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PHOTOAFFINITY LABELING OF TURKEY ERYTHROCYTE BETA-ADRENERGIC RECEPTORS: DEGRADATION OF THE M_r = 49,000 PROTEIN EXPLAINS APPARENT HETEROGENEITY

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The potent photoaffinity probe [^{125}I]p-azidobenzylcarazolol was used to identify β-adrenergic receptors from turkey erythrocytes. Two peptides were specifically labeled with apparent M_r = 39,000 and 49,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The ratio of labeling of these peptides was found to be dependent on the method of tissue preparation. Thus, the M_r = 39,000/M_r = 49,000 ratio was 1:2 when labeling was done on intact cells, 1:1 for labeling of crude membranes, and 4:1 for purified membrane preparations. Moreover, incubation of intact cells or either membrane preparation at 37°C led to a diminution of the M_r = 49,000 labeled peptide which was associated with a stoichiometric increase in the M_r = 39,000 form of the receptor. These results suggest that the turkey erythrocyte β_1-adrenergic receptor is a protein of M_r = 49,000 and that the commonly observed M_r = 39,000 peptide is derived from this protein.

A recently developed approach for delineating the structure of β-adrenergic receptors is the covalent incorporation of photoaffinity probes derived from high affinity, radiolabeled β-adrenergic antagonists (1-11). Photoaffinity labeling of mammalian β_1- and β_2-adrenergic receptors in both particulate and purified preparations indicate that these receptors reside predominantly if not exclusively on a M_r = 62,000 - 64,000 peptide (2-4). If protease inhibitors are not included in the receptor preparations, however, degradation products of $M_r \simeq$ 53,000 and 44,000 are additionally seen (1-6). Amphibian β_2-adrenergic receptors appear to be similar to mammalian receptors exhibiting a molecular weight of 58,000 - 62,000 as assessed through purification and photoaffinity labeling techniques (1,3,7,8).

One of the most intensely studied β-adrenergic receptors is that of the turkey erythrocyte. It is currently being employed for numerous investigations of receptor structure (11), desensitization (12) and reconstitution (14-16). Interestingly, in contrast with the mammalian and

[1] The abbreviations used are: [^{125}I]-p-azido-benzylcarazolol, [^{125}I]PABC; bovine serum albumin, BSA; sodium dodecyl sulfate, SDS; polyacrylamide gel electrophoresis, PAGE; isoproterenol, ISO; alprenolol, ALP; phentolamine, PHENT; incubated, INC.

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amphibian receptors, photoaffinity labeling of turkey and other avian erythrocyte β_1-adrenergic receptors identifies two peptides with molecular weights of 38,000 - 42,000 and 45,000 - 50,000 respectively (8-11). The smaller molecular weight peptide is labeled in a 3-4:1 ratio relative to the larger peptide (8-11). Using turkey erythrocytes, these same two peptides have been purified to apparent homogeneity while retaining the same 3-4:1 stoichiometry observed in membranes (11). In these experiments, the inclusion of protease inhibitors did not appear to alter the apparent 3-4:1 ratio of the peptides (11). The structural and functional relationships of these two forms of the receptor have yet to be elucidated. We now report that, as identified with the photoaffinity probe, [^{125}I]-p-azidobenzylcarazolol ([^{125}I]PABC),[1] the ratio of the turkey erythrocyte β_1-adrenergic receptor peptides is highly dependent on the procedure of tissue preparation. Moreover, the M_r = 38,000 - 42,000 peptide appears to be derived from the M_r 45,000 - 50,000 peptide.

METHODS

Tissue Preparations: Turkey erythrocytes were washed three times in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 and then either suspended in 150 mM NaCl, 10 mM glucose, 5 mM EDTA, 17 mM Tris-HCl, pH 7.5 (Buffer A) for whole cell photoaffinity labeling or freeze/thaw lysed for preparation of membranes. Crude membranes were prepared by diluting the lysates with 12.5 mM $MgCl_2$, 1.5 mM EDTA, 75 mM Tris-HCl, pH 7.5 (Buffer B) and centrifuging at 30,000 x g for 10 min. The resulting membrane pellet was washed by resuspension in Buffer B and centrifugation as before. Partially purified plasma membranes were prepared by suspending crude membranes in Buffer B and homogenizing for 5 sec. with a Brinkmann Polytron at maximum speed. The membranes were further homogenized with a dounce homogenizer, underlayed with 50% (w/v) sucrose and centrifuged at 1,200 x g for 20 min. The resulting supernatant was centrifuged at 40,000 x g for 10 min. The final membrane pellet was resuspended in Buffer B for photoaffinity labeling experiments.

Photoaffinity Labeling Experiments: The tissue preparations were diluted to a receptor concentration of 30-50 pM using either Buffer A (intact cells) or Buffer B (crude and purified membranes) and incubated with 30-50 pM [^{125}I]PABC (2,200 Ci/mmol, New England Nuclear) for 90 min at 25°C in the dark. The incubation mixtures were then diluted with either ice-cold Buffer A (cells) or Buffer B (membranes) containing 0.5% fatty acid free bovine serum albumin (BSA) and centrifuged at 500 x g (cells) or 30,000 x g (membranes) for 10 min. The washing with the BSA buffers was repeated two more times and one final wash was performed without BSA. The cells and membranes were then suspended in Buffers A and B, respectively, and irradiated for 90 sec as previously described (1). The irradiated samples were pelleted by centrifugation and finally dissolved in electrophoresis sample buffer.

SDS Polyacrylamide Gel Electrophoresis: Gel electrophoresis was performed according to the method of Laemmli (13) as previously described (1) using 8% homogeneous slab gels. Samples derived from intact cell or crude membrane preparations were sonicated prior to loading on the gel. Upon completion of the electrophoresis run, gels were dried prior to autoradiography at -80°C for 2-3 days. Subsequent to autoradiography, covalently incorporated ^{125}I was quantitated by slicing the gel into 2 mm sections and counting in a Packard Gamma Counter.

RESULTS

Since the turkey erythrocyte β_1-adrenergic receptor appears to exist in two distinct molecular forms as evidenced in both membranes and purified

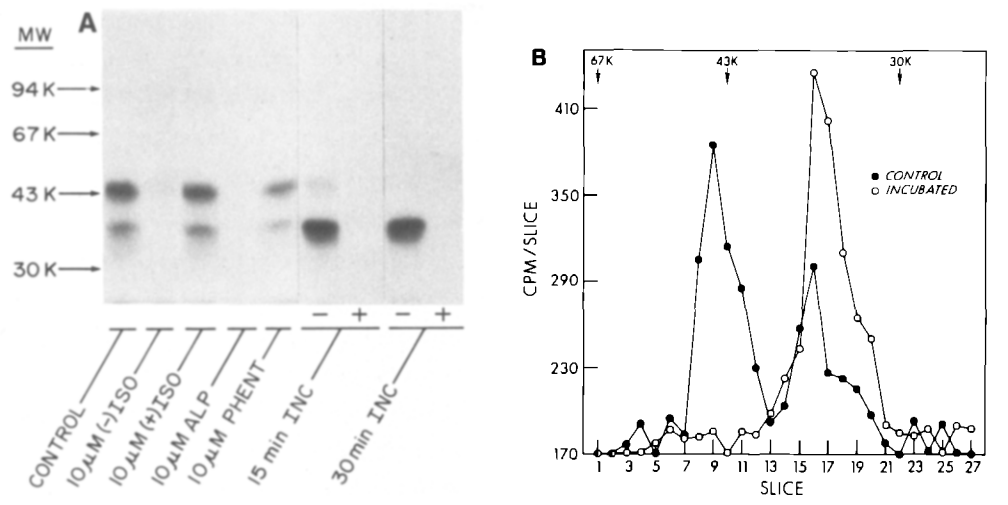

Figure 1. Photoaffinity labeling of intact turkey erythrocytes with [^{125}I]PABC. A, Intact turkey erythrocytes were incubated with [^{125}I]PABC alone (control) or in the presence of the indicated compounds as described in the Methods. After photolysis, some samples that had been labeled in the absence (—) or presence (+) of 10 μM alprenolol were lysed by freeze/thaw and incubated at 37°C for the indicated times before further processing. B, The control lane and the 30 min incubation (—) lane was sliced and counted as described in the Methods. In the control lane the M_r = 39,000 and 49,000 peptide bands contained 290 and 569 specifically incorporated cpm, respectively, while in the incubated lane the M_r = 39,000 peptide contained 795 cpm.

preparations (11), we wondered if similar results would be obtained from photoaffinity labeling the receptors on the surface of intact cells. Figure 1 shows the results of such an experiment. As seen in Fig. 1a, [^{125}I]PABC incorporates into two polypeptides with apparent M_r = 39,000 and 49,000 on SDS-PAGE. Covalent incorporation of [^{125}I]PABC into both polypeptides is stereospecifically blocked by the agonist isoproterenol. 10 μM (-)isoproterenol completely inhibits photoincorporation while 10 μM (+)isoproterenol is without effect (Fig. 1a). The β-adrenergic antagonist alprenolol also completely inhibits labeling of the peptides whereas an equal concentration of phentolamine, an α-adrenergic antagonist, affords only minor protection (Fig. 1a). These results substantiate that both peptide bands labeled with [^{125}I]PABC exhibit identical β-adrenergic specificity in agreement with previously published results (1,11).

Contrary to previous observations with purified membranes, however, the M_r = 39,000 and 49,000 peptides in intact cells are not photolabeled in ratio of 3-4:1 but instead exhibit a M_r = 39,000/M_r = 49,000 ratio of 1:2 as determined from slicing and counting the gels (Fig. 1b). Moreover, if crude membranes are prepared from [^{125}I]PABC labeled cells and incubated at 37°C, there is a rapid disappearance of the M_r = 49,000 labeled peptide (Fig. 1a). Figure 1b shows that concomitant with the M_r = 49,000 peptide disappearance,

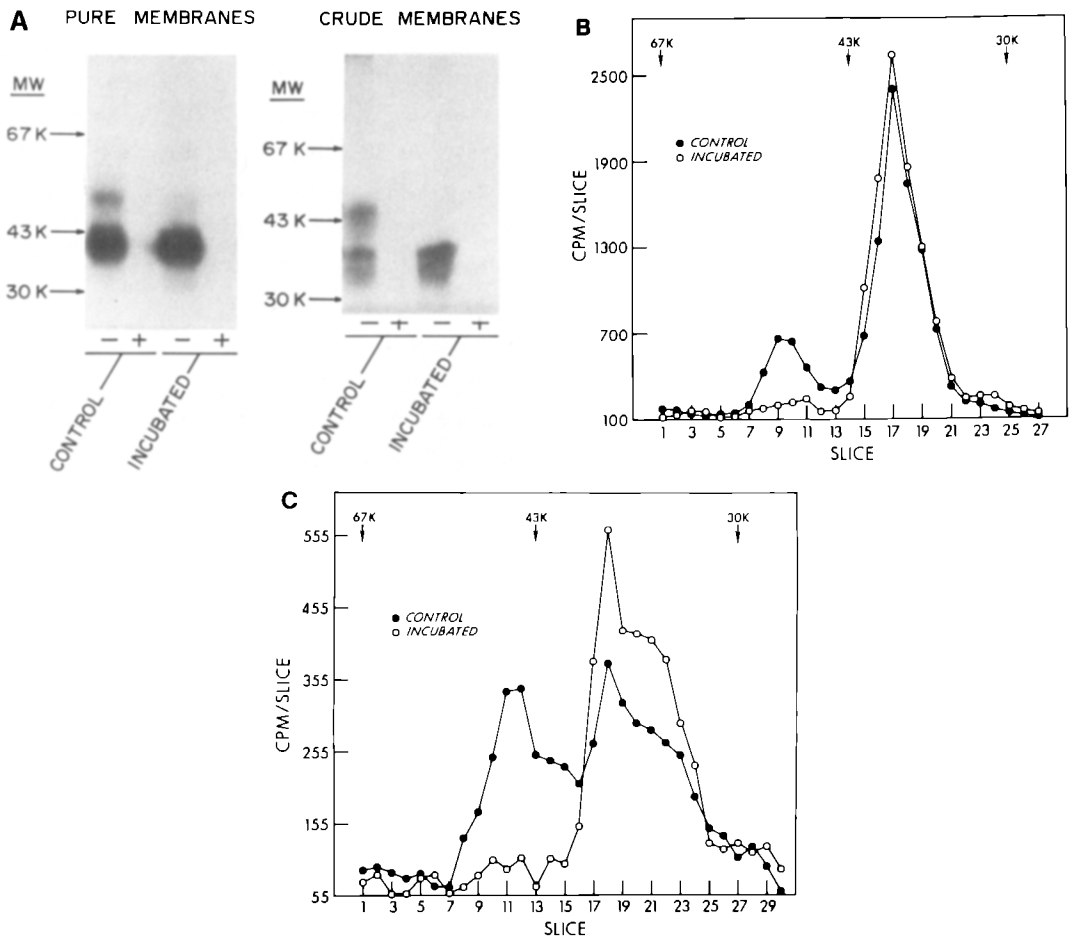

Figure 2. Photoaffinity labeling of turkey erythrocyte membrane preparations with [^{125}I]PABC. A, Purified or crude membranes were incubated with [^{125}I]PABC alone (—) or with 10μM alprenolol (+) as described in the Methods. After photolysis, some control and protected membrane samples were incubated at 37°C for 30 min as indicated. B, The (—) purified membrane lanes in A were sliced and counted as described in Fig. 1b. In the control lane the M_r = 39,000 and 49,000 peptides contained 7,080 and 1,750 cpm, respectively, whereas after incubation the M_r = 39,000 peptide contained 8,410 cpm. C, the (—) crude membrane lanes in A were sliced and counted with the following results: control, M_r = 39,000 - 1,602 cpm, M_r = 49,000 - 1,326 cpm; incubated, M_r = 39,000 - 2,556 cpm.

there is an increase in the amount of peptide that migrates at M_r = 39,000. In fact, the amount of specifically incorporated radioactivity in the incubated sample (M_r = 39,000) accounts for 93% of the total radioactivity in the control sample (M_r = 39,000 + M_r = 49,000) suggesting that the higher molecular weight peptide has been quantitatively converted to the lower molecular weight moiety.

Figure 2 shows the results of photoaffinity labeling β-adrenergic receptors in turkey erythrocyte membrane preparations. In contrast to the findings obtained using intact cells, when purified membranes are photo-

labeled, the M_r = 39,000 and 49,000 peptides are present in a ratio of 4:1, respectively (Fig. 2a,b). These results are identical to those previously observed (1,11). If, however, the membranes are subjected to a 37°C incubation subsequent to the labeling, then only the M_r = 39,000 peptide is seen (Fig. 2a). Quantification of the ^{125}I in Figure 2b indicates that the loss of specifically incorporated radioactivity in the M_r = 49,000 peptide is associated with an equivalent increase of specifically incorporated radioactivity in the M_r = 39,000 peptide.

Figure 2 also shows the results of photoaffinity labeling β-adrenergic receptors in crude membranes. As seen, these results are intermediate between those observed using intact cells or purified membranes. In this case the M_r = 39,000/M_r = 49,000 peptide ratio is about 1:1 (Fig. 2a,c). In addition, if the photolabeled membranes are incubated at 37°C then there is a complete disappearance of the M_r = 49,000 labeled peptide (Fig. 2a). As with the intact cell (Fig. 1b) and purified membrane preparations (Fig. 2b) the disappearance of the M_r = 49,000 peptide is associated with a stoichiometric increase in the M_r = 39,000 form of the receptor (Fig. 2c).

DISCUSSION

The major findings of the present study are: 1) that the ratio of labeling of the two molecular weight forms of the turkey erythrocyte β_1-adrenergic receptor is variable; 2) that this ratio is highly dependent on the procedure of tissue preparation and 3) that the smaller molecular weight receptor peptide is derived from the larger molecular weight form. This latter conclusion is suggested by the observation that upon incubation there is not just a simple disappearance of the M_r = 49,000 peptide but a concomitant and equivalent increase in the M_r = 39,000 peptide. The simplest explanation for these results is that the M_r = 39,000 peptide represents a proteolyzed form of the M_r = 49,000 receptor.

The nature of the putative protease involved in these phenomena is at present obscure. A variety of protease inhibitors including phenylmethylsulfonyl fluoride, benzamidine, bacitracin, soybean trypsin inhibitor, N-ethylmaleimide and EDTA have previously been shown to be without effect in altering the ratio of these two receptor forms (11). The protease also appears to be highly compartmentalized within the erythrocyte and released only upon vigorous disruption. Thus, intact cells show mostly the M_r = 49,000 peptide, crude membranes from gently lysed cells exhibit equal proportions of M_r = 49,000 and 39,000 peptides while vigorously homogenized purified membranes show mostly the M_r = 39,000 peptide.

Previously Shorr *et al.* (11) observed that the ratio of the two turkey erythrocyte receptor peptides was constant throughout purification or photo-

affinity labeling of purified receptor or membrane preparations. However, Shorr et al. employed only one form of purified membrane preparation and in separate experiments we have ascertained that the "proteolysis" effect is lost subsequent to receptor solubilization (data not shown). It is interesting that in the study of Shorr et al. (11), preliminary characterization of the two peptides by partial protease digestion suggested a large degree of similarity between them.

The current findings thus suggest that the native turkey erythrocyte β-adrenergic receptor is most likely a $M_r \tilde{=} 49,000$ protein. This differs from recent findings which suggest that mammalian β_1 - and β_2-adrenergic receptors and amphibian β_2-receptors exhibit higher molecular weights of between 58,000 and 64,000 (1-8). Although we cannot categorically exclude the possibility that the turkey erythrocyte $M_r = 49,000$ peptide is in turn generated from a higher molecular weight form of the receptor, we consider this unlikely especially in light of the intact cell labeling experiments. Moreover, in all avian species examined to date, including duck, turkey and pigeon, photoaffinity labeling of erythrocyte membranes has failed to provide any evidence for a β-adrenergic receptor peptide larger than $M_r \tilde{=} 50,000$ (8-11). The evolutionary relationship of these avian and mammalian β-adrenergic receptors is an interesting subject for further study.

An important yet unanswered question relates to the functionality of the two molecular weight forms of the turkey β_1-adrenergic receptor. Although the $M_r = 39,000$ form appears to be derived from the $M_r = 49,000$ peptide, the lower molecular weight peptide is still capable of binding antagonist ligands as evidenced here through photoaffinity labeling with [^{125}I]PABC. Moreover, Shorr et al. (11) have resolved the two polypeptides and demonstrated that they interacted identically with both agonist and antagonist ligands. Whether or not the $M_r = 39,000$ peptide is as efficacious as the $M_r = 49,000$ peptide in coupling with the guanine nucleotide binding protein and activating adenylate cyclase, however, remains to be determined. This question is especially relevant with respect to recent experiments employing turkey erythrocyte β-adrenergic receptor preparations with which to reconstitute hormone-sensitive adenylate cyclase systems (14-16). Differing results might be anticipated if the $M_r = 39,000$ and 49,000 peptides are functionally dissimilar and if different ratios of these peptides are employed in the reconstitution procedure.

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