the silver reagent. An aliquot of non-reduced receptor (10 ng) was alkylated and subjected to electrophoresis to provide an internal standard (lane 7).

avian counterparts, are activated by cleavage of intramolecular disulfide(s). Earlier, the effect of dithiothreitol was shown to be on the receptor rather than on G_s by treating the receptor with dithiothreitol prior to reconstitution under anaerobic conditions such that G_s was not exposed to a significant concentration of the reducing agent (12). Such a control was not possible in this case because chemical reduction of the mammalian receptor with thiols caused it to be too labile to survive this process (data not shown). Nevertheless, the effect of dithiothreitol on receptor-G_s vesicles shown in Fig. 8 appears to be caused by a direct effect on the receptor, both by analogy with our previous work and because no significant activating effects of dithiothreitol on G_s have been observed (12).

Reduction of intramolecular disulfides in the β-adrenergic receptor has at least three measurable effects on the molecule. First, the thiol-reduced and alkylated receptor displays a significantly retarded electrophoretic mobility in SDS when compared to that of the non-reduced receptor. The reduced and alkylated turkey erythrocyte β-adrenergic receptor also displays a markedly slower electrophoretic mobility in SDS when compared to its non-reduced counterpart (data not shown). Second, the thiol-reduced receptor is far less stable with respect to its ability to bind β-adrenergic ligands or its ability to activate G_s (3–5, 12). Third, both avian and mammalian β-adrenergic receptors are functionally activated following chemical reduction of their intramolecular disulfides by thiols. The thiol-reduced receptors are able to catalyze exchange of guanine nucleotides by G_s in the absence of β-adrenergic ligands or in the presence of antagonist ligands (Fig. 8, see also Ref. 12).

The present study takes advantage of the change in electrophoretic mobility of the mammalian receptor in SDS upon reduction with thiols in an effort to determine whether these receptors possess intramolecular disulfides in their normal cellular environment. Immunoblots of cell membrane proteins that have been separated by SDS-PAGE indicate that the β-adrenergic receptors display the faster electrophoretic mobility associated with the presence of disulfide bridges. The M_r 55,000 species of the receptor likewise was observed in immunoblots of membranes prepared from cells harvested and disrupted under anaerobic conditions and in the presence of high concentrations of agents which readily alkylate free sulfhydryls of proteins. These stringent precautions virtually preclude the artifactual formation of disulfide bridges or thiol-disulfide interchange by the receptor. Additionally, the functional reconstitution of isoproterenol-stimulated G_s activation was performed with purified mammalian receptors that also display the faster electrophoretic mobility in SDS associated with the presence of intramolecular disulfides. Thus, the species of re-

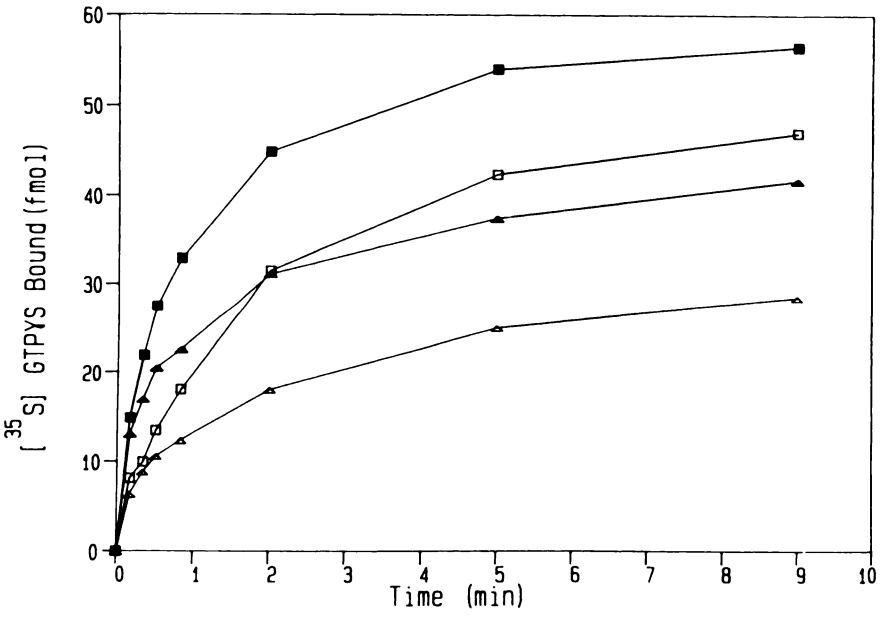

Fig. 8. Activation of S49 lymphoma cell β-adrenergic receptors by dithiothreitol. Receptors purified from S49 lymphoma cells and G_s purified from rabbit liver were co-reconstituted into phosphatidylethanolamine/phosphatidylcholine/cholesterol vesicles, as described by Brandt and Ross (24). Vesicles were incubated at 0° for 60 min in the assay buffer, but without GTPγS, in the presence (■, ▲) or absence (□, △) or 5 mM dithiothreitol. Such incubation is optimal for the reductive activation of the avian erythrocyte receptor (12). Each batch of vesicles was then assayed for the binding of [^{35}S]-GTPγS (200 nM final concentration) in the presence of either 0.01 mM (−)-isoproterenol (■, □) or 0.001 mM (−)-propranolol (▲, △). Each data point represents vesicles that contained 3.0 fmol of receptor. The data are an average of duplicate time courses obtained using a single batch of vesicles and are representative of experiments performed using three separately prepared batches of vesicles.

ceptor observed *in situ* by immunoblotting displays the same electrophoretic mobility as the species mediating agonist-stimulated activation of G_s in the reconstitution assay.

It is not clear from the data shown here or elsewhere (12) that a single disulfide mediates all three effects. However, further studies using proteolytic fragments of the β-adrenergic receptor suggest that, at most, 2 disulfides, out of 19 available cysteinyl residues in the avian receptor (28), mediate all three phenomena (29). The conservation of the cysteinyl residues in the transmembrane-spanning domains predicted for avian (28), hamster (30), and human (31, 32) β-adrenergic receptors, as well as for the porcine cerebral and cardiac muscarinic acetylcholine receptors (33, 34), suggests that these residues may play a critical role(s) in the structure and function of G-protein-linked membrane receptors (15). Identification of the cysteinyl residues that are involved in the regulatory disulfide(s) should help us determine whether oxidation and reduction of cysteine are involved in regulation of the function of the β-adrenergic receptor or whether redox chemistry may be involved in the mechanism of action of β-adrenergic catecholamines.

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