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THE EFFECT OF REDUCING AGENTS ON THE STRUCTURE OF MAMMALIAN β-ADRENERGIC RECEPTORS

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Under reducing conditions (5% β-mercaptoethanol) the mammalian β-adrenergic receptor binding site from both β_1 (porcine heart membranes) and β_2 receptors (hamster lung and rat erythrocyte membranes) appears to reside on peptides of M_r 62,000-65,000 as determined by photoaffinity labeling with p-azido-m-[^{125}I]iodobenzylcarazolol and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When similar experiments are performed in these same systems under a variety of non-reducing conditions, there are minimal changes in the apparent molecular weight of both the β_1- and β_2-adrenergic receptor binding subunits and no specifically labeled higher molecular weight proteins are observed suggesting that there are no disulfide linked subunits in mammalian β-adrenergic receptors.

Mammalian β-adrenergic receptors can be divided into two subtypes termed β_1 and β_2 based on their relative affinities for a series of catecholamines (1). Photoaffinity labeling and purification techniques have now demonstrated that the binding subunits from both mammalian β_1- and β_2-adrenergic receptor subtypes reside on proteins of M_r = 62,000-65,000 (2-4). Although the proteins derived from each receptor subtype have similar overall apparent molecular weights, peptide mapping techniques have revealed differences in the primary structure of the β_1- and β_2-adrenergic receptor binding subunits (5). Using purified mammalian β_2-adrenergic receptors, it can be demonstrated that not only do these peptides recognize and bind catecholamines with the appropriate pharmacologic specificity, but these receptor peptides can be reconstituted into cell membranes deficient in β-adrenergic receptors to produce membranes with catecholamine-responsive adenylate cyclase activity (6,7). These studies have all determined the apparent molecular weight of these peptides under reducing conditions with β-mercaptoethanol using the gel electrophoresis methods of Laemmli (8).

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Some hormone receptors such as the insulin receptor are known to consist of several subunits linked by disulfide bonds (9). Under non-reducing conditions proteins of apparent molecular weight 520,000 and 350,000 can be detected (9). Under reducing conditions the 520,000 M_r peptide produces peptides of M_r 210,000, 135,000 and 95,000 and the 350,000 M_r peptide produces subunits of 135,000 and 95,000 (9). A recent report utilizing bovine lung membranes has suggested that the β_2-adrenergic receptor subunit from that tissue has an apparent molecular weight of 59,000 and that the mammalian lung β_2 receptor is a dimer of two identical subunits of 59,000 linked by disulfide bonds (10).

To determine if the mammalian β_1- and β_2-adrenergic receptors are composed of multiunit structures linked by disulfide bonds we have performed photoaffinity labeling of β_1- and β_2-adrenergic receptors and subjected the labeled peptides to SDS-PAGE under a variety of reducing and non-reducing conditions. We report herein that there is no evidence for disulfide linked subunits of the mammalian β-adrenergic receptor.

MATERIALS AND METHODS

Materials: Para-azido-m-[^{125}I]iodobenzylcarazolol ([^{125}I]pABC) was obtained from New England Nuclear Corp., Boston, Mass. Electrophoresis reagents were generally from Bio-Rad Laboratories. The enzyme inhibitors leupeptin, pepstatin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride (PMSF) were all obtained from Sigma Chemical Co., 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), and α-lactalbumin (14,000) were from Pharmacia. X-ray film and developing solutions were from Kodak. Intensifying screens (Cronex Lightening Plus) were from DuPont.

Methods: Porcine myocardial and rat erythrocyte membranes were prepared as recently described (2). Hamster lung membranes were prepared as recently described (3) except the following proteinase inhibitors were included: 2 mM EDTA, 5 μg/ml leupeptin, and 10^{-4} M PMSF. [^{125}I]pABC labeling of all membranes was performed as recently described (2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on slab gels with a buffer system as described by Laemmli (8). The acrylamide concentration was varied from 5½-10% and the β-mercaptoethanol (5% final concentration) was either present or absent as indicated. Electrophoresis was performed at 20 mA for approximately 6-8 hours (until tracking dye had just exited the bottom of the separating gel). Films were exposed to the dried gels for 2-5 days at -70°C and were developed manually.

RESULTS AND DISCUSSION

When β_2-adrenergic receptors from hamster lung membranes and rat erythrocyte membranes are photolabeled with [^{125}I]pABC alone or in the

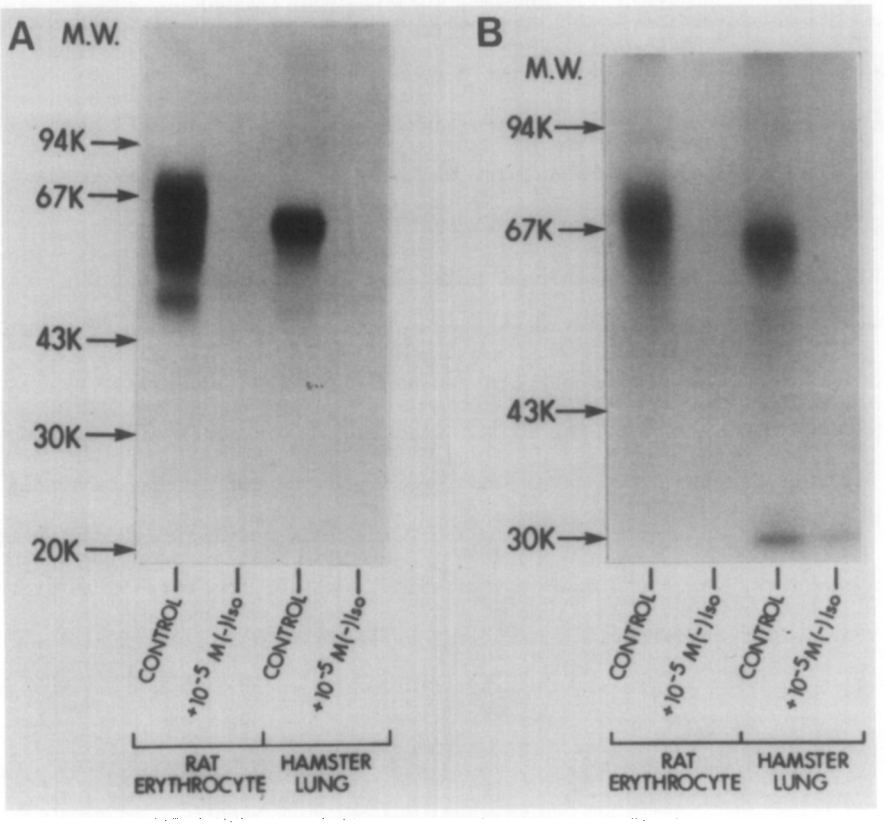

Figure 1 - Photoaffinity labeling of hamster lung and rat erythrocyte β_2-adrenergic receptors with [^{125}I]pABC. Aliquots of each membrane preparation were first labeled in the presence or absence (control) of 10^{-5} M (-)isoproterenol and then solubilized in SDS buffer with (A) or without (B) 5% β-mercaptoethanol as described in Methods. The samples were then electrophoresed on a 8% acrylamide gel. The molecular weights shown (MW) are x 1000 (K) and were determined with iodinated protein standards. (-)Iso, (-)isoproterenol.

presence of 10^{-5} M (-)isoproterenol and electrophoresed under reducing conditions (5% β-mercaptoethanol), the predominant specifically labeled peptides have M_r of 64,000 and 65,000 respectively (see Figure 1A). We have previously demonstrated that the smaller M_r peptides are likely proteolytic products of the major (∿ 60,000) M_r peptide and that their relative proportions can be altered by proteinase inhibitors (particularly EDTA and leupeptin) (2,3). All these peptides have been shown to display all the appropriate pharmacologic specificity and stereospecificity characteristic of a β_2-adrenergic receptor (2,3). When aliquots of these same [^{125}I]pABC labeled membranes are solubilized (5-10% SDS) under non-reducing conditions

and electrophoresed the major peptides visualized by autoradiography demonstrate an M_r of 70,000 for the erythrocyte β_2 receptor and 68,000 for the hamster lung β_2-adrenergic receptor (Figure 1B). It can be seen that there is no evidence for any larger molecular weight peptides under these conditions. In addition, varying the acrylamide concentration in the separating gel from 5½-10% failed to reveal any higher molecular weight labeled peptides. Since the exclusion limit for a 5½% acrylamide slab gels is greater than 750,000 daltons it is unlikely that we have excluded any disulfide linked subunits of the $M_r \cong 60,000$ peptides. The slight increase in the apparent molecular weight of these labeled peptides under non-reducing conditions is consistent with the notion that under non-reducing conditions intramolecular disulfide bonds may exist which could alter the ability of SDS to bind to the protein and hence alter the protein's mobility on SDS-PAGE (9).

As mentioned above, the binding subunit peptides from these β_2-adrenergic receptors display the appropriate pharmacologic specificity of a β_2-adrenergic receptor. In addition, purified preparations of the hamster and guinea pig lung β_2-adrenergic receptor (containing only 64,000 M_r proteins) have now been reconstituted into lipid vesicles and fused with a cell lacking β-adrenergic receptors (Xenopus laevis erythrocyte). Following these procedures the membranes now demonstrate catecholamine-sensitive adenylate cyclase activity (6,7). These facts, along with a lack of evidence for a disulfide linked oligomeric structure of the mammalian β_2-adrenergic receptors, suggests that the $M_r \cong 64,000$ peptides may contain both the "binding" and "coupling" domains of the β_2-adrenergic receptor.

When the β_1-adrenergic receptors from porcine myocardial membranes are photolabeled with [^{125}I]pABC alone, or in the presence of 10^{-5} M (-)isoproterenol, and then solubilized and electrophoresed under reducing conditions a peptide of $M_r = 62,000$ is specifically labeled. It demonstrates all of the appropriate pharmacologic characteristics of a β_1-adrenergic receptor (Figure 2A and ref. 2). Several non-specifically labeled peptides can also be seen. It should be noted that a peptide of $M_r \sim 95,000$ is

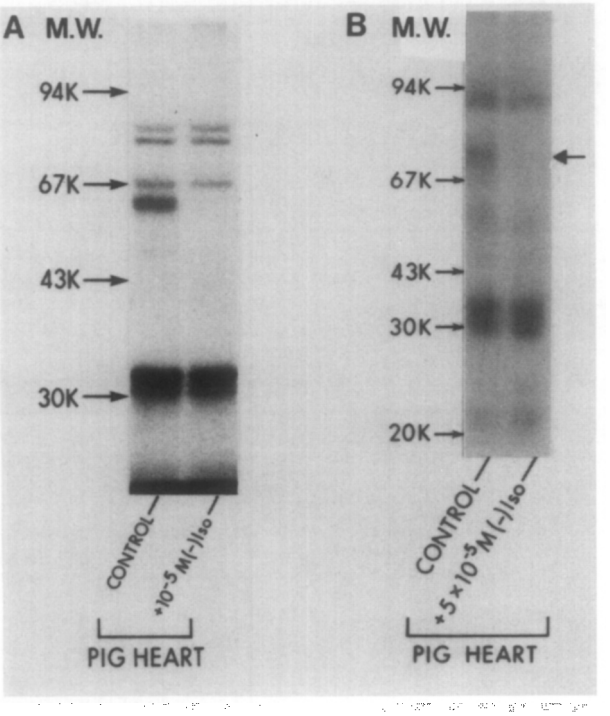

Figure 2 - Photoaffinity labeling of porcine myocardial β_1-adrenergic receptors with [^{125}I]pABC. Aliquots of these membranes were first labeled in the presence and absence (control) of 5 x 10^{-5} M (-)isoproterenol and then solubilized in SDS buffer with (A) or without (B) 5% β-mercaptoethanol as described in Methods. The samples were then electrophoresed on a 8% acrylamide gel. The molecular weights shown (MW) are x 1000 (K) and were determined with iodinated protein standards. The large arrow in panel B indicates the receptor peptide. (-)Iso, (-)isoproterenol.

photolabeled and can be partially and inconsistently protected from photoincorporation of [^{125}I]pABC with adrenergic ligands but this peptide does not display all the appropriate pharmacologic characteristics of a β_1-adrenergic receptor. When the same experiments are performed under non-reducing conditions a single, appropriately "protected" peptide with an M_r of approximately 72,000 can be seen (Figure 2B). Thus, we find no evidence for a disulfide linked subunit structure of the β_1-adrenergic receptor.

These data suggest that the functional activities of mammalian β_1- and β_2-adrenergic receptors may reside on a single peptide of $M_r \sim$ 60,000-65,000.

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