Biochem. J. (1989) 263, 533–538 (Printed in Great Britain)

Localization of β-adrenergic receptors in A431 cells *in situ*

Effect of chronic exposure to agonist

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The status of β-adrenergic receptors was investigated in A431 cells exposed to chronic stimulation by the β-adrenergic agonist, (−)-isoproterenol. Specific binding of β-adrenergic antagonist (−)-[^{125}I]iodocyanopindolol declined to 60–80% below control values within 12 h of agonist treatment. This decline in ligand binding was also observed in high-speed membrane fractions prepared from agonist-treated cells. Immunoblots probed with anti-receptor antibodies revealed both that β-adrenergic receptors from untreated and treated cells migrated as 65000-M_r peptides and that the cellular complement of receptor was unchanged. Indirect immunofluorescence localization of β-adrenergic receptors was comparable in control (untreated) cells and cells challenged with (−)-isoproterenol for 1, 12, or 24 h. Thus receptor complement, migration on SDS/polyacrylamide-gel electrophoresis, and localization *in situ* are largely unaffected by agonist stimulation. Receptor binding of antagonist radioligands, in contrast, is markedly down-regulated in cells stimulated chronically with β-adrenergic agonists. These data argue in favour of agonist-induced alteration(s) in the conformation of the receptor that preclude radioligand binding rather than agonist-induced receptor sequestration and/or degradation.

INTRODUCTION

Desensitization, the waning of a hormonal response following chronic exposure to agonist, is a well-established phenomenon (Hertel & Perkins, 1984; Sibley & Lefkowitz, 1985). Although the precise molecular basis for β-catecholamine-induced desensitization remains obscure, major advances have been made in our understanding of several features of the process. Implicated in the process of desensitization are a reduction in the number of receptors identified by radioligand binding (Su *et al.*, 1980), 'uncoupling' of β-adrenergic receptors from the stimulatory guanine-nucleotide-binding regulatory protein of adenylate cyclase, G_s (Su *et al.*, 1980), receptor sequestration (Chuang & Costa, 1979; Harden *et al.*, 1980; Alho *et al.*, 1988), receptor down-regulation (Shear *et al.*, 1976) and phosphorylation (Sibley *et al.*, 1987). When cells are challenged with a β-adrenergic agonist, the binding of radiolabelled antagonists declines rapidly (Harden, 1983) and functional uncoupling of receptor and G_s occurs (Su *et al.*, 1980). The rapid partial loss of radioligand binding is reversible and may result from an agonist-induced conformational change in the receptor that precludes binding of antagonist ligands. Alternatively, receptor sequestration away from the plasma membrane may be promoted by agonist binding. Over prolonged incubation with agonist, cells often display a loss of the cellular complement of receptors, a process termed 'down-regulation' (Su *et al.*, 1980). Agonist-promoted down-regulation of receptor mRNA appears to be one component responsible for this loss of receptor (Hadcock & Malbon, 1988*a*).

Although radioligand binding techniques provide a means with which the number and affinity of β-adrenergic receptors can be determined (Stiles *et al.*, 1984), receptors with binding sites either occupied by agonist or inaccessible to the radioligand are not detected. Furthermore, radioligand binding cannot provide information on the conformation and integrity of receptors. The present study takes advantage of the availability of well-characterized anti-receptor antibodies to study both immunochemically and immunocytochemically the effect of chronic stimulation by agonist on β-adrenergic receptors (see the preceding paper, Wang *et al.*, 1989).

EXPERIMENTAL

The materials and methods are described in detail in the preceding paper (Wang *et al.*, 1989). A431 cells were grown in 150 mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (0.1 mg/ml), penicillin (0.06 mg/ml), and heat-treated fetal bovine serum (5%). Cells were plated at 10^6/ml and harvested prior to reaching confluence. For desensitization, cells were stimulated chronically with the β-adrenergic agonist (−)-isoproterenol at 10 μM. Membrane fractions were prepared as described (Wang *et al.*, 1989), except that the membranes were collected from 1000 *g* post-nuclear supernatants by centrifugation at 160000 *g* for 2 h in a Beckman Ti-50 rotor.

RESULTS

Radioligand binding analysis

The status of β-adrenergic receptors available for radioligand binding was assessed using the high-affinity radiolabelled antagonist (−)-[^{125}I]iodocyanopindolol

Abbreviations used: G-protein, guanine-nucleotide-binding protein; ICYP, iodocyanopindolol; PAGE, polyacrylamide-gel electrophoresis.
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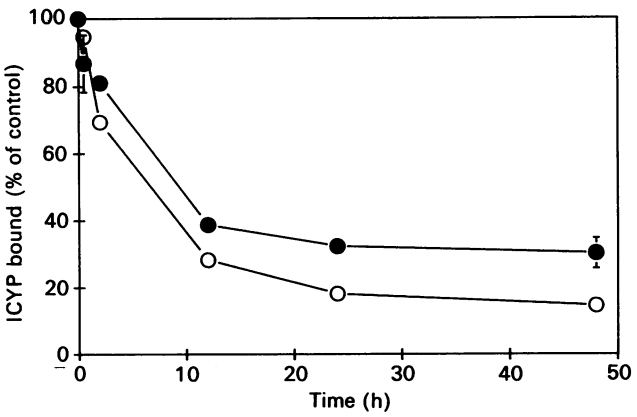

Fig. 1. Specific [^{125}I]ICYP binding to A431 cells: effects of chronic treatment with (−)-isoproterenol

Cells (●) that were pre-confluent were incubated with β-adrenergic agonist [10 μM-(−)isoproterenol] for different periods of time before analysis of radioligand binding. About 50000 cells were used in each assay tube. The concentration of radioligand was 1 nM. Specific binding of [^{125}I]ICYP was determined in cells incubated with agonist for the indicated times. The data are expressed as mean values of two separate experiments. Each determination was performed in triplicate. Specific ICYP binding was also measured in a high-speed (160000 *g*, 2 h) membrane fraction of a post-nuclear supernatant (○). The assay was performed with 20 μg of membrane protein and 1 nM-ICYP, as described (Wang *et al.*, 1989). The data are expressed as mean values (±S.E.M.) of triplicate determinations from a representative experiment. For many data points, the S.E.M. is smaller than the symbol.

(ICYP). Radioligand binding was performed with intact A431 cells and membranes prepared from these cells (Fig. 1). In assays employing intact cells, specific ICYP binding declined following a challenge with β-adrenergic agonist [(−)-isoproterenol, 10 μM]. Within 12 h, the level of ICYP binding declined to 40% of control values in (−)-isoproterenol-treated cells. Specific ICYP binding was also determined in high-speed pellets of a 1000 *g* post-nuclear supernatant. Specific ICYP binding in the membrane fractions of cells treated with (−)-isoproterenol declined with time (Fig. 1). After 12 h of exposure to agonist, ICYP binding had declined to 20% of control values.

Immunoblotting analysis

Both the M_r on SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) and the abundance of β-adrenergic receptors in control as compared with (−)-isoproterenol-treated cells were evaluated independently of ligand binding by immunoblotting. Crude membranes (Wang *et al.*, 1989) were subjected to SDS/PAGE and transferred to nitrocellulose, and these blots were then probed with an anti-peptide antibody (CM8-3) to the β-adrenergic receptor (Fig. 2*a*). Comparable immunoreactive staining of a 65000-M_r species was observed in blots of membranes from untreated (lanes 1 and 3) and (−)-isoproterenol-treated (lanes 2 and 4) cells. Immunostaining of membrane fractions of untreated and agonist-treated cells was evaluated both at 0.2 mg (lanes 1 and 2) and at 0.4 mg (lanes 3 and 4) of membrane protein per SDS/PAGE lane. The patterns of immunoreactive staining were unaltered in membranes from (−)-isoproterenol-treated cells. No major or novel

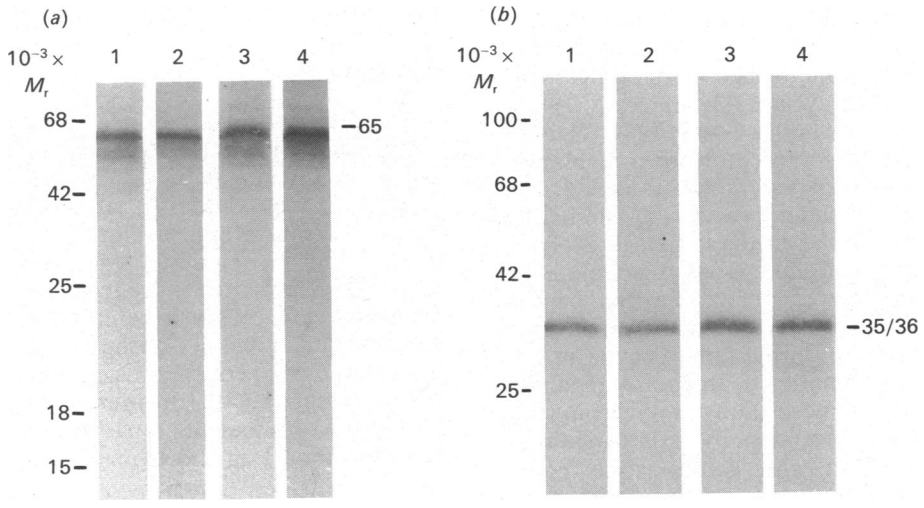

Fig. 2. Immunoblotting of β-adrenergic receptors and G_β-subunits of A431 cells: effect of chronic treatment with (−)-isoproterenol

A431 cells were incubated in Dulbecco's modified Eagle's medium. To half of the cell plates, 10 μM-(−)-isoproterenol was added for 24 h before the cells were harvested. Low-speed (33000 *g*, 20 min) 'crude' membrane fractions of untreated cells (lanes 1 and 3) and agonist-treated cells (lanes 2 and 4) were prepared as described previously (Wang *et al.*, 1989). Membrane proteins were subjected to SDS/PAGE and the separated proteins were transferred to nitrocellulose and probed at 1:400 dilution by either anti-receptor antibody CM8-3 (*a*) or anti-G_β-subunits antibody (G_β-6) (*b*). Immunoblots stained for β-adrenergic receptor represent 0.2 mg (lanes 1 and 2) and 0.4 mg (lanes 3 and 4) of membrane protein/lane (*a*). Immunoblots stained for β-subunits of G-proteins represent 0.1 mg (lanes 1 and 2) and 0.2 mg (lanes 3 and 4) of membrane protein/lane (*b*). Immune complexes were made visible using a radioiodinated goat anti-rabbit IgG second antibody in tandem with autoradiography (36 h).

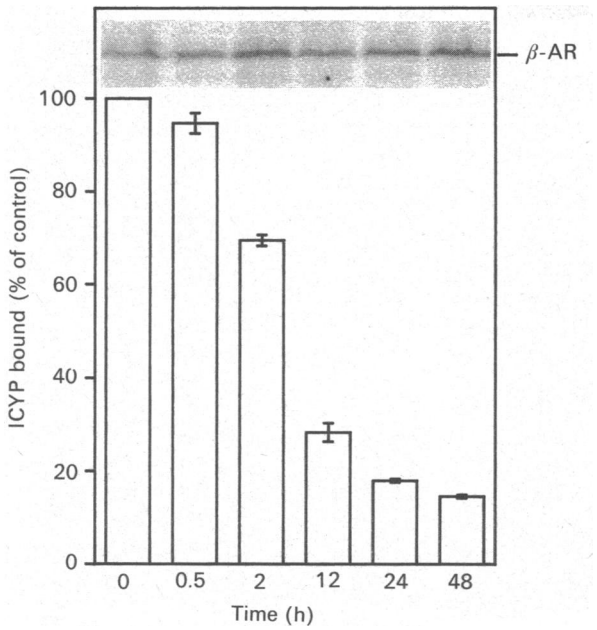

Fig. 3. Correlation between specific ICYP binding and immunoreactive β-adrenergic receptors in a high-speed membrane fraction prepared from A431 cells treated with β-adrenergic agonist

Immunoblotting of β-adrenergic receptors (β-AR; inset) and ICYP binding are displayed. Pre-confluent A431 cells were challenged with 10 μM-(−)-isoproterenol for up to 48 h. Cells were homogenized and the nuclei were removed by a low-speed centrifugation (1000 *g*, 5 min). The supernatant was then centrifuged for 2 h at 160000 *g* to collect both cell membranes and light vesicles. Specific ICYP binding was determined at the indicated time points as described in the legend to Fig. 1. The data are expressed as mean values (±S.E.M.) of triplicates from a representative experiment. Inset: membranes (0.2 mg) were subjected to SDS/PAGE, transferred to nitrocellulose and the blots then probed with anti-receptor antiserum CM8-3 at a final dilution of 1:400. Immune complexes were made visible using a radioiodinated goat anti-rabbit IgG second antibody in tandem with autoradiography (36 h).

immunoreactive species of lower M_r were observed in membranes of agonist-treated cells. Immunoblots of whole-cell homogenates from untreated as compared with agonist-treated cells stained with anti-receptor antibodies also revealed similar levels of immunoreactivity (results not shown).

To evaluate an independent membrane marker, immunoblots of untreated cells (lanes 1 and 3) and (−)-isoproterenol-treated cells (lanes 2 and 4) were probed with antibodies against the β-subunits common to G-proteins, like G_s and G_i (Fig. 2*b*). Aliquots of 0.1 mg (lanes 1 and 2) and 0.2 mg (lanes 3 and 4) of membrane protein per SDS/PAGE lane were subjected to immunoblotting. The β-subunits of several G-proteins (G_s, G_o and G_is) migrate on SDS/PAGE as polypeptides with M_r values of 35000 and 36000 (Sternweis & Robishaw, 1984; Evans *et al.*, 1987). Specific staining of blots of A431 cell membranes with antisera raised against purified human placental β-subunits (G_β-6) was confined to species with M_r of 35000/36000 in blots from untreated and agonist-treated cells alike. Furthermore, immunostaining of G-protein β-subunits was comparable in blots from agonist-treated and untreated A431 cells.

Specific ICYP binding and β-adrenergic receptor levels of A431 cells challenged with the (−)-isoproterenol for various times up to 48 h were analysed by radioligand binding and immunoblotting respectively (Fig. 3). The steady-state level of β-adrenergic receptor protein was not significantly altered in membrane fractions of cells exposed to 10 μM-agonist for periods from 0.5 to 48 h (see inset of Fig. 3). Specific ICYP binding, in contrast, declined to less than 30% of control values in cells treated with agonist for 12 h. After 48 h of challenge with agonist, specific ICYP binding was less than 15% of that observed for untreated cells, while the level of immunoreactive staining of β-adrenergic receptors remained essentially unchanged.

Indirect immunofluorescence

We investigated the distribution of the β-adrenergic receptors in control and (−)-isoproterenol-treated cells by performing indirect immunofluorescence in fixed, intact cells using anti-receptor antiserum CM8-3 (Fig. 4). Phase-contrast and epifluorescence microscopy of fixed, intact cells that were not exposed to the β-adrenergic agonist are shown in Figs. 4(*a*) and 4(*b*) respectively. Punctate immunofluorescence staining was observed throughout the cell surface. Epifluorescence of fixed intact cells previously exposed for 1 h to a combination of (−)-isoproterenol (10 μM) and the potent β-adrenergic antagonist propranolol (10 μM) is displayed in Fig. 4(*c*). Epifluorescence of cells exposed to (−)-isoproterenol alone is displayed in Fig. 4(*d*). The immunofluorescence of cells exposed for 1 h to agonist with (Fig. 4*c*) or without (Fig. 4*d*) an antagonist was equivalent to that of untreated cells (Fig. 4*b*). When the exposure to agonist and antagonist in combination (Fig. 4*e*), or to agonist alone (Fig. 4*f*) was extended to 12 h, the staining patterns were again similar. Comparable levels of immunofluorescence were observed in cells treated without agonist, with agonist alone, or with agonist in combination with an antagonist at these times, and after 24 and 48 h of exposure (results not shown). Untreated and agonist-treated A431 cells that were fixed and permeabilized with detergents (Wang *et al.*, 1989) and stained with anti-receptor antibodies also displayed comparable levels of immunofluorescence (results not shown).

DISCUSSION

Elucidation of the molecular mechanisms of desensitization remains an important goal in regulatory biology. The phenomenon by which cells attenuate their responses in the face of a chronic stimulation by agonist is widely observed in nature, from photoadaptation to chemotaxis in bacteria. As a model for the study of adaptation, desensitization of catecholamine responses in vertebrates has been the focus of intense research. Several hypotheses have been proposed to explain the attenuation in the response to chronic stimulation (for a review, see Sibley & Lefkowitz, 1985). In the present study immunochemical and immunocytochemical analyses were employed in tandem with radioligand binding to evaluate the status of a prominent member of G-protein-linked receptors, the β-adrenergic receptor, in A431 cells chronically stimulated with (−)-isoproterenol.

Using indirect immunofluorescence and a monoclonal

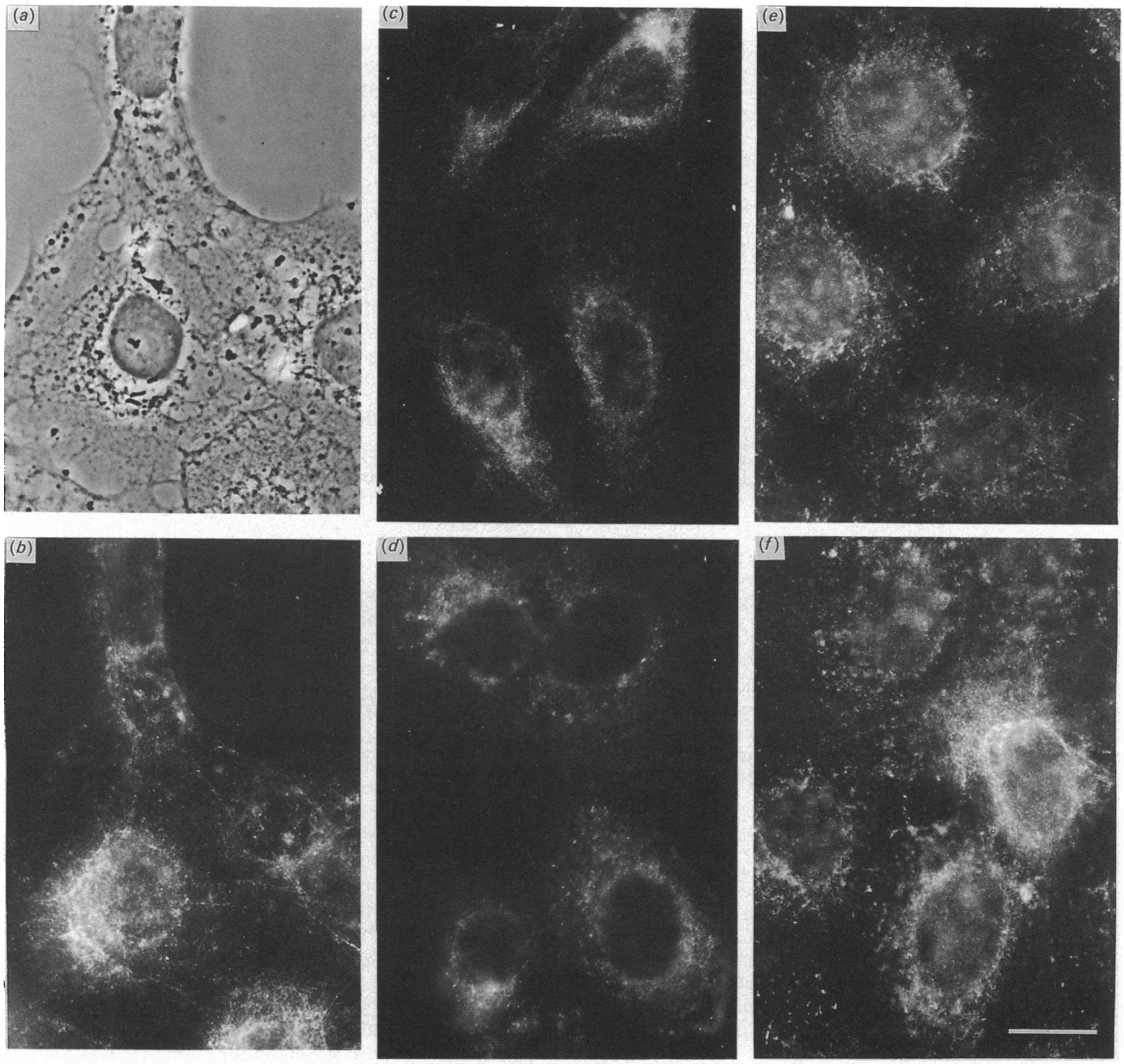

Fig. 4. Indirect immunofluorescence of control and agonist-treated A431 cells stained with antibodies to β-adrenergic receptors

Phase-contrast (*a*) and epifluorescence (*b–f*) images are displayed and are representative of several hundred images obtained of untreated and agonist-treated A431 cells. Untreated cells (*a, b*), cells treated with 10 μM-(−)-isoproterenol in combination with 10 μM-propranolol (*c, e*) or (−)-isoproterenol alone (*d, f*), for 1 h (*c, d*) or 12 h (*e, f*) were fixed and then stained with anti-peptide antiserum CM8-3 specific for the β-adrenergic receptor, and then employed in the indirect immunofluorescence study. Bar, 25 μm.

antibody generated against a synthetic peptide that corresponds to a cytoplasmic domain of the deduced hamster β_2-adrenergic receptor sequence (Dixon *et al.*, 1986). Strader *et al.* (1987) examined the status of β-adrenergic receptors in desensitized transfected mouse L-cells. The L-cells, transfected with an expression vector harboring the cDNA encoding the hamster β_2-adrenergic receptor, stably express these receptors. Permeabilization of cells with detergent was required when using antibodies directed against a cytoplasmic domain of the receptor. The entire complement of receptor would presumably be available for antibody recognition in fixed, permeabilized cells. Specific immunofluorescence was reported to be virtually absent in cells treated for 30–60 min with (−)-isoproterenol (Strader *et al.*, 1987). Two possible explanations to account for the loss of specific immunofluorescence observed in agonist-treated cells are (i) receptor loss or degradation and (ii) alterations in receptor conformation that preclude antibody recognition. Our immunoblotting data provide no evidence to suggest either a loss or degradation of receptor in desensitized cells. Cells treated with (−)-isoproterenol for 1, 12 or 24 h display a normal complement of β-adrenergic receptors. Immunoblots of membranes from control (untreated) and agonist-treated A431 cells display equivalent levels of β-adrenergic receptors. β-Adrenergic

receptors from both control and desensitized cells display an M_r of 65000 when subjected to SDS/PAGE. No novel lower-M_r species of receptor were identified in immunoblots of membrane fractions from cells treated with (−)-isoproterenol. Furthermore, recovery of receptors in cells that have been desensitized for periods of less than 12 h has been shown by radioligand binding to occur rapidly after removal of agonist ligand, even in the presence of cycloheximide (Doss *et al.*, 1981; Hertel & Staehelin, 1983; Limas & Limas, 1984; Mahan *et al.*, 1985*b*). At early times, enhanced loss or degradation of β-adrenergic receptors does not appear to play a major role in agonist-induced desensitization in A431 cells. Our data do not preclude the possibility of an agonist-induced post-translational modification of the receptor, such as phosphorylation. A change in the phosphorylation state of the receptor would probably escape detection under the conditions employed.

Receptor sequestration in response to chronic stimulation by agonist has been proposed as an early component of desensitization (Chuang & Costa, 1979; Harden *et al.*, 1980; Su *et al.*, 1980; Hertel & Perkins, 1984). Agonist-induced redistribution of β-adrenergic receptors from membrane to 'light vesicle' fractions has been reported (for review, see Hertel & Perkins, 1984). According to this proposal, the loss in radioligand binding typically observed in desensitization results from the redistribution of receptors to a light vesicle membrane fraction that cannot be collected on low-speed centrifugation (Harden *et al.*, 1980). If redistribution of receptors among subcellular fractions were truly responsible for the decline in radioligand binding observed in membrane fractions of agonist-treated cells, specific ICYP binding in a pool of these membrane fractions prepared from (−)-isoproterenol-treated cells should be identical to that of untreated cells. Specific ICYP binding in high-speed membrane fractions from post-nuclear supernatants was analysed and found to decline to 20% of control values for A431 cells challenged with (−)-isoproterenol for 12 h (Figs. 1 and 3). These data argue in favour of an agonist-induced change in receptor conformation that precludes subsequent binding of antagonist ligands.

Receptor redistribution to light vesicle fractions was previously shown to be dependent upon the method employed for cell disruption (Strader *et al.*, 1984). Although displaying the attenuation of agonist-stimulated adenylate cyclase characteristic of desensitization, membrane fractions obtained from agonist-treated cells by several commonly employed methods of homogenization failed to display receptor redistribution (Strader *et al.*, 1984). Likewise, agonist-induced desensitization of β-adrenergic receptors has been shown to be unaffected by treatment with various chemical agents (Hertel *et al.*, 1985; Kassis *et al.*, 1985), lectin binding (Kassis & Fishman, 1984; Kassis *et al.*, 1986), or reductions in temperature (Cook *et al.*, 1987) that block receptor redistribution from membrane to light vesicle fractions.

We took advantage of the availability of well-characterized anti-receptor antibodies (Moxham *et al.*, 1986, 1988; George *et al.*, 1988; Hadcock & Malbon, 1988; Wang *et al.*, 1989) to investigate the localization of β-adrenergic receptors *in situ* in agonist-treated cells. Indirect immunofluorescence provided the means for ascertaining the distribution of the receptor independently of radioligand binding. Localization *in situ* revealed comparable patterns of staining of β-adrenergic receptors in (−)-isoproterenol-treated and untreated A431 cells. Although indirect immunofluorescence cannot provide the number of receptors in intact cells, it can provide a relative index of receptor complement. A 2-fold change in β-adrenergic receptor levels in glucocorticoid-treated hamster vas deferens cells (DDT_1 MF-2) was demonstrated using indirect immunofluorescence and confirmed by radioligand binding analysis (Hadcock & Malbon, 1988*b*). Using this same strategy we observed no obvious change in the fluorescence signal of fixed, intact cells treated with (−)-isoproterenol for 1, 12, or 24 h. ICYP binding in agonist-treated cells, in contrast, declined to 20% of the control by 24 h.

Results of acid elution of radioligands from agonist-treated S49 mouse lymphoma cells suggest that although uncoupled from G_s the receptors have not been internalized by 15 min (Mahan *et al.*, 1985*a*). The abundance and distribution of another G-protein-linked receptor, the α_1-adrenergic receptor, has been reported to be unchanged in DDT_1MF-2 cells exposed to adrenaline (Cowlen & Toews, 1987). Pretreatment with agonist for periods of up to 30 min reduced adrenaline-stimulated phosphatidylinositol turnover by 50% without altering the cellular distribution of the receptor. In an analogous study, Ross *et al.* (1988) have shown recently that neither the number of nicotinic acetylcholine receptors nor the amount of receptor available for staining by anti-receptor antibodies is altered following chronic exposure of cultured embryonic chick muscle cells to agonist. Interestingly, agonist treatment promoted a redistribution of the cell-surface receptors from larger to smaller aggregates (Ross *et al.*, 1988).

Guillet *et al.* (1985) reported on the use of a monoclonal antibody (mAb2B4) to β-adrenergic receptors. Indirect immunofluorescence studies of desensitized cells with this antibody revealed a decline in fluorescence signal in cells treated with agonist. Radioligand binding declined by 50% after a 30 min challenge with agonist. The loss in epifluorescence of agonist-treated cells at 30 min, in contrast, was complete. Strader *et al.* (1987) reported a complete loss of receptor-specific immunofluorescence in agonist-treated cells, although these cells still displayed 50% of their complement of receptors as measured by radioligand binding. Zemcik & Strader (1988) recently reported a similar observation in agonist-treated hamster vas deferens cells (DDT_1-MF-2). In these cells, pretreatment with isoproterenol for 1 h reduced radioligand binding by 60% while abolishing specific immunofluorescence. Both the observations of Strader and coworkers (Strader *et al.*, 1987; Zemcik & Strader, 1988) and those of Guillet *et al.* (1985) suggest that agonist-induced loss of antibody recognition rather than loss of receptor *per se* may explain the decrease in indirect immunofluorescence.

Localization of β-adrenergic receptors *in situ* in A431 cells provides an alternate experimental model for the study of desensitization of G-protein-linked receptors. The data presented here are the first to establish that the M_r on SDS/polyacrylamide gels, cellular complement, and localization *in situ* of a G-protein-linked receptor are largely unaffected in cells following a 1–24 h challenge with agonist. Thus, what has been previously reported in the literature as 'down-regulation of receptor' is, in fact,

'down-regulation of ligand binding'. Kashles & Levitzki (1987) characterized the β-adrenergic receptor dependent adenylate cyclase in A431 cells. The patterns of desensitization observed in these cells are not unlike that reported for other cell types (see Sibley & Lefkowitz, 1985, and references cited therein).

Defining the structural basis for agonist-induced down-regulation of ligand binding capacity is an important goal towards the development of a greater understanding of the molecular mechanism(s) of desensitization. Receptor phosphorylation (Sibley & Lefkowitz, 1985) and intramolecular disulphide bridges (Malbon *et al.*, 1987) have each been implicated as possible candidates for the regulation of receptor function.

We thank Dr. John K. Northup (Department of Pharmacology, Yale University, New Haven, CT, U.S.A.) for the antibodies against G-protein β-subunits. In addition, we thank Ms. Jeanne Yokelson for her expert assistance in the photography. This work was supported by United States Public Health Services grants DK30111, DK25410, and K04-AM00786 from the National Institutes of Health (to C.C.M.), and in part by grant DCB-8615969 from the NSF (to M.B.).

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Received 13 February 1989/8 May 1989; accepted 16 May 1989

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