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PHOTOAFFINITY LABELLING OF MAMMALIAN BETA-ADRENERGIC RECEPTORS: METAL-DEPENDENT PROTEOLYSIS EXPLAINS APPARENT HETEROGENEITY

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The beta-adrenergic receptors in membranes from rat and hamster lungs have been studied using the photoaffinity label p-azido-m-[^{125}I]-iodobenzyl-carazolol. Previous work with several beta adrenergic photoaffinity probes has suggested heterogeneity of the labelled beta adrenergic receptor peptides with 2-3 receptor peptides generally being identified. We now report that rat and hamster lung membranes prepared either in the presence or absence of protease inhibitors reveal striking differences in the ratios of photoaffinity labelled peptides. In the rat lung the inclusion of protease inhibitors in the membrane preparation changes the ratios of the 64,000, 53,000 and 44,000 molecular weight peptides from 28:42:30 to 72:16:12. Similarly, in hamster lung membranes there is evidence of multiple photoaffinity labelled peptides in preparations without protease inhibitors while only one peptide (app. M_r = 64,000) is labelled in preparations with protease inhibitors. Of the inhibitors tested EDTA and EGTA were the most active in preventing appearance of multiple labelled peptides suggesting that metal-dependent proteolysis may be involved in the generation of apparent receptor peptide heterogeneity.

The technique of photoaffinity labelling has recently proven to be very useful in studies of beta-adrenergic receptor systems (1-7). This approach not only enables one to study the molecular size of the receptor in membrane preparations but is also extremely useful in characterizing the receptor at various stages of purification. In two systems, the frog erythrocyte (6) and the turkey erythrocyte (8), it has been shown, using photoaffinity labelling and purification techniques, that the purified beta-adrenergic receptor peptides are in fact identical to those labelled in the membranes.

During the course of these studies by several laboratories it has become apparent that significant heterogeneity exists in photoaffinity labelling patterns of the receptor in several different systems. Evidence of heterogeneity has been shown in turkey erythrocytes (4,7,8), duck and pigeon erythrocytes (1,5), rat reticulocytes (7), S49 cells (3) and rat and rabbit

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lung (7). Indeed, every beta-adrenergic receptor system that has been studied to date, with the exception of the frog erythrocyte (2,5-7), has been shown to have multiple photoaffinity labelled peptides. Speculations as to the basis of the heterogeneity have included multiple types of receptors, multiple receptor subunits, subtype differences ($beta_1$ vs. $beta_2$) and proteolysis. In this paper we present evidence that the heterogeneity of beta-adrenergic receptor peptides observed in two mammalian systems, rat and hamster lung membranes, is in fact due predominantly to proteolysis.

MATERIALS AND METHODS

Materials: p-Azido-m-[^{125}I]-iodobenzylcarazolol, [^{3}H](-)dihydroalprenolol, (±)[^{125}I]cyanopindolol and Na[^{125}I] were from New England Nuclear Corporation, Boston, Mass. Rats (Sprague Dawley) were from Charles River Breeding Laboratories, Wilmington, Mass. while hamsters were from Harlan Sprague Dawley, Inc., Indianapolis, IN. EDTA[1] was from Mallinckrodt while EGTA, N-ethylmaleamide, p-hydroxymercuribenzoate, aprotinin, antipain, chymostatin, leupeptin, 2,2'dithiodipyridine, phosphoramidone, soybean trypsin inhibitor, pepstatin, benzamidine and PMSF were from Sigma Chemical Company, St. Louis, Mo. Premixed electrophoresis standards (phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), alpha-lactalbumin (14,400)) were from Pharmacia. Electrophoresis reagents were from Bio-Rad Laboratories. X-ray film (XAR-5) and developing solutions were from Kodak. Intensifying screens (Cronex Lightning Plus) were from Dupont. Alprenolol hydrochloride was a generous gift from Hassle Pharmaceutical Company of Sweden. Other biochemical reagents were usually from Sigma. Chemicals were usually from Aldrich and all solvents and reagents were of the highest grade available.

Membrane Preparations: Rat and hamster lung membranes were prepared from fresh lungs which were immediately frozen in liquid nitrogen upon excision. The frozen lungs were thawed and minced in 20 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4 containing various protease inhibitors. The suspension was homogenized with two 10 sec. bursts of a tissue disruptor (Brinkmann Polytron) at maximum speed. The homogenate was centrifuged at ∿ 500 x g for 10 min. and the supernatant was then recentrifuged at 40,000 x g for 15 min. The pelleted membranes were washed twice before being homogenized with a Teflon-glass homogenizer in 5 volumes of the starting buffer. All preparations were used fresh and routinely contained 0.6 ± 0.1 pmol/mg receptor in the rat lung and 1.4 ± 0.2 pmol/mg in the hamster lung. The concentration of membrane proteins was determined by the method of Lowry et al. (9) using bovine serum albumin as standard. Receptor binding site concentration was determined by [^{3}H]DHA binding (10).

Membrane-labelling Experiments: The labelling procedures used were similar to those of Lavin et al. (7). Briefly, membranes were diluted to a receptor concentration of 30-50 pM using a buffer identical to that used in the membrane preparation. The [^{125}I]pAIBC was diluted in 10% ethanol, 5 mM HCl before addition to the membranes yielding a final [^{125}I]pAIBC concentration

[1]The abbreviations used are: [^{125}I]pAIBC, p-azido-m-[^{125}I]-iodobenzylcarazolol; EDTA, ethylene dinitrilo tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; [^{3}H]DHA, [^{3}H]dihydroalprenolol; [^{125}I]CYP, [^{125}I]cyanopindolol.

of 100 pM and an ethanol concentration of < 0.1%. Membrane suspensions were incubated for 90-120 min. in the dark at 25°C. Following the incubation the pelleted membranes were washed twice by centrifugation in buffer containing 0.1% BSA and once in incubation buffer before resuspension in the same buffer. The final suspension was photolyzed for 60 sec., 12 cm from a Hanovia 450 W medium pressure mercury arc lamp filtered with 5 mm of Pyrex glass. Following centrifugation of the photolyzed suspension the pellet was resuspended into electrophoresis sample buffer (defined below).

Sodium Dodecyl Sulfate Gel Electrophoresis: Gel electrophoresis was performed according to the method of Laemmli (11) using 8% homogeneous slab gels with the exception that samples were denatured in 8% SDS, 10% glycerol, 5% beta-mercaptoethanol, 50 mM Tris-HCl, pH 6.5 (sample buffer). Upon completion of the electrophoresis run, gels were dried using a Bio-Rad (Model 224) gel dryer prior to autoradiography at -80°C with Kodak XAR-5 film using one intensifying screen typically for 12-36 hours. Autoradiographs were scanned using a Zeineh scanning densitometer (Biomed Instruments, Inc.).

RESULTS AND DISCUSSION

Photoaffinity labelling of beta-adrenergic receptors in rat lung membranes prepared without protease inhibitors leads to specific labelling of bands at apparent molecular weights of 64,000, 53,000 and 44,000 as shown in Fig. 1. These bands are found in a 28 ± 5 : 42 ± 2 : 30 ± 5 ratio (n = 11) and are similar to those previously observed by Lavin et al. (7). Additionally, in experiments using frozen membranes a labelled peptide at M_r = 30,000 is frequently observed (7).

Possible proteolysis occurring during the rat lung membrane preparations was studied by including a variety of protease inhibitors in the homogenization buffer. The effect of serine protease inhibitors, soybean trypsin inhibitor and benzamidine, combination serine and thiol protease inhibitors, PMSF and leupeptin, a carboxyl protease inhibitor, pepstatin and a metal protease inhibitor, EDTA, were studied as shown in Fig. 2. It is apparent that EDTA has a dramatic effect on the labelling pattern in the rat lung leading to band ratios of 72 ± 8 : 16 ± 3 : 12 ± 6 (n = 8) for the 64,000, 53,000 and 44,000 molecular weight peptides, respectively. Leupeptin also produces a measurable change in the band ratios: 41 ± 7 : 42 ± 2 : 17 ± 6 (n = 4). Additionally, the effect of several other protease inhibitors including aprotinin, antipain, chymostatin, EGTA, N-ethylmaleamide, beta-hydroxymercuribenzoate, 2,2'dithiodipyridine and phosphoramidone was

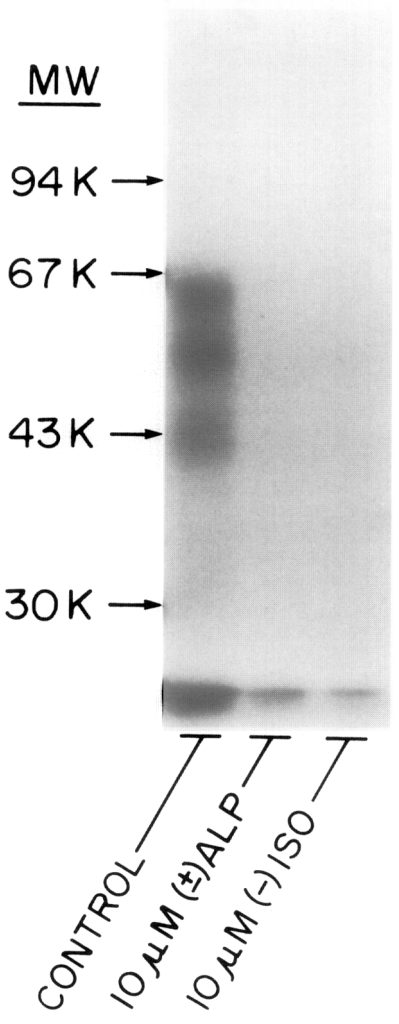

Fig. 1. Photoaffinity labelling of rat lung membranes with [^{125}I]pAIBC. Rat lung membranes (30 pM receptor) prepared in the absence of protease inhibitors were incubated with [^{125}I]pAIBC (100 pM) alone or in the presence of (±)alprenolol (10 μM) or (-)isoproterenol (10 μM). Photolysis and electrophoresis conditions were as described in Materials and Methods.

studied. Overall, only EDTA, EGTA and leupeptin were found to have measurable effects on the ratios of the labelled bands.

Since EDTA and EGTA had such dramatic effects on the labelling patterns further studies were carried out to determine if these agents actually inhibited proteolysis of the receptor or simply altered the incorporation of label into different peptides. To assess this, membranes were prepared with and without EDTA and then labelled in the presence or absence of EDTA. In samples from membranes prepared in the absence of EDTA, the presence or

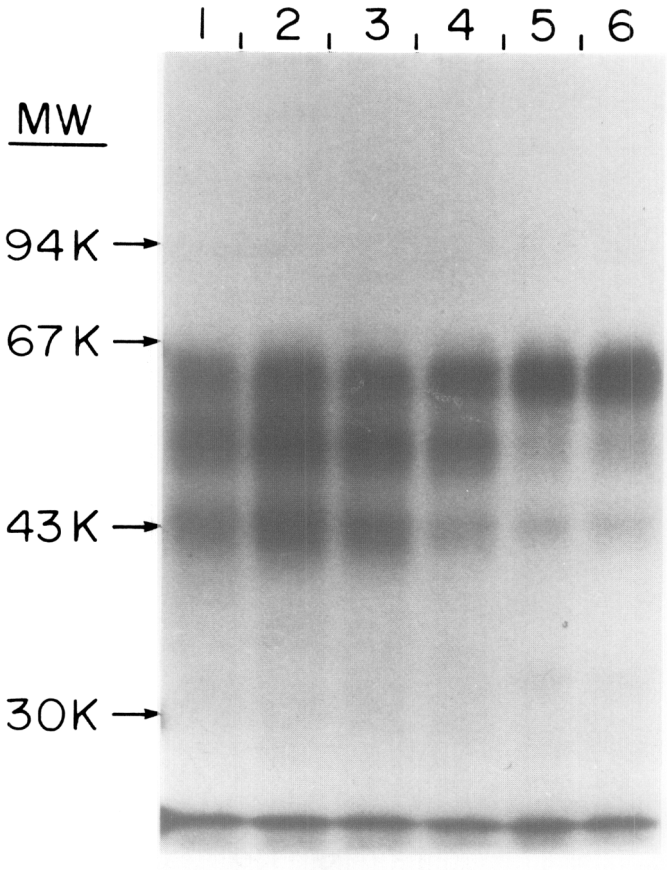

Fig. 2. Effect of protease inhibitors on [^{125}I]pAIBC photoaffinity labelling patterns of rat lung membranes. Rat lung membranes (30-50 pM receptor) were prepared as described in Materials and Methods in buffer containing the following protease inhibitors: lane 1, none; lane 2, 5 µg/mL soybean trypsin inhibitor, 100 µM benzamidine and 100 µM PMSF; lane 3, 5 µg/mL pepstatin; lane 4, 5 µg/mL leupeptin; lane 5, 5 mM EDTA; lane 6, 5 µg/mL soybean trypsin inhibitor, 100 µM benzamidine, 5 µg/mL leupeptin and 5 mM EDTA. Membranes were incubated with [^{125}I]pAIBC (100 pM) and were photolyzed and electrophoresed as in Materials and Methods.

absence of 5 mM EDTA in the photoaffinity labelling procedures did not change the pattern of labelling obtained (i.e. 28:42:30). With samples from membranes prepared in the presence of EDTA a different pattern was observed (a major peptide of M_r 64,000 as shown in Fig. 2). However, the presence or absence of EDTA during photoaffinity labelling procedures did not alter this pattern (data not shown). These data suggest that EDTA does not affect the photoincorporability of the probe into the different peptides and that the presence of EDTA may be essential only during the membrane preparation procedures. To further explore this point, Ca^{2+}, Zn^{2+} or Mg^{2+}, which

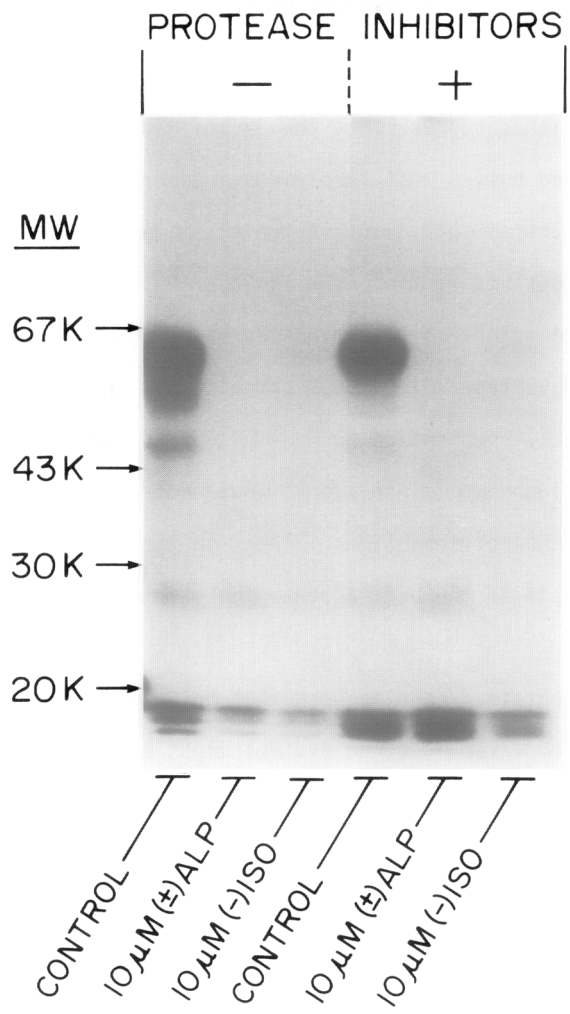

Fig. 3. Effect of protease inhibitors on [^{125}I]pAIBC photoaffinity labelling patterns of hamster lung membranes. Hamster lung membranes (50 pM receptor) were prepared as described in Materials and Methods in buffer containing either no protease inhibitors or 5 μg/mL soybean trypsin inhibitor, 100 μM benzamidine, 5 μg/mL leupeptin and 5 mM EDTA. Membranes were incubated with [^{125}I]pAIBC (100 pM) alone or in the presence of (±)alprenolol (10 μM) or (-)isoproterenol (10 μM). Photolysis and electrophoresis conditions were as described.

are potential activators of metal dependent proteases, were added at a concentration of 1 mM during the photoaffinity labelling procedures to membrane samples prepared in the presence of EDTA. Even under these conditions, the labelling pattern remained unaltered and featured the predominant M_r 64,000 peptide. Finally, the same peptide(s) evidenced by photoaffinity labelling can be obtained by purification procedures with the

ratios of the purified peptides again being dramatically altered by the presence of EDTA during the membrane preparation (unpublished observations). These lines of evidence suggest that the putative protease involved is not only metal-activated but is most likely not a membrane-bound enzyme.

Fig. 3 demonstrates that quantitatively similar results could be obtained with preparations derived from hamster lungs. These membranes contain > 95% $beta_2$ adrenergic receptors as determined by radioligand binding studies with [^{125}I]CYP (12) and subtype selective antagonists as described in (13). It is apparent that only one peptide at M_r = 64,000 is seen when protease inhibitors are included while additional lower molecular weight peptides are present in the absence of inhibitors. Thus, in the hamster lung, as in the rat lung, proteolysis of the beta-adrenergic receptor occurs during membrane preparation.

The findings obtained here suggest that heterogeneity of beta-adrenergic receptor peptides observed in other systems might also be due to proteolysis. Since the various proteolyzed forms of the receptors are all specifically labelled by [^{125}I]pAIBC, they each contain the intact ligand binding site of the beta-adrenergic receptor. Whether such receptor proteolysis also occurs with some physiological significance in vivo or whether it is simply an in vitro phenomenon remains to be determined. These observations, however, further support the notion that the mammalian $beta_2$-adrenergic receptor is contained primarily if not exclusively on a peptide of apparent M_r = 62-65,000.

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