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Fluorescent localization of the β-adrenergic receptor on DDT-1 cells

Down-regulation by adrenergic agonists

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Continuous incubation of cultured cells with β-adrenergic agonists results in the desensitization of adrenergic responsiveness accompanied by the down-regulation of cell surface β-adrenergic receptors (βAR). Previous studies have relied on measurements of ligand binding activity for the detection of the βAR in the cell. In the present study, we have raised a monoclonal antibody to a synthetic peptide corresponding to amino acid numbers 226–239 of the hamster β_2AR. This antibody was used to localize the βAR in hamster smooth-muscle DDT-1 cells by immunofluorescence, without regard for the ability of the receptor to bind ligands. The βAR was found to be localized primarily at the plasma membrane of these cells, with a non-homogeneous pattern of distribution. A rapid loss of βAR-specific immunofluorescence, which paralleled receptor down-regulation as measured by ligand-binding activity, was seen with β-adrenergic agonists, but not with antagonists. In addition, a transient increase in fluorescence was observed after short times of exposure of the cells to agonists. This fluorescence increase may reflect a ligand-induced conformational change in the receptor.

INTRODUCTION

Chronic exposure of cells to β-adrenergic agonists results in a decrease in the cellular responsiveness to further agonist challenge, via desensitization of the β-adrenergic receptor (βAR) (Perkins, 1983; Sibley & Lefkowitz, 1985). This process involves a functional uncoupling of the receptor from its effector adenylate cyclase system, which correlates in some systems with receptor phosphorylation and/or a decrease in the cell-surface receptor-specific binding of adrenergic ligands (Su *et al.*, 1980; Strasser *et al.*, 1984; Sibley *et al.*, 1984, 1985; Mahan *et al.*, 1985; Kassis & Sullivan, 1986). The decrease in ligand binding appears to result primarily from a sequestration of the receptor away from the cell surface. It has been shown in several cell lines that exposure of intact cells to adrenergic agonists results in a loss of the βAR from the plasma membrane of the cell and the concomitant appearance of β-adrenergic binding in membrane fractions distinct from the plasma membrane. In other cell types, the βAR binding which is lost from the cell surface during desensitization cannot be recovered intracellularly, implying that receptor degradation or an irreversible loss of ligand binding activity can also occur. These studies have led to a model for receptor down-regulation whereby receptors are initially sequestered away from the cell surface with their ligand binding intact, after which the ligand binding activity is lost and the receptors can no longer be detected within the cell.

To date, studies on agonist-mediated receptor down-regulation have depended on ligand binding to measure receptor loss, as the specificity and affinity of available antibodies to the βAR were not sufficient to allow direct visualization of the β-receptor protein in mammalian cells. Thus, only receptors which maintained their ligand binding capability could be detected during the desensitization process, leaving the role of possible receptor inactivation unexplored. In the present study, we have developed a monoclonal antibody to a defined region of the primary sequence of the βAR protein. This antibody has been used in immunofluorescence experiments to localize the βAR in the plasma membrane of hamster smooth-muscle DDT-1 cells, without regard for its ability to bind ligands. Our results indicate that the βAR protein itself is lost from the cell membrane during agonist-mediated desensitization, and further suggest the presence of ligand-induced conformational changes in the βAR in these cells.

EXPERIMENTAL

Monoclonal antibody production

A peptide with the sequence YAKRQLQKIDKSEGR, corresponding to amino acids 226–239 of the hamster βAR, was synthesized and coupled to thyroglobulin for use as an antigen, as described previously (Dixon *et al.*, 1986). *Balb/c* mice were immunized with this conjugate in six subcutaneous injections over a period of 7 weeks, with a final intravenous injection of the pure peptide

Abbreviations used: βAR, β-adrenergic receptor; e.l.i.s.a., enzyme-linked immunosorbent assay; CYP, cyanopindolol; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate.

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administered 3 days before fusion. Spleen cells from the immunized mouse were fused with SP2/0 myeloma cells in the presence of polyethylene glycol and dimethyl sulphoxide, as described previously (St. Groth & Scheidegger, 1980). The hybrid cells were grown in microtitre plates and the tissue culture supernatants were screened for antibodies to both the synthetic peptide and to the βAR itself. Colonies testing positive for immunoreactivity to the βAR were subcultured and cloned by limiting dilution. Ascites were produced from these clonal cell lines in *Balb/c* mice and the IgM fraction of the protein was purified from ascites by h.p.l.c.–DEAE chromatography and used at a protein concentration of 0.1 mg/ml (Gemski *et al.*, 1985). Non-immune mouse IgM was prepared from ascites isolated from mice injected with the SP2/0 control cells and were purified as described above for the specific antibody and used at the same concentration.

Screening assays

E.l.i.s.a. Dynatec Immulon II microtitre plates were coated with 10 μg/ml of the synthetic peptide in 50 mM-sodium bicarbonate, pH 9.6, for 16 h at 4 °C (Douillard & Hoffman, 1983). After washing three times in phosphate-buffered saline (PBS) containing 0.05% Tween-20, individual tissue-culture supernatants from the fused cells were added and incubated for 1 h at 37 °C. The plates were then further washes three times with PBS/Tween, and incubated with anti-(mouse IgG), conjugated to alkaline phosphatase (Miles) for 1 h at 37 °C. After three washes with PBS/Tween, the plates were incubated for 30 min at 23 °C with *p*-nitrophenyl phosphate substrate at a concentration of 1 mg/ml. The absorbance of the solution at 410 nm was then determined with a Dynatec Microplate Reader.

Immunoprecipitation of radiolabelled βAR. Hamster lung membranes were prepared by the method of Benovic *et al.* (1984). Membranes (3 mg/ml) were incubated with 250 pM-^{125}I-cyanopindolol (^{125}I-CYP) (New England Nuclear) for 1 h at 25 °C, pelleted at 40000 *g* for 20 min, washed, and resuspended in 10 mM-Tris (pH 7.2)/0.1 M-NaCl/2% digitonin (Boehringer/Mannheim). After extraction at 4 °C for 1 h, the membranes were removed by centrifugation, and the free ^{125}I-CYP separated from the soluble extract by chromatography on G-50 Sepharose in 10 mM-Tris/0.15 M-NaCl/0.1% digitonin. The column eluate contained the ^{125}I-CYP-labelled βAR, which was stable to freezing and storage at −70 °C. This ^{125}I-CYP-labelled βAR preparation was incubated with the tissue culture supernatants from the fused cells for 2 h at 23 °C, before precipitation of the ^{125}I-CYP-βAR-antibody complex with 50% saturated $(NH_4)_2SO_4$ as previously described (Strader *et al.*, 1983). Radioactivity incorporated into the immune precipitate was detected with a gamma counter. Alternatively, pure hamster-lung βAR was radiolabelled with ^{125}I and used directly in immunoprecipitation (Strader *et al.*, 1983). SDS/polyacrylamide-gel electrophoresis was performed according to Laemmli (1970).

Cell culture

DDT-1 MF-2 smooth-muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 4.5 g of glucose/l containing 10% foetal-calf serum/1% penicillin/streptomycin/1% glutamine, in a humidified atmosphere of CO_2/air (2:23). At confluence, cell monolayers were dispersed with 1 mM-EDTA in PBS and the cells subcultured at a 1:10 dilution. Cells were used for experiments at confluence, 2–3 days after subculturing.

Immunofluorescence

DDT-1 cells were grown to confluence in DMEM on chamber slides (Miles), washed in PBS, and fixed in 3.7% formalin/1% ethyldimethyldiaminopropyl carbodi-imide in PBS for 10 min at 23 °C. After permeabilization in 0.1% Triton X-100 for 5 min, cell monolayers were incubated with goat γ-globulin at 4 mg/ml for 1 h at 23 °C, to block non-specific sites on the cell membranes (Willingham *et al.*, 1978). Cells were washed with PBS before incubation with 200 μl of either the purified monoclonal antibody or control antibody at 0.1 mg/ml for 1 h at 23 °C. After further washing with PBS, the monolayer was incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-(mouse IgG) (Cappel) at a 1:50 dilution for 1 h at 23 °C. Cells were then washed extensively in PBS and the fluorescence observed using a Leitz Diaplan fluorescence microscope, and photographed with a Leitz Vario-Orthomat camera system on the manual setting, using a constant exposure time for all samples in a given experiment, as given in the Figure legends.

For studies involving the time course of incubation with various hormones, the monolayer was incubated in DMEM at 37 °C in CO_2/air (2:23) with 10^{-5} M-(−)isoproterenol, 10^{-5} M-(−)alprenolol, or 10^{-7} M-prazocin for the times indicated in the Figure legends, quickly washed with cold PBS, and the cells fixed immediately as described above.

Down-regulation of the βAR on DDT-1 cells

DDT-1 cells were grown to confluence in DMEM in T-75 cm^2 flasks. For treatment with isoproterenol, cell monolayers were incubated with 10 ml of conditioned media, containing 0.2 mM-sodium metabisulphite and 10^{-5} M-(−)isoproterenol for the times indicated in the Figure legends at 37 °C in an atmosphere of CO_2/air (2:23). At the end of the incubation time, the monolayers were washed three times with PBS, and the cells lysed by incubation for 15 min at 4 °C in 1 mM-Tris, pH 7.2. Cell membranes were scraped from the flask and pelleted by centrifugation at 40000 *g* for 15 min. The membrane pellet was resuspended to a protein concentration of approx. 1 mg/ml in 75 mM-Tris (pH 7.5)/12.5 mM-$MgCl_2$/1.5 mM-EDTA (TME buffer), and the binding of ^{125}I-CYP was measured in 0.25 ml of TME, containing 25 μg of membrane protein, with 250 pM-^{125}I-CYP as previously described (Benovic *et al.*, 1984). Alternatively, the down-regulation was carried out in cell monolayers grown on 24-well plates. In this case, the isoproterenol was washed away with 5 × 0.5 ml of DMEM containing 20 mM-Hepes (pH 7.5)/0.5% bovine serum albumin, and the ^{125}I-CYP binding performed in 0.25 ml of this buffer directly on the cell monolayer for 1 h at 4 °C using 60 pM-^{125}I-CYP. These conditions have been demonstrated to only allow measurement of cell surface βAR (Linden *et al.*, 1984; Mahan *et al.*, 1985). Non-specific binding in all cases was defined in the presence of 10 μM-alprenolol.

RESULTS

Characterization of monoclonal antibodies

Monoclonal antibodies were raised against a synthetic peptide corresponding to amino acids 226–239 of the hamster β_2AR (Dixon *et al.*, 1986). Antibody-producing clones were chosen on the basis of an e.l.i.s.a. which detected antibody against the synthetic peptide itself, and confirmed by immunoprecipitation of a ^{125}I-CYP-βAR complex, as shown in Fig. 1. Three monoclonal antibodies were raised by this procedure, of which antibody βAR(226–239)3–1 was the most reactive by immunofluorescence. This antibody, which is an IgM, was able to specifically immunoprecipitate pure hamster-lung βAR either in the presence or the absence of bound ligand, as assessed by SDS/polyacrylamide-gel electrophoresis (Fig. 2), and the antibody had no effect on the subsequent ligand binding or adenylate cyclase activation by the receptor (results not shown). The immunoprecipitation of the βAR was completely blocked by prior incubation of the antibody with the synthetic peptide used as the antigen (Fig. 3).

Immunofluorescence of the βAR on intact cells

Antibody βAR(226–239)3–1 was used in indirect immunofluorescence to localize the βAR in DDT-1 cells. As shown in Figs. 4 and 5, incubation of fixed permeabilized cells with the antibody followed by FITC-conjugated second antibody produced an intense specific fluorescence compatible with the reported presence of 60000 βAR/cell (Norris *et al.*, 1987). The specific immunofluorescence was primarily localized at the plasma membrane of the cells, with some clustering observed near the ends of the long processes of the cells extending away from the cell body, indicating that the population of βAR was distributed non-uniformly along the cell surface. Incubation with antibody βAR(226–239)3–1 in the presence of an excess of the synthetic peptide used as the antigen, or with the non-immune mouse antibody, gave only a dull background fluorescence attributable to non-specific uptake of the fluorescent label by the cells, with no localization to the cell membranes (see Figs. 4 and 5).

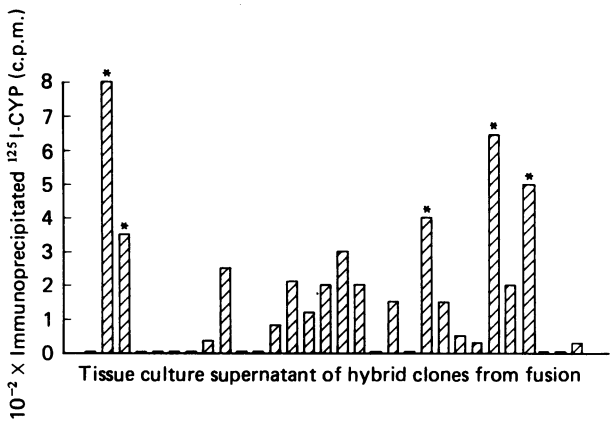

Fig. 1. Immunoprecipitation of ^{125}I-CYP-labelled βAR with tissue-culture supernatants from antibody-producing cells

Monoclonal antibody production is described in the Experimental section. Tissue-culture supernatants (65 μl) containing antibodies to the peptide antigen, as assessed by e.l.i.s.a., were incubated in 75 μl of PBS/0.1% bovine serum albumin/0.1% digitonin, for 16 h at 4 °C with solubilized βAR from hamster-lung membranes, which had been labelled with ^{125}I-CYP as described under in the Experimental section, before precipitation of the immune complex for 30 min at 4 °C with 50% $(NH_4)_2SO_4$. ^{125}I-CYP-labelled βAR (2000 c.p.m.) was used in each assay tube. Clones positive for the production of antibodies recognizing the native βAR, marked with an asterisk in the Figure, were subcloned by limiting dilution and used for further characterization as described in the text.

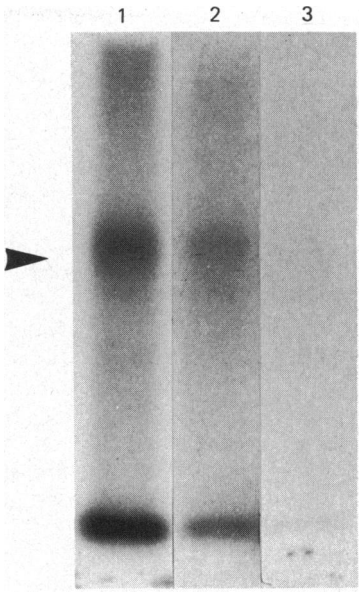

Fig. 2. Immunoprecipitation of ^{125}I-labelled βAR with antibody βAR (226–239)3–1

Purified ^{125}I-labelled βAR (5000 c.p.m.) (shown in lane 1) was incubated with: lane 2, 25 μl of tissue culture supernatant from monoclonal antibody-producing cells; or lane 3, 25 μl of HT media containing 1 μg of mouse immunoglobulin/ml. After 2 h at 23 °C, antibodies were precipitated with 0.1 ml of Protein A–Sepharose, the pellets were washed three times, and the proteins in samples were separated by SDS/polyacrylamide-gel electrophoresis. The arrow at the left indicates the position of pure ^{125}I-βAR. The results shown are representative of four similar experiments.

Effects of isoproterenol on βAR distribution on DDT-1 cells

Exposure of the cells to the β-adrenergic agonist isoproterenol caused striking changes in the immunofluorescence patterns seen with antibody βAR(226–239)3–1, as shown in Fig. 4. Incubation of the cells with isoproterenol for short times (1–3 min) resulted in an increase in the intensity of specific cellular immunofluorescence. This increased fluorescence was a transient phenomenon, with the fluorescence returning to control levels after approx. 5 min of incubation with the agonist. Treatment of the cells with the antagonist alprenolol also caused a slight transient increase in fluorescence, which was noticeably less intense than that observed with the agonist isoproterenol (Fig. 4). After the first 3–5 min, continued exposure of the cells to isoproterenol resulted in a decrease in the intensity of

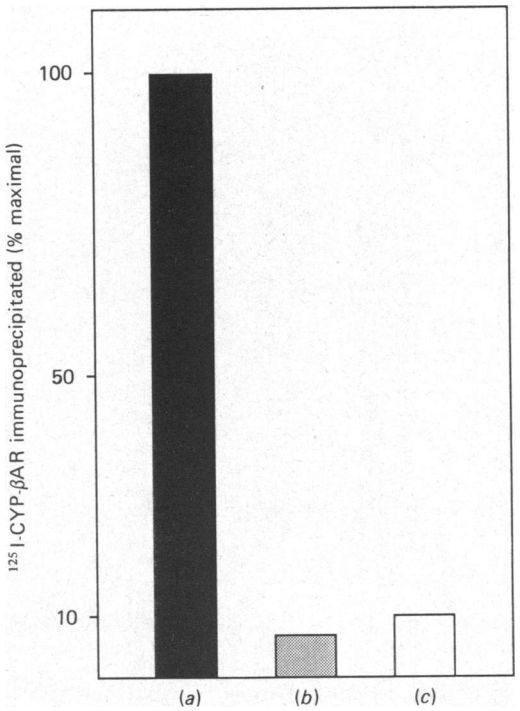

Fig. 3. Blockade of specific immunoprecipitation of ^{125}I-CYP-labelled βAR

^{125}I-CYP-βAR (2000 c.p.m.) was incubated with: (*a*) 50 μl of purified antibody βAR(226–239)3–1; (*b*) 50 μl of antibody βAR(226–239)3–1 previously incubated with synthetic peptide (226–239) (1 mg/ml) for 12 h at 4 °C; or (*c*) 50 μl of negative control SP2/0 antibody. After 2 h at 23 °C, precipitation of the antibody was performed at 4 °C with 50 % saturated $(NH_4)_2SO_4$ for 30 min as described in the legend to Fig. 1. Radioactivity incorporated into the immune precipitate was detected using a gamma counter. Maximal immunoprecipitation by antibody βAR(226–239)3–1 was 800 c.p.m.

observed immunofluorescence, with the immunoreactive receptor dropping to barely detectable levels after 30 min with isoproterenol. Closer inspection of the receptor-specific immunofluorescence during the course of this down-regulation revealed no apparent change in the pattern of receptor distribution on the cell surface. The receptors appeared to be lost from both the clusters and the non-clustered regions of the cell surface at equal rates, with no intermediate capping or patching of the immunoreactive protein observed during this process. In contrast with these results, cells incubated with alprenolol showed no decrease in specific immunofluorescence, demonstrating the agonist specificity of this receptor down-regulation. The specificity of the observed changes in βAR-specific immunofluorescence is further

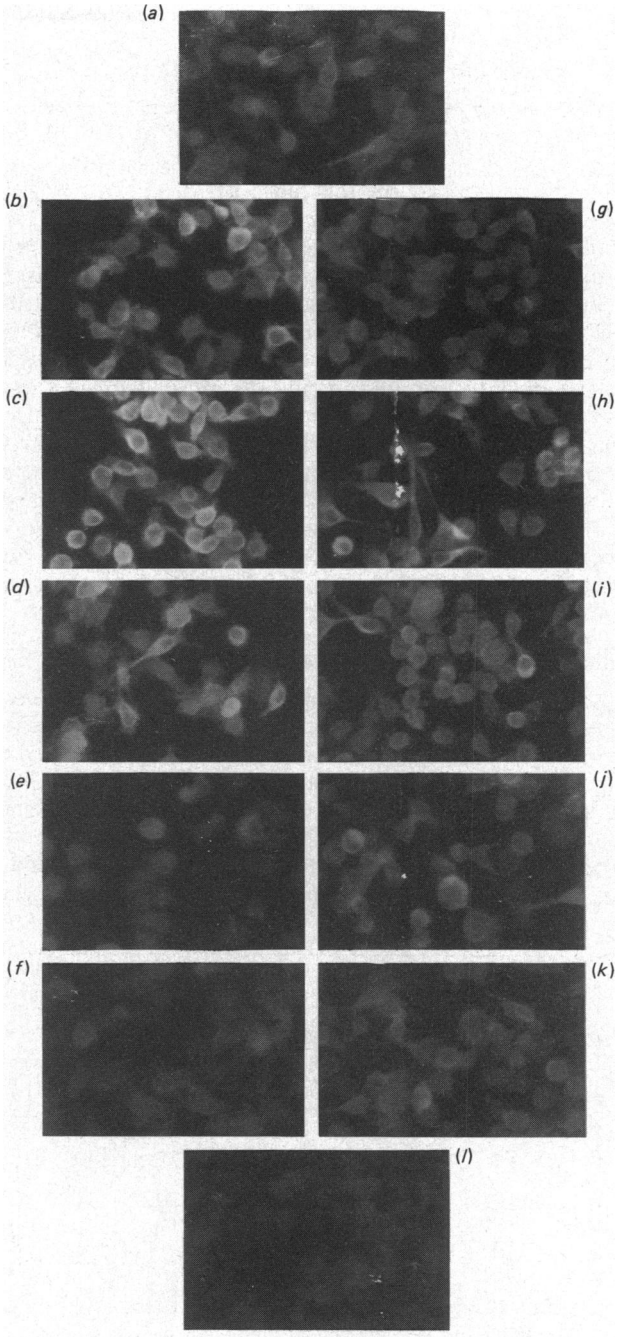

Fig. 4. Time course of down-regulation by isoproterenol, as visualized with immunofluorescence

Cell monolayers were treated with 10 μM-isoproterenol in DMEM for the times indicated below, then fixed, permeabilized, and incubated with 200 μl of 0.1 mg of antibody βAR(226–239)3–1/ml [except in the case of Panel (l), where the antibody used was control mouse IgM], washed, and incubated with a 1:50 dilution of FITC-conjugated goat anti-(mouse IgG) as described in the Experimental section. Panel (*a*) Cell monolayer before exposure to isoproterenol. Panels (*b–f*) Cell monolayers were exposed to isoproterenol for 1, 3, 5, 10 and 30 min respectively, washed quickly with PBS, then fixed, permeabilized, and incubated with antibody βAR(226–239)3–1 as described in the text. Panels (*g–k*) Cell monolayers were exposed to alprenolol for 1, 3, 5, 10, and 30 min respectively, before fixation, permeabilization, and incubation with βAR(226–239)3–1. Panel (*l*) Cell monolayer treated with control IgM, with no exposure to isoproterenol. These results were indistinguishable from those in which antibody βAR(226–239)3–1 was first absorbed with the synthetic peptide (not shown). All photographs were taken under the same conditions with a constant exposure time of 15 s. The results shown are from a single time course of ligand treatment; the results are representative of five separate experiments.

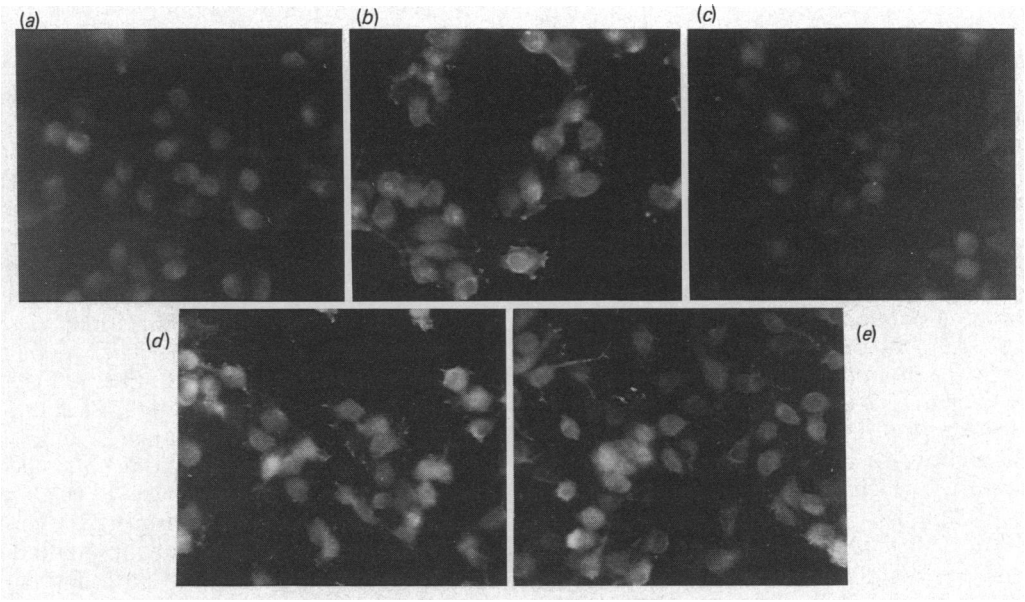

Fig. 5. Agonist-specificity of the attenuation of immunofluorescence

DDT-1 cell monolayers were treated with various reagents in DMEM as indicated below for 1 h at 37 °C, then fixed, permeabilized, and treated with antibody as described in the Experimental section. Panel (*a*) Untreated cells incubated with 200 μl of 0.1 mg of control antibodies/ml. Panel (*b*) Untreated cells incubated with 200 μl of 0.1 mg of βAR(226–239)3–1/ml. The specific immunofluorescence was completely abolished by prior absorption of the antibody with the synthetic peptide antigen (results not shown). Panels (*c–e*) Cells were treated with 10 μM-isoproterenol, 10 μM-alprenolol, or 0.1 μM-prazocin, respectively, for 1 h in DMEM at 37 °C before fixation and incubation with 200 μl of 0.1 mg of βAR(226–239)3–1/ml. All cells were photographed under identical conditions with a constant 15 s exposure. Results shown are from a single experiment, representative of at least two separate experiments.

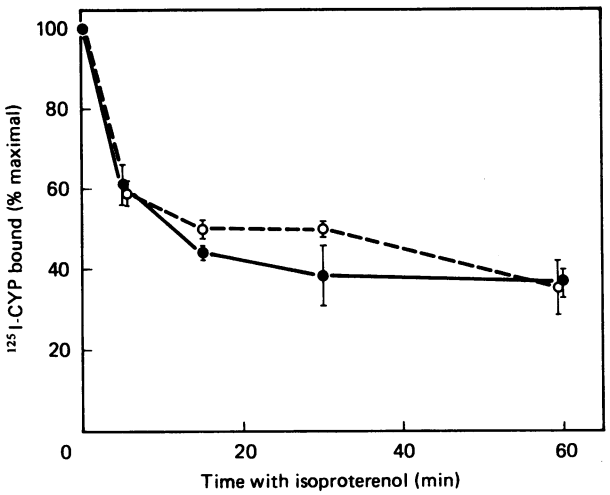

Fig. 6. Down-regulation of βAR on DDT-1 cells, as assessed by ligand binding

Experimental procedures are detailed in the Experimental section. Cell monolayers were incubated with isoproterenol at 37 °C for the times indicated. Cells were then washed and the density of βAR measured either by direct binding of ^{125}I-CYP (60 pM) to the cell monolayer for 1 h at 4 °C (○), or by the binding of ^{125}I-CYP (250 pM) to a membrane preparation from the cells (●). Non-specific binding was assessed in the presence of 10 μM-alprenolol. Total binding of ^{125}I-CYP to the intact cell monolayers was 3 fmol/1 cm well, with approx. 30% non-specific binding. Total binding of ^{125}I-CYP to the membrane preparation was 80 fmol/mg of protein with approx. 8% non-specific binding. Results shown are means ± S.D. from two separate experiments, with each determination done in triplicate.

demonstrated in Fig. 5, in which the effects on the immunofluorescence of a 1 h incubation of the cells with various adrenergic ligands are compared. The loss of immunoreactive receptors from the cell was apparent after incubation of the cells with isoproterenol, but not with alprenolol or the α_1-antagonist prazocin.

The changes in fluorescence were correlated with alterations in ligand binding to the cellular βAR, to facilitate comparison of these results with those obtained in previous studies. Intact DDT-1 cells were treated with isoproterenol and their membrane-associated βAR were measured by the binding of ^{125}I-CYP to both the surface of intact cells and to a membrane fraction prepared from the cells. As shown in Fig. 6, exposure of DDT-1 cells to the agonist isoproterenol resulted in a down-regulation of ^{125}I-CYP binding. The binding was lost from the cell surface and the membrane preparation at approximately the same rate, suggesting that the receptors lost from the cell did not accumulate intracellularly. The onset of this loss of binding sites was immediate upon addition of the agonist, with a half-maximal decrease occurring after approx. 10 min of exposure of the cells to isoproterenol. A comparison of Figs. 4 and 6 reveals that the loss of ligand binding from the cell paralleled the loss of specific immunofluorescence observed with antibody βAR(226–239)3–1 during the time range of 5–60 min of treatment with isoproterenol. However, the transient increase in immunofluorescence observed during the first 3 min of incubation with the agonist was not reflected in a corresponding enhancement of ^{125}I-CYP binding to the cell, and, in fact, occurred during a period in which the ligand binding to the plasma membrane decreased by approx. 30%.

DISCUSSION

Agonist-mediated desensitization of the βAR has important physiological consequences and has been extensively studied in many cell systems. The desensitization of receptor activity appears to be mediated in part by the loss of βAR binding sites from the cell surface. To date, this process has, by necessity, been characterized only by measuring the ligand binding activity of the βAR, leaving the possible formation of inactive receptors unexplored. In the present study, the sequence information obtained from cloning the gene for the mammalian βAR (Dixon *et al.*, 1986) was used to produce monoclonal antibodies which cross-react with the native βAR protein. We have used these monoclonal antibodies to localize the βAR protein in DDT-1 cells and to provide a measure of agonist-mediated down-regulation of the βAR which is independent of its ability to bind adrenergic ligands.

The localization of the βAR in hamster DDT-1 cells with monoclonal antibody βAR(226–239)3–1 revealed that the receptor resides primarily on the plasma membrane of the cell, with an additional component of specific immunofluorescence localized more diffusely, apparently within the cell. This additional fluorescence might be due to unprocessed receptor precursors within the cell. Experiments designed to investigate changes in βAR-specific immunofluorescence upon treatment with an agonist were performed on fixed, permeabilized DDT-1 cells. The fixation was necessary in these studies to avoid perturbations in the distribution of the receptor during the long antibody incubation which followed the shorter incubations with agonist. For other experiments utilizing untreated cells, fixation and permeabilization of the cells before antibody treatment markedly enhanced the level of specific immunofluorescence, suggesting a cytoplasmic exposure of this region of the βAR. However, further studies utilizing antibodies to a variety of receptor epitopes will be necessary to establish the extramembrane exposure of the receptor.

Treatment of the cells with agonist resulted in a rapid down-regulation of ligand binding to the βAR, which correlated with a decrease in specific immunofluorescence with the antibody to the βAR. The parallel decrease in immuofluorescence and binding activity suggests that the loss of binding observed in these cells upon βAR down-regulation corresponds with degradation or complete denaturation of the protein, rather than with simple inactivation of the binding site. We did not observe any agonist-mediated changes in the cellular distribution of the βAR before its loss from the cell, suggesting that, if sequestration of receptors is involved in down-regulation in these cells, it causes only subtle changes in the subcellular distribution of the protein. This observation was confirmed by ^{125}I-CYP binding, which revealed that no measurable level of internalized receptor accumulates at any time during the down-regulation process.

During the first few min of exposure of cells to isoproterenol, an increase in specific immunofluorescence was observed, with no corresponding increase in ligand-binding activity. This initial potentiation of receptor immunofluorescence was also observed to a much lesser extent with antagonist treatment. Thus, this transient increase in immunofluorescence may reflect some component of the ligand binding process, rather than any agonist-specific desensitization. One explanation for the enhancement of immunofluorescence would be a conformational change in the receptor occurring during the agonist binding and activation process. The region of the βAR recognized by the antibody lies in a long hydrophilic stretch of amino acids which is located between two hydrophobic regions (Dixon *et al.*, 1986). By analogy with the commonly accepted model of rhodopsin, with which the βAR shares structural and sequence homology, the peptide used as the antigen in the present study would correspond to a cytoplasmic loop between the fifth and sixth hydrophobic transmembrane helices of the protein (Dixon *et al.*, 1986; Findlay *et al.*, 1986). A mutant of the βAR in which a segment of this loop immediately *C*-terminal to this antigenic region was deleted resulted in a loss of agonist-stimulated adenylate cyclase activity with no change in agonist or antagonist binding to the receptor (Dixon *et al.*, 1987). This mutant receptor, expressed in mouse L cells, did not down-regulate in response to adrenergic agonists, as shown both by ligand binding and by immunofluorescence with antibody βAR(226–239)3–1 (Strader *et al.*, 1987). The results of the present study are consistent with a model in which this region of the βAR is involved in conformational changes during the processes of ligand binding and receptor activation.

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