Biochemical Characterization of Phosphorylated β-Adrenergic Receptors from Catecholamine-Desensitized Turkey Erythrocytes

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ABSTRACT: Isoproterenol-induced desensitization of turkey erythrocyte adenylate cyclase is accompanied (1) by a decrease in the mobility of β-adrenergic receptor proteins, specifically photoaffinity labeled with ^{125}I-(*p*-azidobenzyl)carazolol (^{125}I-PABC), on sodium dodecyl sulfate (SDS)–polyacrylamide gels and (2) by a 2–3-fold increase in phosphate incorporation into the β receptor [Stadel, J. M., Nambi, P., Shorr, R. G. L., Sawyer, D. F., Caron, M. G., & Lefkowitz, R. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3173]. Analysis of ^{32}P-labeled β receptors partially purified by affinity chromatography and subsequently hydrolyzed in 6 N HCl revealed that the β receptor from control erythrocytes contained only phosphoserine and that agonist-promoted phosphorylation of the receptor in desensitized cells occurred on serine residues. Comparison of limited-digest peptide maps of ^{125}I-PABC-labeled β receptors from control and desensitized erythrocytes reveals distinctly different sensitivities of the two β receptors to cleavage by chymotrypsin and *Staphylococcus aureus* protease. The altered mobility of the ^{125}I-PABC-labeled β receptor from desensitized erythrocytes was eliminated when 5 M urea was included in the SDS–polyacrylamide gels. Limited-digest peptide mapping of ^{32}P-labeled β receptors from control and desensitized cells with the protease papain identified a unique phosphorylated peptide in desensitized preparations. Our results are consistent with the hypothesis that the altered mobility of β-receptor proteins on SDS gels following desensitization is due to changes in conformation promoted by prolonged exposure to agonists.

The specific binding interactions between β-adrenergic receptors and agonist agents promote the rapid activation of the enzyme adenylate cyclase. Prolonged occupancy of these receptors by agonists, however, results in an attenuated responsiveness of the enzyme to a fresh challenge by these compounds. This regulatory process is termed desensitization or refractoriness and has been studied in a wide variety of cell types (Gavin et al., 1974; Mukherjee et al., 1975; Lefkowitz et al., 1980; Su et al., 1980; Harden, 1983). The investigations of adenylate cyclase coupled β-adrenergic receptor systems indicate the existence of multiple mechanisms of desensitization (Lefkowitz et al., 1980; Su et al., 1980; Harden, 1983). These include the "uncoupling" of receptors from the adenylate cyclase complex and the "down regulation" of receptors or the loss of assayable receptors from the cell surface. However, the precise molecular events that form the basis of desensitization remain to be elucidated.

The β-adrenergic receptor–adenylate cyclase complex of the turkey erythrocyte provides a useful model system in which to study both agonist-promoted activation of the cyclase enzyme and agonist-induced desensitization. Prolonged incubation of these erythrocytes with catecholamines results in an uncoupling of the β receptor from the cyclase, and this process appears to be mediated, in part, by cyclic AMP (Stadel et al., 1981; Simpson & Pfeuffer, 1980). Catecholamine-induced desensitization of adenylate cyclase in turkey erythrocytes promotes a stable modification of the β receptors. This can be assessed by comparing the electrophoretic mobility in SDS[1]–polyacrylamide gels of β-receptor proteins from control and desensitized cells (Stadel et al., 1982, 1983). The β-receptor proteins were specifically identified by prior photoaffinity labeling with ^{125}I-(*p*-azidobenzyl)carazolol (^{125}I-PABC). The β-receptor proteins from desensitized cells displayed a decreased mobility on SDS–PAGE. The apparent molecular weights of the receptor proteins increased from ≃38 000 and ≃50 000 for the control to ≃42 000 and ≃52 000 following desensitization. Agonist also promotes an increased phosphorylation of β receptors in these erythrocytes and the phosphorylated β receptors demonstrate the same mobility on SDS–polyacrylamide gels as ^{125}I-PABC-labeled receptors from desensitized cells (Stadel et al., 1983; Sibley et al., 1984). It is possible that the phosphorylation state of the β receptor is responsible for the altered behavior of receptor proteins during SDS–PAGE and for the uncoupling of the β receptor from the adenylate cyclase complex. Thus, this study was undertaken to further characterize the modification(s) of β-adrenergic receptors that accompanies (accompany) catecholamine-induced desensitization of adenylate cyclase in intact turkey erythrocytes.

Experimental Procedures

Materials

^{125}I-CYP (2200 Ci/mmol), [^{32}P]orthophosphate, and ^{125}I-PABC (2200 Ci/mmol) were purchased from New England Nuclear Corp. Chymotrypsin and papain were purchased from Worthington Biochemical Corp. *Staphylococcus aureus* protease and HCl for protein hydrolysis were obtained from Pierce Chemical Co. Phosphoamino acid standards were purchased from Sigma Chemical Co.

Methods

Catecholamine-Induced Desensitization of Turkey Erythrocytes. Turkey erythrocytes were prepared and incubated in the presence or absence of 1.0 μM isoproterenol for 4 h at 37 °C as described (Hoffman et al., 1979). The cells were

[1] Abbreviations: SDS, sodium dodecyl sulfate; ^{125}I-CYP, ^{125}I-cyanopindolol; ^{125}I-PABC, ^{125}I-(*p*-azidobenzyl)carazolol; PAGE, polyacrylamide gel electrophoresis; *S. aureus*, *Staphylococcus aureus*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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then washed 3 times to remove the agonist.

Turkey Erythrocyte Membranes. Partially purified turkey erythrocyte membranes were prepared as described previously (Stadel et al., 1980). Membranes were resuspended (≃5 mg/mL) in buffer containing 250 mM sucrose, 75 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, 1.5 mM EDTA, and 0.5 mM dithiothreitol and stored under liquid N_2. Crude turkey membranes were used in adenylate cyclase assays (Hoffman et al., 1979) to determine the extent of catecholamine-induced desensitization of the enzyme.

Photoaffinity Labeling of Turkey Erythrocyte β-Adrenergic Receptors with ^{125}I-PABC. β-Adrenergic receptors in membranes from control and desensitized turkey erythrocytes were covalently labeled with ^{125}I-PABC as described (Stadel et al., 1982).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) as described (Stadel et al., 1982).

^{32}P Labeling of Turkey Erythrocyte β-Adrenergic Receptors. Five milliliters of washed turkey erythrocytes was resuspended to 20% hematocrit in buffer (157.5 mM NaCl, 2.5 mM KCl, 11.1 mM glucose, 10 mM Hepes, pH 7.4) as described (Stadel et al., 1983; Alper et al., 1980a,1980b). The cells were incubated with [^{32}P]orthophosphate (0.5 mCi/mL) for 20 h at 42 °C. At the completion of these incubations, sodium metabisulfite (0.2 mM final concentration) was added to each flask of cells and isoproterenol (1 μM) was added to test flasks, and the incubation was continued for another 4 h. The cells were then washed 3 times to remove the agonist and free [^{32}P]orthophosphate. The cells were lysed by hypotonic shock in 10 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ and then washed 3 times in a buffer containing 75 mM Tris-HCl (pH 7.5) and 25 mM $MgCl_2$. The washed cell lysates were solubilized with 2% digitonin, and the soluble β receptors were partially purified by affinity chromatography over an alprenolol–Sepharose column (1.5 × 12 cm) (Stadel et al., 1983; Shorr et al., 1982). β-Receptor activity in solubilized preparations was quantified by the binding of the radiolabeled antagonist ligand ^{125}I-CYP. ^{32}P-Labeled β-receptor preparations were concentrated over Amicon YM-30 membranes prior to peptide mapping.

Peptide Mapping of ^{125}I-PABC- and ^{32}P-Labeled β Receptors from Control and Desensitized Turkey Erythrocytes. Limited-digest peptide maps were generated according to the method of Cleveland et al. (1977) with the following modifications. The ^{125}I-PABC- or ^{32}P-labeled β receptors were electrophoresed in the first dimension on a 10% polyacrylamide gel. For ^{125}I-PABC-labeled β receptors, equal amounts of solubilized membrane protein from control and desensitized preparations were loaded onto the gel (300–500 μg). For ^{32}P-labeled β receptors, equal amounts of receptor activity (1.5–1.9 pmol) were loaded onto the gel. The protein bands corresponding to the M_r ≃38 000 receptor protein from control erythrocyte membranes and the M_r ≃42 000 receptor protein from desensitized erythrocyte membranes were located in the slab gel according to their relative mobilities compared to covalently prestained protein standards (Bethesda Research Laboratories) run in the same gel lanes. That the appropriate band was excised from the gel was confirmed by autoradiography. The gel slices containing the ^{125}I-PABC or ^{32}P-labeled receptor proteins were then embedded into the second-dimension slab gel by polymerizing the stacking gel around them. The stacking gels contained the proteases at the concentrations given in the figures. The second-dimension gels were electrophoresed for approximately 1 h at 20-mA constant current/gel without cooling, allowing the samples to proceed through the stacking gel and permitting proteolysis to occur. As the samples entered the 12-cm 15 or 18% polyacrylamide separating gels, the current was lowered to 5 mA/gel and the electrophoresis continued for 16 h until the dye front migrated to the bottom of the gel. The gels were then dried and exposed to Kodak XAR-5 film in the presence of an intensifying screen for 1–3 weeks. When *S. aureus* protease was used, the second-dimension slab gel could be electrophoresed for an additional 3 h after the dye front reached the bottom of the gel without losing any of the peptides generated; this procedure enhanced the resolution of the peptide maps.

Urea/SDS–Polyacrylamide Gel Electrophoresis of ^{125}I-PABC-Labeled β Receptor from Control and Desensitized Turkey Erythrocytes. The ^{125}I-PABC-labeled β-adrenergic receptors were electrophoresed on a 12-cm, 10% polyacrylamide gel. Equal amounts of solubilized membrane protein from control and desensitized samples were loaded onto the gel (300–400 μg/lane). The M_r ≃38 000 (control) and M_r ≃42 000 (desensitized) receptor proteins were located in the slab gel according to their relative mobilities compared to the covalently prestained protein standards run in the same gel lanes. The appropriate gel slice was placed in an ISCO sample concentrator and electroeluted at 3 W for 4 h. The concentrated samples were diluted with SDS sample buffer (Stadel et al., 1982; Laemmli, 1970), and solid urea was added to make the final urea concentration 5 M. The ^{125}I-PABC-labeled β receptors from control and desensitized preparations were then loaded onto a second SDS–polyacrylamide gel containing 5 M urea in both the stacking and separating gels. The gel was run for 16 h at 7-mA constant current. The gels were then fixed in a solution of 30% methanol and 10% acetic acid for 1 h and subsequently washed in 10% acetic acid for 30 min to remove urea. The gel was then dried and exposed to Kodak XAR-5 film in the presence of intensifying screens for 1–2 weeks.

Identification of [^{32}P]Phosphoamino Acids after Acid Hydrolysis of the β-Adrenergic Receptors Isolated from Control and Desensitized Turkey Erythrocytes. ^{32}P-Labeled β-adrenergic receptors from control and desensitized turkey erythrocytes were partially purified by affinity chromatography over alprenolol–Sepharose. The affinity-purified ^{32}P-labeled β receptors were analyzed by SDS–polyacrylamide gel electrophoresis and appeared to be the only labeled proteins detected by autoradiography of the gel (data not shown), indicating these preparations were suitable for [^{32}P]phosphoamino acid analysis. Equal amounts of β receptors (0.5–0.9 pmol), as determined by ^{125}I-CYP binding, were precipitated by 30% trichloroacetic acid in the presence of 75 μg of bovine γ-globulin as carrier. The precipitates were washed twice with ice-cold ethanol and then once with an ice-cold mixture of ethanol and diethyl ether (1:1 v/v). The precipitate was then hydrolyzed in 6 N HCl for 2 h at 115 °C under N_2. The HCl was removed under reduced pressure, and the hydrolysates were dissolved in H_2O containing phosphoamino acid standards at 1 mg/mL. Protein hydrolysates were analyzed on cellulose thin-layer plates according to the procedure of Hunter and Sefton (1980). Electrophoresis was conducted at pH 1.9 for 60 min at 1.5 kV in glacial acetic acid/formic acid/H_2O, 78:25:897 (v/v). Ascending chromatography in the second dimension was performed with isobutyric acid/0.5 M NH_4OH, 5:3 (v/v). The marker phosphoamino acids were detected by ninhydrin staining. The cellulose plate was then exposed to Kodak XAR-5 film for 1–2 weeks in the presence of inten-

sifying screens. ^{32}P incorporation into phosphoamino acids was quantified by desensitometric scanning of autoradiograms on a Beckman DU-8 spectrophotometer.

RESULTS

Identification of [^{32}P]Phosphoamino Acids following Acid Hydrolysis of the β-Adrenergic Receptor. Prolonged exposure of turkey erythrocytes to isoproterenol leads to a 40–50% decrease in the catecholamine responsiveness of the adenylate cyclase (Hoffman et al., 1980; Stadel et al., 1981; Simpson & Pfeuffer, 1980). The desensitization-promoted change in electrophoretic mobility of the β receptor and the associated increase in phosphorylation of the receptor proteins have been shown to correlate with the attenuation of adenylate cyclase responsiveness to agonist (Stadel et al., 1983; Sibley et al., 1984).

To gain insight into the mechanism of agonist-promoted phosphorylation of the β receptor in intact turkey erythrocytes, we determined which amino acid residues were phosphorylated by the following experiment. Turkey erythrocytes were preincubated with [^{32}P]orthophosphate for 20 h at 42 °C (Stadel et al., 1983; Alper et al., 1980a,b). These cells were then further incubated for 4 h in the presence of 1.0 μM isoproterenol to desensitize the cells or in the absence of the catecholamine as the control. The β receptors from treated and control cells were then partially purified by affinity chromatography over alprenolol–Sepharose (Stadel et al., 1982; Shorr et al., 1982). Equal amounts of ^{32}P-labeled receptors from the control and desensitized preparations were precipitated with 30% trichloroacetic acid, dissolved in 6 N HCl, and hydrolyzed for 2 h at 115 °C. The hydrolysates were then analyzed on cellulose thin-layer plates by electrophoresis in the first dimension followed by ascending chromatography in the second dimension. Autoradiography indicated the only [^{32}P]phosphoamino acid detected in the control receptor sample was [^{32}P]phosphoserine (data not shown). Similarly, the analysis of the hydrolysate of the β receptor from desensitized cells also only revealed [^{32}P]phosphoserine (data not shown). The radioactivity in phosphoserine increased 2–3-fold following desensitization, which corresponds closely to the increased phosphorylation of the β-receptor proteins as determined in earlier studies (Stadel et al., 1983; Sibley et al., 1984).

Peptide Mapping of ^{125}I-PABC-Labeled β Receptors from Control and Desensitized Turkey Erythrocytes. Previous studies have shown that the decreased mobility of ^{125}I-PABC-labeled receptor proteins on SDS–PAGE closely correlates with the extent of desensitization of the adenylate cyclase to stimulation by agonist (Stadel et al., 1983; Sibley et al., 1984). One proposed mechanism for this observed mobility change is covalent modification, such as phosphorylation, which stabilizes a conformational change in the receptor resulting in a slower mobility of the receptor proteins during electrophoresis. To test this hypothesis, we have employed the Cleveland et al., (1977) method of partial proteolytic digestion to peptide map ^{125}I-PABC-labeled β receptors from control and desensitized erythrocytes. This study focuses on the smaller, $M_r \simeq 38\,000$–$42\,000$, β-receptor proteins because they were more extensively labeled with ^{125}I-PABC (Stadel et al., 1982; Lavin et al., 1982) and thus provided a very consistent pattern during the mapping. Figure 1 compares the sensitivity of the β receptor from control (C) erythrocytes and from desensitized (D) cells to proteolytic cleavage by increasing concentratons of chymotrypsin. Chymotrypsin preferentially catalyzes hydrolysis of peptide bonds involving aromatic amino acids. Two observations are notable. First, the receptor protein from control cells is more sensitive to the protease than is its

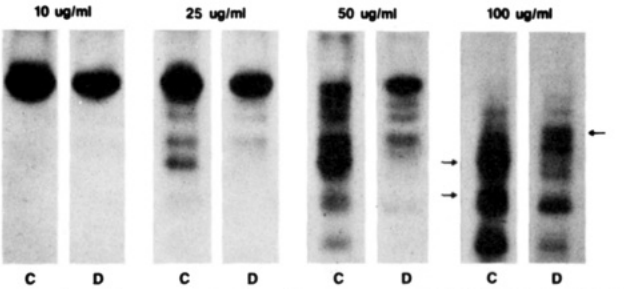

FIGURE 1: Chymotryptic digestion patterns of ^{125}I-PABC-labeled β-adrenergic receptor proteins from control (C) and desensitized (D) turkey erythrocytes. The β receptors in membranes from control and desensitized erythrocytes were labeled with ^{125}I-PABC (Stadel et al., 1982). The β-receptor proteins were then purified in a SDS–10% polyacrylamide gel. The receptor bands were excised and embedded into the stacking gel for protease cleavage as described under Experimental Procedures. The β-receptor proteins were digested by the indicated concentrations of chymotrypsin in the stacking gel and then electrophoresed on a 12-cm, 15% polyacrylamide gel. The gels were dried and then exposed to Kodak XAR-5 film in the presence of intensifying screens for 2–3 weeks. The arrows indicate unique peptides. The experiment was repeated 2 times with similar results.

counterpart from the desensitized preparation. This is particularly apparent when 50 μg/mL chymotrypsin is used. Second, the protein patterns generated by chymotryptic cleavage of the ^{125}I-PABC-labeled control and desensitized receptors show distinct differences (see arrows in Figure 1). These differences in the peptide maps are primarily qualitative, although quantitative differences are also observed. These results are consistent with the notion that a conformational change in the β-adrenergic receptor accompanies catecholamine-induced desensitization in turkey erythrocytes. These peptide mapping data were further investigated by employing a second protease, *Staphylococcus aureus* protease. This enzyme cleaves proteins specifically at aspartic and glutamic acid residues. As shown in Figure 2, the β receptor from control erythrocytes was again more susceptible to cleavage by this protease than was the desensitized receptor. Although fewer peptides were generated with *S. aureus* protease rather than with chymotrypsin and a considerable homology exists between the peptide maps of the control and desensitized preparation, a distinct peptide was generated from the desensitized receptor that is not apparent in the peptide map of the control receptor (see Figure 2, arrow).

Urea/SDS–Polyacrylamide Gel Electrophoresis of ^{125}I-PABC-Labeled β Receptors from Control and Desensitized Turkey Erythrocytes. Although the peptide mapping data are consistent with putative conformational differences between β receptors from control and desensitized erythrocytes, other interpretations of the results are possible. To further investigate the possible relationship between the altered mobility on SDS–PAGE of ^{125}I-PABC-labeled β receptors from desensitized erythrocytes and a conformational change in the β receptor, the receptor proteins were denatured in the presence of SDS and urea. In these experiments, the ^{125}I-PABC-labeled β receptors from both control and isoproterenol-desensitized erythrocytes were electroeluted from a 10% SDS–polyacrylamide gel. Solid urea was added to the electroeluted sample to a final concentration of 5 M. These samples were subsequently electrophoresed on an SDS–PAGE system containing 5 M urea. As seen in Figure 3A, electrophoresis in the presence of 5 M urea virtually eliminated the mobility differences between the ^{125}I-PABC-labeled receptor proteins from control and desensitized preparations. Both receptor proteins demonstrate an apparent M_r of 38 000, which is identical with that for ^{125}I-PABC-labeled β-receptor protein from control cells

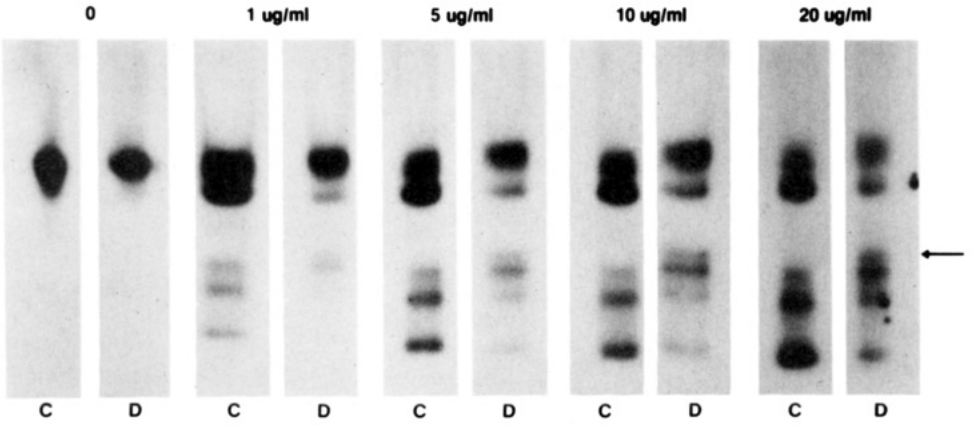

FIGURE 2: *S. aureus* proteolytic digestion patterns of ^{125}I-PABC-labeled β-adrenergic receptor proteins from control (C) and desensitized (D) turkey erythrocytes. The β receptors in membranes from control and desensitized erythrocytes were labeled with ^{125}I-PABC (Stadel et al., 1982). The proteolytic digestion using *S. aureus* protease at the concentrations indicated and subsequent peptide mapping were carried out as described under Experimental Procedures and the legend to Figure 1. The arrow indicates a unique peptide in the desensitized preparation. The experiment was repeated 3 times with comparable results.

on SDS–PAGE in the absence of urea. The denaturing effect of the urea on the β receptor from desensitized erythrocytes is reversible. This is shown in Figure 3B. Samples of ^{125}I-PABC-labeled β receptor supplemented with urea to 5 M show the normal, slower mobility ($M_r \simeq 42\,000$) when run on an SDS–PAGE system that does not contain no urea. These data strongly suggest that the altered mobility of the β-receptor proteins following desensitization is due to changes in conformation.

Peptide Mapping of ^{32}P-Labeled β Receptors from Control and Desensitized Turkey Erythrocytes. The protease papain, which nonspecifically catalyzes the hydrolysis of peptide bonds, generated identical peptide patterns from ^{125}I-PABC-labeled β-receptor proteins of control and desensitized cells (Figure 4, lanes 1 and 2). This result is in contrast to the observations with the site-specific proteases chymotrypsin and *Staphylococcus aureus* protease (see Figures 1 and 2). On the same gel, peptides derived from ^{32}P-labeled β receptors of control and desensitized erythrocytes were compared. The bulk of the ^{32}P-labeled peptides from the β receptor of control and desensitized cells run as broad bands centered at $M_r \simeq 1800$. Increased phosphorylation of this band was observed in the desensitized preparations. A unique phosphorylated peptide ($M_r \simeq 2800$) appeared to be generated from the β receptor of desensitized erythrocytes (Figure 4, lane 4, arrow), suggesting a topographically distinct phosphorylation site.

Discussion

Reversible phosphorylation is an important mechanism for the regulation of metabolic activity. The modulatory effects of this type of covalent modification are best illustrated in studies of the enzymes that control glycogen metabolism (Krebs & Beavo, 1979). Recent reports indicate that hormones such as epidermal growth factor and insulin promote the phosphorylation of their cell surface receptors (Cohen et al., 1980; Kasuga et al., 1982). The site of hormone-induced phosphorylation is tyrosine for both of these receptors, and the mechanisms of modification appear to be autocatalytic in each case (Cohen et al., 1980, 1982; Kasuga et al., 1983). The functional significance of hormone-promoted phosphorylation of either the insulin or the epidermal growth factor receptor remains to be elucidated. In contrast, we find that the β-adrenergic receptor of turkey erythrocytes is endogenously phosphorylated on serine and that the agonist isoproterenol promotes additional phosphate incorporation into serine residues only. The purified β receptor from this tissue does not appear to autophosphorylate.[2] The time course over which agonist promotes both phosphorylation of the β receptor (Sibley et al., 1984), as well as an alteration in electrophoretic mobility of β-receptor proteins on SDS–PAGE (Stadel et al., 1983), correlates closely with the desensitization of agonist–stimulated adenylate cyclase activity. These data suggest a functional link between agonist–promoted phosphorylation on serine residues of the β-adrenergic receptor and the desensitization process.

The first direct evidence that a modification of the β receptor accompanies desensitization of turkey erythrocyte adenylate cyclase stems from the observation that photoaffinity-labeled receptor proteins from desensitized erythrocytes have an altered mobility on SDS–PAGE when compared to control receptor proteins (Stadel et al., 1982). This altered behavior of ^{125}I-PABC-labeled receptor proteins from desensitized cells can still be observed subsequent to purification of the β receptor, indicating that a direct and stable modification of these receptors accompanies the desensitization process (Stadel et al., 1983; Strulovici et al., 1984). Since membrane proteins such as glycophorin have been shown to retain considerable secondary structure in SDS detergent solution (Tanford, 1975), it is possible that conformational changes in the β receptor following desensitization could explain the reduced mobility of the receptor proteins during SDS–PAGE. Examination of the mobility of the photoaffinity-labeled β receptors on urea/SDS–PAGE supports this notion. The data suggest that agonist-induced desensitizaton results in changes in receptor conformation that can subsequently be reversed in the presence of 5 M urea. Such agonist-promoted conformational changes might expose unique sites for proteolytic cleavage. Indeed, by comparing Cleveland-type peptide maps of the lower molecular weight β-receptor proteins from control and desensitized erythrocytes that were labeled at the ligand binding site with ^{125}I-PABC, we have demonstrated distinct differences in these receptors. Prolonged agonist occupancy of the β receptor promotes a modification of the receptor, possibly mediated by phosphorylation, making it more resistant to proteolysis by chymotrypsin or *S. aureus* protease and resulting in the generation of qualitatively distinct peptide patterns.

An alternative explanation for the peptide mapping data could be that β receptors from control and desensitized erythrocytes incorporate the photoaffinity label into different sites. Although this possibility cannot be excluded, this in-

[2] J. M. Stadel and R. G. L. Shorr, unpublished observation.

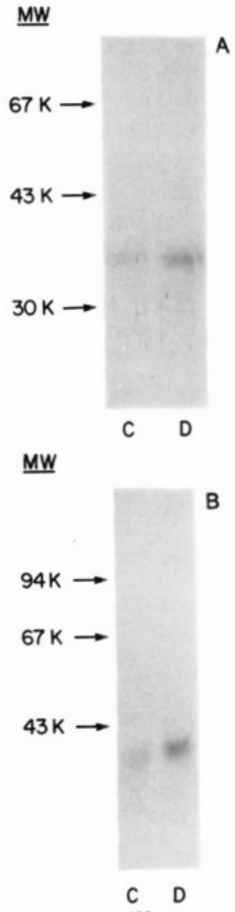

FIGURE 3: Urea/SDS–PAGE of ^{125}I-PABC-labeled β-adrenergic receptor proteins from control (C) and desensitized (D) turkey erythrocytes. Turkey erythrocytes were preincubated in the presence or absence of 1 μM isoproterenol for 3 h at 37 °C and plasma membranes were prepared from these cells as described under Experimental Procedures. The β receptors in the membranes from control and desensitized cells were covalently labeled with ^{125}I-PABC (Stadel et al., 1982). The β-receptor proteins were then separated in a SDS–10% polyacrylamide gel. The receptor bands (M_r ≃38 000, control; M_r ≃42 000, desensitized) were excised and the receptor proteins electroeluted from the gel slices. The eluted samples were diluted with SDS sample buffer, and solid urea was added to a final concentration of 5 M. The samples were then analyzed on SDS–PAGE containing 5 M urea (A) or in the absence of added urea (B) (12-cm, 10% polyacrylamide) as described under Experimental Procedures. The gels were then fixed, dried, and exposed to Kodak XAR-5 film in the presence of intensifying screens for 1–2 weeks. Molecular weight standards (Pharmacia) are phosphorylase (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), and soybean trypsin inhibitor (M_r 20 000). The experiment was repeated 3 times with similar results.

terpretation of the data is unlikely for the following reasons. First, the binding affinity of the turkey erythrocyte β receptor for antagonist and agonists (in the presence of guanine nucleotides, i.e., low-affinity state) is unaltered by the desensitization process (Hoffman et al., 1979; Stadel et al., 1981; Simpson & Pfeuffer, 1980). However, following desensitization, the β receptor is "uncoupled" from the stimulatory guanine nucleotide regulatory protein of the adenylate cyclase as evidenced by its impaired ability to form a high-affinity nucleotide-sensitive complex with agonist (Stadel et al., 1981; Simpson & Pfeuffer, 1980). Second, the efficiency of incorporation of the ^{125}I-PABC into the β-receptor proteins is apparently unaltered by desensitization. These data suggest that desensitization modifies a domain of the receptor involved in coupling to the other components of the adenylate cyclase complex without significantly perturbing the ligand binding domain of the receptor. The receptor modification associated

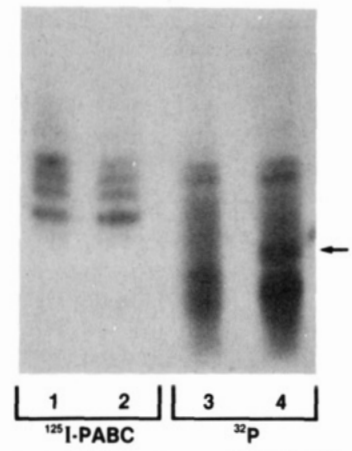

FIGURE 4: Papain digestion patterns of ^{125}I-PABC- and ^{32}P-labeled β-adrenergic receptor proteins from control and desensitized turkey erythrocytes. The ^{125}I-PABC- and ^{32}P-labeled β-adrenergic receptors were prepared from control and desensitized turkey erythrocytes as described under Experimental Procedures. The proteolytic digestion using papain (4 μg/mL) and subsequent peptide mapping was carried out as described under Experimental Procedures and the legend to Figure 1 with the exception that an 18% polyacrylamide gel was used to resolve the peptides. Equal amounts of membrane protein (400 μg/lane) for the ^{125}I-PABC-labeled β receptors from control (lane 1) and desensitized (lane 2) cells and equal amounts of ^{32}P-labeled β receptors (1.8 pmol/lane) from control (lane 3) and desensitized (lane 4) cells were used. The arrow indicates a unique phosphorylate peptide generated from the desensitized preparation. The molecular weight of the ^{32}P-labeled peptides was estimated by extrapolation of a calibration curve on the basis of the molecular weight standards (Bethesda Research Laboratories) ovalbumin (M_r 43 000), α-chymotrypsin (M_r 25 700), β-lactoglobulin (M_r 18 400), lysozyme (M_r 14 300), bovine tripsin inhibitor (M_r 6200), and insulin (M_r 3000) run on a companion gel without protease. The experiment was repeated 2 times with comparable results.

with desensitization may be very specific and localized. Thus, it is not surprising that the peptide maps of ^{125}I-PABC-labeled receptors show considerable homology between control and desensitized preparations while, at the same time, very distinct peptides are observed to be generated. The altered mobility of the β-receptor proteins on SDS gels, the reversal of these mobility changes by urea, and the differences in the peptide maps are all consistent with the notion of an agonist-promoted conformational change in the β receptor following desensitization.

Papain-promoted limited-digestion peptide mapping of the ^{32}P-labeled β receptor from control and desensitized erythrocytes provides additional information concerning the agonist-induced posttranslational modification of the β-adrenergic receptors in these cells. Maximum desensitization of turkey erythrocytes is accompanied by a 3-fold increase in covalently incorporated phosphate (Stadel et al., 1983; Sibley et al., 1984). Additional phosphate appears to be incorporated near the site of basal phosphorylation, i.e., the phosphorylation site observed in the control receptor preparation. Phosphate may also be incorporated into a serine residue distinct from this first site as suggested by the appearance of a unique phosphorylated peptide derived from the desensitized samples. Consequently, we postulate that agonist binding to the turkey erythrocyte β receptor promotes a conformational change in the receptor protein, thus exposing serine residues for phosphorylation. Such an agonist-promoted conformational change has been observed in the β-adrenergic receptor purified from hamster lung (Benovic et al., 1985). The covalent modification may stabilize conformational changes in the receptor initiated by the binding of the agonist. The spatial relationship of the phosphorylation sites on the β receptor is currently under

investigation in our laboratory by limit-digest peptide mapping techniques. Our observations provide evidence that conformational changes in the β receptor, which are associated with increased serine phosphorylation, may perturb the interactions of the receptor with the other components of the adenylate cyclase complex, thus attenuating the ability of β-adrenergic agonists to activate the effector enzyme.

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Registry No. L-Serine, 56-45-1; isoproterenol, 7683-59-2.

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