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Catecholamine-Induced Desensitization of Adenylate Cyclase Coupled β-Adrenergic Receptors in Turkey Erythrocytes: Evidence for a Two-Step Mechanism

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Received February 3, 1987; Revised Manuscript Received May 11, 1987

ABSTRACT: Preincubation of turkey erythrocytes with isoproterenol is associated with (1) 50–60% attenuation of agonist-stimulated adenylate cyclase activity, (2) altered mobility of the β-adrenergic receptor on sodium dodecyl sulfate–polyacrylamide gels, and (3) increased phosphorylation of the β-adrenergic receptor. Using a low-cross-linked polyacrylamide gel, the β-adrenergic receptor protein from isoproterenol-desensitized cells, labeled with ^{32}P or with the photoaffinity label ^{125}I-(*p*-azidobenzyl)carazolol, can be resolved into a doublet ($M_r \simeq 37\,000$ and $M_r \simeq 41\,000$) as compared to a single $M_r \simeq 37\,000$ β-adrenergic receptor protein from control erythrocytes. The appearance of the doublet was dependent on the concentration of agonist used to desensitize the cells. Incubation of erythrocytes with dibutyryl-cAMP did not promote formation of the doublet but decreased agonist-stimulated adenylate cyclase activity 40–50%. Limited-digestion peptide maps of ^{32}P-labeled β-adrenergic receptors using papain revealed a unique phosphopeptide in the larger molecular weight band ($M_r \simeq 41\,000$) of the doublet from the agonist-desensitized preparation that was absent in the peptide maps of the smaller band ($M_r \simeq 37\,000$), as well as control or dibutyryl-cAMP-desensitized receptor. These data provide evidence that maximal agonist-induced desensitization of adenylate cyclase coupled β-adrenergic receptors in turkey erythrocytes occurs by a two-step mechanism.

The biological effects of catecholamines are initiated by binding to cell surface receptors. The physiological responses resulting from β-adrenergic stimulation are mediated by adenosine cyclic 3′,5′-phosphate (cAMP)[1] which is produced from substrate ATP by the membrane-bound enzyme adenylate cyclase (Robison et al., 1971; Lefkowitz et al., 1983). Prolonged occupancy of β-adrenergic receptors by agonist leads to desensitization, i.e., an attenuated responsiveness of the adenylate cyclase to a fresh challenge of agonist (Lefkowitz et al., 1983; Harden, 1983). Studies of the β-adrenergic receptor–adenylate cyclase complex have provided insights into the molecular mechanisms underlying this regulatory process.

The β-adrenergic receptor–adenylate cyclase complex in avian erythrocytes has served as a model system in which to investigate receptor–cyclase coupling and regulation. Fol-

[1] Abbreviations: ^{125}I-PABC, ^{125}I-(*p*-azidobenzyl)carazolol; ^{125}I-CYP, ^{125}I-cyanopindolol; A, acrylamide monomer; B, bis(acrylamide); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; dbcAMP, dibutyryl-cAMP; ISO, (−)-isoproterenol; cAMP, adenosine cyclic 3′,5′-phosphate.

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lowing preincubation of turkey erythrocytes with isoproterenol, agonist-stimulated adenylate cyclase activity was attenuated 50–60% (Hoffman et al., 1979; Simpson & Pfeuffer, 1980; Stadel et al., 1981). Analogues of cAMP could partially mimic agonist-induced desensitization in these cells (Simpson & Pfeuffer, 1980; Stadel et al., 1981). Exposure of avian erythrocytes to phorbol diesters also leads to an uncoupling of β-adrenergic receptors from adenylate cyclase (Sibley et al., 1984a; Kelleher et al., 1984).

Biochemical characterization of β-adrenergic receptors from agonist-desensitized turkey erythrocytes showed the receptor proteins, specifically labeled with the photoaffinity label ^{125}I-(*p*-azidobenzyl)carazolol (^{125}I-PABC), had an altered mobility on SDS–PAGE compared to receptor proteins from control erythrocytes (Stadel et al., 1982, 1983, 1986). The alteration in apparent molecular weight of the β-adrenergic receptor proteins ($M_r \simeq 38\,000$ for control vs. $M_r \simeq 42\,000$ for desensitized) correlated with the desensitization of the adenylate cyclase (Stadel et al., 1983).

Agonist-induced desensitization of turkey erythrocytes also promoted a 2–3-fold increase in the phosphate content of the β-adrenergic receptors (Stadel et al., 1983; Sibley et al., 1984b). Limited-digestion peptide mapping of ^{32}P-labeled β-adrenergic receptors from desensitized cells revealed a unique [^{32}P]phosphopeptide ($M_r \simeq 2800$) which was absent in the control (Stadel et al., 1986). The desensitized ^{32}P-phosphorylated β-adrenergic receptor showed the same decreased mobility on SDS–PAGE as that observed following ^{125}I-PABC labeling of the receptor from desensitized erythrocytes (Stadel et al., 1983, 1986; Sibley et al., 1984b). The alteration in mobility of the β-adrenergic receptor on SDS–PAGE following desensitization appears to reflect conformational changes in the receptor protein that may be stabilized by phosphorylation (Benovic et al., 1985; Stadel et al., 1986).

In previous studies, it was noted that the ^{125}I-PABC-labeled $M_r \simeq 42\,000$ β-adrenergic receptor protein from desensitized erythrocytes could occasionally be resolved into a doublet by SDS–PAGE (Stadel et al., 1982). In the present study, we have further investigated this preliminary observation, and the results are discussed in terms of mechanistic implications for the process of desensitization.

Materials and Methods

Materials

^{125}I-PABC (2200 Ci/mmol), [^{32}P]orthophosphate, and ^{125}I-CYP (2200 Ci/mmol) were purchased from New England Nuclear Corp. Papain was purchased from Worthington Biochemical Corp. Dibutyryl-cAMP was purchased from Sigma. Whole turkey blood was obtained from Featherdown Farms, Raleigh, NC. Digitonin was purchased from Gallard Schlesinger.

Methods

Desensitization of Turkey Erythrocytes. Turkey erythrocytes were prepared and incubated with the indicated concentrations of (−)-isoproterenol or dbcAMP for 3 h at 37 °C as described (Hoffman et al., 1979; Stadel et al., 1981). The cells were washed 3 times to remove the desensitizing agents. Membranes for adenylate cyclase assays and for photoaffinity labeling experiments were prepared as described (Stadel et al., 1982).

Adenylate Cyclase Assays. Adenylate cyclase assays were performed as described previously (Hoffman et al., 1979) using 50-μL incubation volumes and 0.12 mM ATP as substrate. Incubations were for 10 min at 37 °C. Agonist-stimulated adenylate cyclase activities were measured in the presence of 10^{-4} M (−)-isoproterenol. Protein was determined by the method of Bradford (1976).

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

^{32}P Labeling of Turkey Erythrocyte β-Adrenergic Receptors. Turkey erythrocytes were preincubated with [^{32}P]-orthophosphate (0.5 mCi/mL) for 18 h at 37 °C (Stadel et al., 1983, 1986; Sibley et al., 1984b). The cells were then exposed to desensitizing agents for an additional 3 h. The preincubation period did not affect the extent of desensitization of adenylate cyclase or the patterns of ^{125}I-PABC-labeled β-adrenergic receptor proteins analyzed by SDS–PAGE. The erythrocytes were then washed and lysed as previously described (Stadel et al., 1983). The washed cell lysates were then solubilized with 2% digitonin, and the soluble β-adrenergic receptors were partially purified by affinity chromatography over an alprenolol–Sepharose affinity column (1.5 × 12 cm) (Stadel et al., 1983; Sibley et al., 1984b). β-Adrenergic receptor activity in the soluble preparations was quantified by the binding of the radiolabeled antagonist ^{125}I-CYP. Affinity chromatography yielded 1–2 pmol of partially purified β-adrenergic receptor. ^{32}P-labeled β-adrenergic receptor preparations were concentrated over Amicon YM-30 membranes and lyophilized prior to electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) with a ratio of acrylamide monomer (A) to bis(acrylamide) cross-linker (B) = 37.5 or according to Dreyfuss et al. (1984) with A/B = 111.7.

Peptide Mapping of ^{32}P-Labeled β-Adrenergic Receptors from Control and Desensitized Turkey Erythrocytes. Limited-digestion peptide maps were generated according to the method of Cleveland et al. (1977) with the following modifications (Stadel et al., 1986). The ^{32}P-labeled β-adrenergic receptors were isolated by electrophoresis in the first dimension on a 12.5% acrylamide gel (A/B = 111.7). Equal amounts of β-adrenergic receptor activity from control and desensitized cells were loaded onto the gel (1–2 pmol). The protein bands corresponding to $M_r \simeq 37\,000$ for the control and the $M_r \simeq 37\,000$ and 41 000 doublet for the agonist-desensitized preparations were located in the slab gel by their relative mobility compared to covalently prestained standards (Bethesda Research Laboratories). The gel slices containing the ^{32}P-labeled β-adrenergic receptor proteins were then embedded into the second-dimension slab gel by polymerizing the stacking gel around them. The gel slices were oriented in the stacking gel such that the bands of the doublet could be peptide mapped individually. The stacking gel contained the protease papain (4 μg/mL). The second-dimension gels (18% polyacrylamide; A/B = 37.5) were electrophoresed as described previously (Stadel et al., 1986). The ^{32}P-labeled β-adrenergic receptors appeared to be completely proteolyzed as all the radioactivity entered the separating gels. The gels were then dried and exposed to Kodak XAR-5 film in the presence of an intensifying screen for 1–3 weeks.

Results

Figure 1 shows that by using a ratio of acrylamide monomer (A) to bis(acrylamide) cross-linker (B) of 111.7 (Dreyfuss et al., 1984), i.e., a low-cross-linked polyacrylamide gel, the β-adrenergic receptor protein from desensitized turkey erythrocyte was consistently resolved into a doublet on SDS–

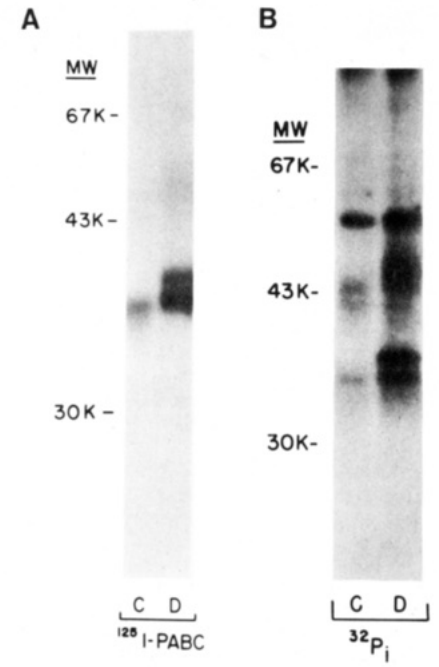

FIGURE 1: SDS–PAGE of ^{125}I-PABC and ^{32}P-labeled β-adrenergic receptors from control (C) and isoproterenol-desensitized (D) turkey erythrocytes. (A) Turkey erythrocytes were incubated with and without 10^{-6} M isoproterenol for 3 h at 37 °C. Membranes were prepared from the cells and labeled with ^{125}I-PABC as described under Methods. (B) Turkey erythrocytes were preincubated with [^{32}P]P_i for 20 h at 37 °C as described under Methods. The ^{32}P-labeled cells were incubated with and without 10^{-6} M isoproterenol for 3 h. The ^{32}P-labeled β-adrenergic receptors were solubilized with digitonin and partially purified by affinity chromatography over alprenolol–Sepharose. The ^{125}I-PABC- and ^{32}P-labeled β-adrenergic receptors were solubilized in SDS sample buffer and electrophoresed on a 12.5% polyacrylamide gel (A/B = 111.7). The gel was dried and exposed to Kodak XAR-5 film for 7 days in the presence of intensifying screens. The molecular weight standards (Pharmacia) are bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and carbonic anhydrase (M_r 30 000). The experiment was repeated 4 times with similar results.

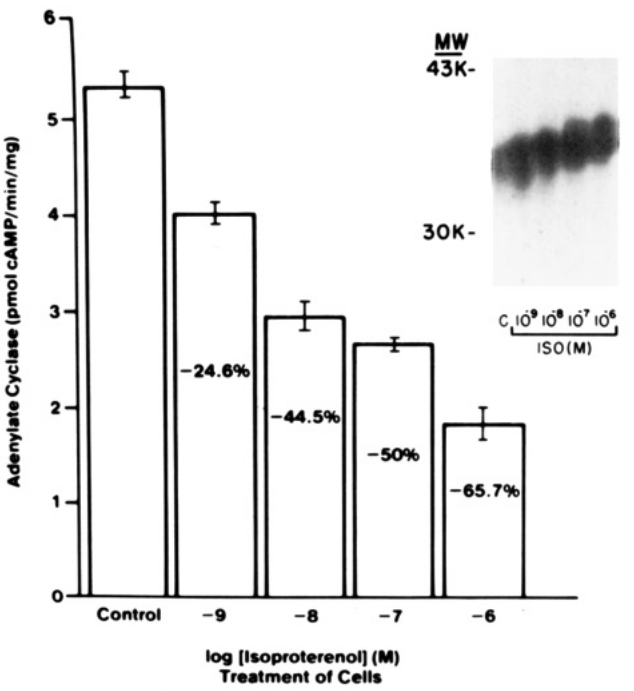

FIGURE 2: Dose relationship of isoproterenol preincubation of turkey erythrocytes on attenuation of agonist-stimulated adenylate cyclase activity and resolution of ^{125}I-PABC-labeled β-adrenergic receptors into a doublet on SDS–PAGE. Turkey erythrocytes were incubated with the indicated concentrations of isoproterenol for 3 h at 37 °C. The cells were washed and membranes prepared as described under Methods. Membranes were assayed for adenylate cyclase activity in the presence of 10^{-4} M isoproterenol as described under Methods. Values in the bars represent the percent desensitization. Inset: Membranes were labeled with ^{125}I-PABC as described under Methods and then solubilized in SDS sample buffer. Electrophoresis was carried out on a 32 cm, 12.5% polyacrylamide slab gel (A/B = 111.7). The gel was dried and exposed to Kodak XAR-5 film for 5 days in the presence of intensifying screens. Molecular weight standards are ovalbumin (M_r 43 000) and carbonic anhydrase (M_r 30 000). The experiment was repeated twice with comparable results.

PAGE ($M_r \simeq 37\,000$ and 41 000).[2] This is in comparison to the receptor protein from control erythrocytes which runs as a single band ($M_r \simeq 37\,000$). The doublet was only infrequently observed on SDS–PAGE using the formulation of Laemmli (1970; A/B = 37.5), even when the percentage polyacrylamide in the gels was varied between 10% and 15% (Stadel et al., 1982; data not shown). The ability of the low-cross-linked gel to resolve the doublet was independent of whether the β-adrenergic receptor protein was labeled with ^{125}I-PABC (Figure 1A) or ^{32}P (Figure 1B).

A comparison of adenylate cyclase activity and the mobility of ^{125}I-PABC-labeled β-adrenergic receptor proteins on SDS–PAGE as a function of the concentration of isoproterenol used to desensitize the erythrocytes is shown in Figure 2. Preincubation of turkey erythrocytes with increasing concentrations of isoproterenol (10^{-9}–10^{-6} M) led to a progressive loss of agonist-stimulated adenylate cyclase activity. We have previously shown, using a 12.5% polyacrylamide gel (A/B = 37.5), that a correlation exists between β-adrenergic receptor mobility on SDS–PAGE and adenylate cyclase desensitization (Stadel et al., 1983). In the previous studies, the ^{125}I-PABC-labeled β-adrenergic receptor proteins appeared as single bands. In contrast, by employing a 12.5% polyacrylamide gel (A/B = 111.7), the ^{125}I-PABC-labeled β-adrenergic receptor proteins from cells preincubated with 10^{-6} M isoproterenol are resolved into a doublet on SDS–PAGE. Similar results were obtained when ^{32}P-labeled β-adrenergic receptors were analyzed by SDS–PAGE (data not shown).

In a previous report (Stadel et al., 1986), we showed that limited-digestion peptide mapping with papain of ^{32}P-labeled β-adrenergic receptors from control and agonist-desensitized turkey erythrocytes revealed a unique [^{32}P]phosphopeptide of $M_r \simeq 2800$ in desensitized preparations which was not detected in controls. In addition, a [^{32}P]phosphopeptide of $M_r \simeq 1800$ was shown to be found in both preparations. We have applied these techniques (see Methods) in the present studies to characterize further the ^{32}P-labeled β-adrenergic receptor doublet in the desensitized preparations. For these experiments, the ^{32}P-labeled β-adrenergic receptors from control and agonist-desensitized erythrocytes were partially purified by

[2] Previous studies have reported the identification of two turkey erythrocyte β-adrenergic receptor proteins of M_r 38 000–40 000 and 45 000–50 000 (Stadel et al., 1982, 1983, 1986; Lavin et al., 1982; Sibley et al., 1984c). The relative molecular weights of the receptor proteins were determined by SDS–PAGE using the formulation of Laemmli (1970). Both β-adrenergic receptor proteins showed decreased mobility on SDS–polyacrylamide gels following desensitization of the turkey erythrocyte adenylate cyclase, M_r 38 000 vs. 42 000 and M_r 50 000 vs. 53 000 for control and desensitized forms, respectively (Stadel et al., 1982, 1983). However, we have found the extent of labeling of the M_r 50 000 β-adrenergic receptor protein with ^{125}I-PABC to be relatively low and therefore have confined the present study to the lower molecular weight receptor protein. Using the Dreyfuss et al. (1984) SDS–PAGE system, we now find that the β-adrenergic receptor proteins display slightly decreased relative molecular weights to those previously reported. This observation most likely relates to the difficulty in measuring molecular weights of membrane glycoproteins assessed by this technique (Tanford, 1976).

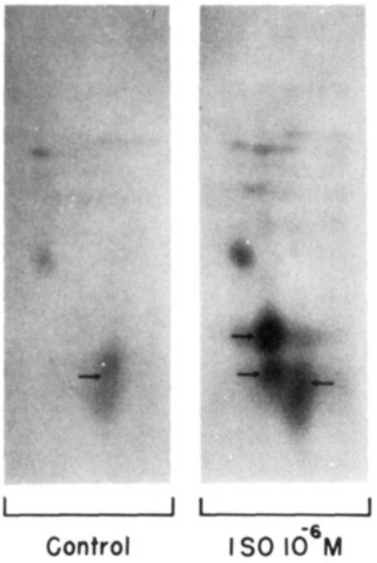

FIGURE 3: Papain digestion pattern of ^{32}P-labeled β-adrenergic receptor proteins from control and isoproterenol-desensitized turkey erythrocytes. Turkey erythrocytes were labeled with [^{32}P]P_i and subsequently incubated with or without 10^{-6} M isoproterenol (ISO) as described under Methods. Agonist-stimulated adenylate cyclase activities were as follows: control = 3.8 ± 0.1 pmol of cAMP min^{-1} mg^{-1} and desensitized = 1.9 ± 0.1 pmol of cAMP min^{-1} mg^{-1} (mean ± SEM). The ^{32}P-labeled β-adrenergic receptors were partially purified by affinity chromatography and subjected to peptide mapping using papain (4 μg/mL) as described under Methods. The gel slice from the desensitized samples is oriented so that the upper band (M_r ≃41 000) of the doublet is to the left (see Results). The arrows point to ^{32}P-labeled β-adrenergic receptor peptides. The experiment was repeated 3 times with similar results.

affinity chromatography over alprenolol–Sepharose followed by SDS–PAGE (A/B = 111.7). The appearance of ^{32}P-labeled β-adrenergic receptor proteins on SDS–PAGE from both the control and desensitized preparations required the specific elution of the affinity resin with β-adrenergic ligands. The ^{32}P-labeled β-adrenergic receptor bands were excised and embedded into a second polyacrylamide gel containing papain (4 μg/mg) in the stacking gel. The orientation of the embedded gel piece was such that the larger M_r ≃41 000 band of the doublet was to the left in Figure 3. The arrows in this figure point to [^{32}P]phosphopeptides derived from the ^{32}P-labeled β-adrenergic receptors. The unique phosphopeptide (M_r ≃2800) from the desensitized receptor preparation was associated only with the upper band of the doublet, whereas both β-adrenergic receptor bands from desensitized erythrocytes, as well as control receptor protein, show the M_r ≃1800 [^{32}P]phosphopeptide.

Comparison of the peptide maps of ^{32}P-labeled β-adrenergic receptors from erythrocytes desensitized by preincubation with 10^{-7} and 10^{-6} M isoproterenol is shown in Figure 4. The extents of desensitization as determined by agonist-stimulated adenylate cyclase activities were 41% for 10^{-7} M isoproterenol-desensitized and 56% for 10^{-6} M isoproterenol-desensitized preparations (control = 8.7 ± 0.8; 10^{-7} M isoproterenol desensitized = 5.1 ± 0.3; 10^{-6} M isoproterenol desensitized = 3.8 ± 0.4; picomoles of cAMP per minute per milligram, mean ± SEM). In all three preparations, an M_r ≃1800 [^{32}P]-phosphopeptide was detected. However, only under conditions of maximal agonist-induced desensitization following exposure to 10^{-6} M isoproterenol was the ^{32}P-labeled β-adrenergic receptor protein clearly resolved into a doublet and the M_r ≃2800 [^{32}P]phosphopeptide observed.

Cyclic AMP and its analogues have been shown to partially mimic isoproterenol in promoting desensitization of the adenylate cyclase in turkey erythrocytes (Simpson & Pfeuffer, 1980; Stadel et al., 1981; Sibley et al., 1984b). To determine if dibutyryl-cAMP (dbcAMP) and isoproterenol induce desensitization through the same mechanism, turkey erythrocytes were preincubated with these agents and the β-adrenergic receptors from these cells photoaffinity labeled with ^{125}I-PABC. Adenylate cyclase assays showed that agonist-stimulated enzyme activity was attenuated 65% for the isoproterenol-treated cells and 40% for the dbcAMP-treated cells (control = 11.2 ± 0.8; isoproterenol desensitized = 3.9 ± 0.2; dbcAMP desensitized = 6.7 ± 0.5; picomoles of cAMP per minute per milligram, mean ± SEM). As seen in Figure 5A following desensitization with isoproterenol (10^{-6} M), the β-adrenergic receptor protein was resolved into a doublet by SDS–PAGE (A/B = 111.7). In contrast, the ^{125}I-PABC-labeled β-adrenergic receptor protein from erythrocytes pretreated with the dbcAMP appeared as a single species on the same gel, similar to the control.

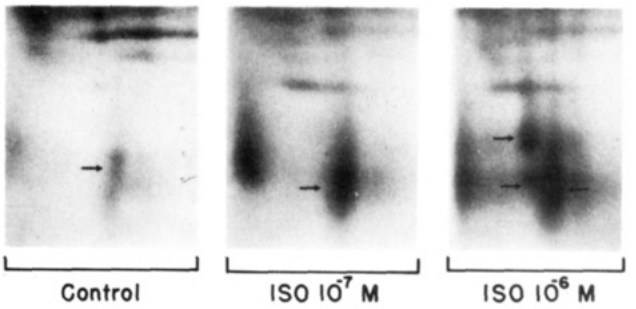

FIGURE 4: Papain digestion patterns of ^{32}P-labeled β-adrenergic receptor proteins from turkey erythrocytes preincubated without and with 10^{-7} or 10^{-6} M isoproterenol. Turkey erythrocytes were labeled with [^{32}P]P_i for 20 h at 37 °C as described under Methods. The cells were then incubated without (control) or with 10^{-7} or 10^{-6} M isoproterenol (ISO) for an additional 3 h. ^{32}P-Labeled β-adrenergic receptors were partially purified by affinity chromatography and solubilized in SDS sample buffer as described under Methods. The peptide mapping was performed as described under Methods with the orientation of the upper band (M_r ≃41 000) of the β-adrenergic receptor doublet toward the left. Arrows indicate phosphopeptides from the ^{32}P-labeled β-adrenergic receptor. The gel was dried and exposed to Kodak XAR-5 film for 10 days. The experiment was repeated twice with similar results.

Comparable results were obtained in experiments when [^{32}P]P_i was used to label the β-adrenergic receptors (Figure 5B). The ^{32}P-labeled β-adrenergic receptor protein from isoproterenol-desensitized cells ran as a doublet while the receptor protein from dbcAMP-desensitized erythrocytes displayed a single band by SDS–PAGE. Adenylate cyclase assays revealed that agonist-stimulated enzyme activity was attenuated 58% for the isoproterenol-desensitized cells and 41% for the dbcAMP-desensitized erythrocytes (control = 7.9 ± 0.9; isoproterenol desensitized = 3.3 ± 0.7; dbcAMP desensitized = 4.7 ± 0.4; picomoles of cAMP per minute per milligram, mean ± SEM). Limited-digestion peptide mapping of ^{32}P-labeled β-adrenergic receptors from dbcAMP-desensitized cells revealed only the M_r ≃1800 [^{32}P]phosphopeptide; the M_r ≃2800 [^{32}P]phosphopeptide was not detected (data not shown). These data provide additional evidence that agonist-induced desensitization of turkey erythrocytes promotes the phosphorylation of a unique site on the β-adrenergic receptor of the turkey erythrocyte.

Discussion

Previous work on agonist-induced desensitization of adenylate cyclase coupled β-adrenergic receptors in the turkey erythrocyte model system established a correlation between the attenuation of the adenylate cyclase and (1) changes in

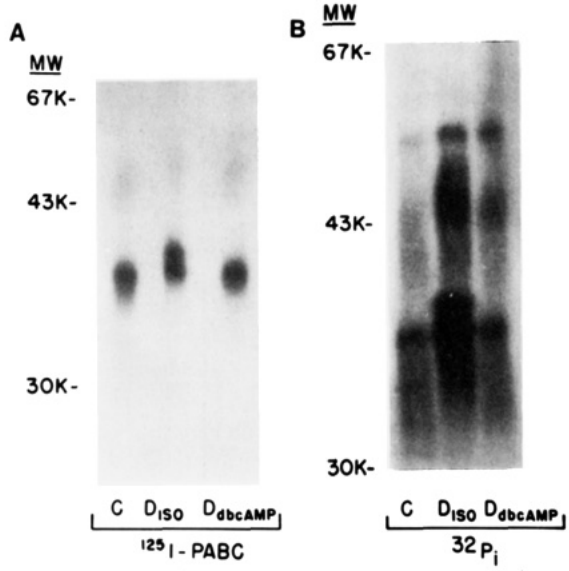

FIGURE 5: SDS–PAGE of ^{125}I-PABC-labeled and ^{32}P-labeled β-adrenergic receptor proteins from turkey erythrocytes preincubated with and without isoproterenol or dbcAMP. (A) Turkey erythrocytes were preincubated without (C) and with 10^{-6} M isoproterenol (D_{ISO}) or 5 mM dbcAMP (D_{dbcAMP}) for 3 h at 37 °C. Membranes were prepared from the cells and labeled with ^{125}I-PABC as described under Methods. (B) Turkey erythrocytes were labeled with [^{32}P]P_i for 20 h at 37 °C as described under Methods. The cells were then incubated without (C) and with 10^{-6} M isoproterenol (D_{ISO}) or 5 mM dbcAMP (D_{dbcAMP}) for an additional 3 h. ^{32}P-Labeled β-adrenergic receptors were partially purified by affinity chromatography as described under Methods. The ^{125}I-PABC-labeled and ^{32}P-labeled β-adrenergic receptor proteins were solubilized in SDS sample buffer and electrophoresed on 32 cm, 12.5% polyacrylamide slab gels (A/B = 111.7). The gels were dried and exposed to Kodak XAR-5 film for 5–7 days in the presence of intensifying screens. Molecular weight standards are described in the legend to Figure 1. The experiments were repeated twice with similar results.

β-adrenergic receptor mobility on SDS–PAGE (Stadel et al., 1982, 1983, 1986; Sibley et al., 1984b) and (2) phosphorylation of the receptor (Stadel et al., 1983, 1986; Sibley et al., 1984b). In the present study, through the use of a low-cross-linked SDS–PAGE system, we have resolved the β-adrenergic receptor protein from isoproterenol-desensitized erythrocytes into a doublet of M_r "oe37 000 and 41 000. Limited-digestion peptide mapping using papain revealed that the doublet represented two distinct populations of β-adrenergic receptors based on [^{32}P]phosphopeptide patterns. Both the $M_r \simeq 37\,000$ and the $M_r \simeq 41\,000$ bands gave rise to an $M_r \simeq 1800$ [^{32}P]phosphopeptide. In addition, a unique $M_r \simeq 2800$ [^{32}P]phosphopeptide was derived from the $M_r \simeq 41\,000$ band.

The formation of the receptor doublet and subsequent identification of a $M_r \simeq 2800$ phosphopeptide occurred only after agonist pretreatment of the cells and correlated with adenylate cyclase desensitization >50%. Preincubation of erythrocytes with dbcAMP resulted in a maximal desensitization of the adenylate cyclase that was quantitatively less than that observed when isoproterenol was used as the desensitizing agent (Simpson & Pfeuffer, 1980; Stadel et al., 1981, 1983; Sibley et al., 1984b). The cAMP analogue did not promote formation of the β-adrenergic receptor doublet nor the phosphorylation of the unique site as observed with agonist. Although we consistently observed the doublet following maximal desensitization by isoproterenol, the relative proportion of the two bands was variable. This may be due to a dynamic interaction between the β-adrenergic receptors in SDS solution. The observation is consistent with an earlier report in which mixing of SDS-solubilized ^{125}I-PABC-labeled β-adrenergic receptor preparations from control and desensitized turkey erythrocytes resulted in a single band of intermediate mobility on SDS–PAGE (A/B ≃37.5) instead of discrete bands corresponding to the individual components (Stadel et al., 1982). It has also been reported that β-adrenergic receptors irreversibly aggregate in SDS solution if samples are only gently heated (Lavin et al., 1982). The potential self-association of membrane proteins in SDS solution is not unique to β-adrenergic receptors. Pure glycophorin has been reported to exhibit multiple bands following analysis by SDS–PAGE, indicating dynamic interactions of protein monomers in SDS solution (Furthmayer & Marchesi, 1976; Martin & Garvin, 1973; Tuech & Morrison, 1974).

Our results suggest that agonist-induced desensitization of β-adrenergic receptors in turkey erythrocytes occurs by a two-step process. We propose that agonist-induced accumulation of intracellular cAMP activates cAMP-dependent protein kinase which phophorylates the β-adrenergic receptor, inhibiting its interaction with other components of adenylate cyclase. The site of induced phosphorylation is near to the site at which the control β-adrenergic receptor is phosphorylated as the control, 10^{-7} M isoproterenol-desensitized, and dbcAMP-desensitized β-adrenergic receptor preparations showed the same $M_r \simeq 1800$ phosphopeptide by peptide mapping. Maximal desensitization by agonist, however, causes the additional phosphorylation of a unique site on the receptor as revealed by peptide mapping. This mechanism is consistent with the observations that maximal desensitization results in an increased incorporation of 1–2 additional mol of phosphate/mol of receptor (Sibley et al., 1984b) and that cAMP analogues only partially mimic agonist-promoted desensitization and receptor phosphorylation. The resolution of two phosphorylated β-adrenergic receptor populations also provides an explanation for why adenylate cyclase activity is not completely inhibited following agonist-induced desensitization.

The molecular mechanism by which maximal agonist-induced desensitization occurs remains to be defined. We have previously shown that prolonged agonist binding to β-adrenergic receptors in turkey erythrocytes results in conformational changes in the receptor protein (Stadel et al., 1986). The altered conformation may expose new sites for phosphorylation. This has been shown for the agonist-dependent phosphorylation of purified/reconstituted hamster lung β-adrenergic receptor by cAMP-dependent protein kinase (Benovic et al., 1985). It is likely that another kinase, distinct from cAMP-dependent protein kinase, may be involved in the phosphorylation of the unique site ($M_r \simeq 2800$) seen by peptide mapping. Studies of desensitization in turkey erythrocyte lysates have shown that a specific inhibitor of cAMP-dependent protein kinase completely inhibited desensitization induced by cAMP but could only block by 50% the desensitization by isoproterenol (Nambi et al., 1985). Therefore, agonist-induced conformational changes in the β-adrenergic receptor may expose sites for phosphorylation by protein kinase C (Sibley et al., 1984a; Kelleher et al., 1984; Nambi et al., 1985), or turkey erythrocytes may possess a receptor-specific kinase as was recently identified in S49 lymphoma cells (Benovic et al., 1986). Our results from the investigation of agonist-induced desensitization of adenylate cyclase coupled β-adrenergic receptors in intact turkey erythrocytes are consistent with a two-step mechanism probably involving more than one protein kinase.

ACKNOWLEDGMENTS

We thank Dr. Frans Stassen for carefully reading the manuscript and C. Kelly for expert secretarial assistance.

Registry No. ISO, 51-31-0; adenylate cyclase, 9012-42-4.

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Occurrence of a Methylated Protein in Chloroplast Ribosomes†

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Received March 24, 1987

ABSTRACT: A ribosomal protein of spinach chloroplast, previously shown to be immunologically homologous to *Escherichia coli* ribosomal protein L2, was purified by using gel filtration, ion-exchange chromatography, and reverse-phase high-performance liquid chromatography separation. Analysis of the N-terminal amino acid sequence of the purified protein and of the fragments obtained by its cyanogen bromide cleavage shows that *N*-methylalanine is the N-terminal residue of this protein. Thus, methyl modification of proteins, whose occurrence in *E. coli* ribosomes is established, is present in chloroplast ribosomes as well. This and the additional significance of the amino acid sequence data reported in this paper are discussed.

The most common modification of both ribosomal protein (r-protein)[1] and rRNA of ribosomes is methylation (Wittmann, 1982; Fellner, 1974). *Escherichia coli* ribosomes contain six methylated proteins, five of them in the large subunit (L3, L11, L12, L16, and L33) and one (S11) in the small subunit (Wittmann-Liebold, 1980). Genetic studies have shown that methyl modifications in *E. coli* are catalyzed by specific methylases (Colson et al., 1979; Isono, 1980). However, as far as is known, the methyl (and other) modifications in themselves are not essential for the basic translational function of the bacterial ribosome (Isono, 1980). It would therefore be important to know, for evaluating their biochemical significance, whether r-protein modifications have been deleted out in ribosomes from organisms that have diverged from bacteria into alternative evolutionary pathways in the very distant past.

Chloroplast ribosomes display the prokaryotic ribosome motif in many of their structural and functional properties even though they exist in the eukaryotic cell and have many of their protein components encoded in the nuclear DNA (Bogorad et al., 1977; Ledoigt & Freyssinet, 1982; Subramanian, 1985). The origin of chloroplast evolution is placed between 500 ×

† This work was supported in part by a grant (Su 90/1-2) from the Deutsche Forschungsgemeinschaft. Dedicated to Dr. George Kalnitsky, The University of Iowa, on his 70th birthday.

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[1] Abbreviations: r-protein, ribosomal protein; rRNA, ribosomal RNA; NM-Ala and *N*-methylalanine, α-*N*-monomethylalanine; SDS, sodium dodecyl sulfate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene thiohydantoin; HPLC, high-performance liquid chromatography.

0006-2960/87/0426-5866$01.50/0