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SHORT COMMUNICATIONS

Subunit structure of rat liver α_1 adrenergic receptor

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A number of different types of detergents have been used in the solubilization of integral membrane proteins such as hormonal receptors. Digitonin has been very useful for the solubilization of receptors, such as the β-adrenergic receptor in an active form [1, 2]. However this detergent gives rise to protein–detergent complexes of artificially high apparent molecular weights [3, 4]. Lubrol PX, which allows solubilization of membrane proteins in a non-aggregated form, will also, however, inhibit the binding of most radioligands used to identify these receptors. One way to overcome this difficulty consists of prelabelling the receptor before solubilization. Using this approach we have recently solubilized the hepatic α-adrenergic receptor covalently bound to an irreversible α-adrenergic antagonist [^{3}H]phenoxybenzamine ([^{3}H]POB).* This ligand has been shown to be a highly specific marker of α-adrenergic receptors in rat liver plasma membranes [5, 6]. The specificity of binding of [^{3}H]POB to rat liver plasma membrane was that expected for an α-adrenoreceptor. Furthermore a close correlation was observed with the potency of various adrenergic drugs on phosphorylase *a* activity in isolated hepatocytes [5, 6]. Under non-denaturing conditions, the molecular weight of the α-adrenergic receptor from rat liver is 96,000. The subunit composition of the receptor can only be achieved using ionic detergents such as sodium dodecyl sulfate. In the present paper, we demonstrate that [^{3}H]POB specifically labels a 44,800 mol. wt peptide, and suggest that the hepatic α-adrenergic receptor is composed of at least two subunits.

Materials and methods

Protein standards such as phosphorylase B (94,000) soybean trypsin inhibitor (21,000), bovine serum albumin (68,000), carbonic anhydrase (30,000), ovalbumin (43,000) were purchased from Bio-Rad Laboratories (Richmond, CA), and β-galactosidase (116,500), catalase (57,000) and lactate dehydrogenase (35,000) were from Sigma Chemical Co. (St Louis, MO). Electrophoretic reagents were also obtained from Bio-Rad Laboratories. Other chemicals were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

Drugs. Phentolamine (Ciba-Geigy, Basel, Switzerland), prazosin (Pfizer, Sandwich, U.K.) yohimbine (Roussel Uclaf, France) and (−)epinephrine (Sigma) were obtained from the suppliers indicated.

Benzyl [^{3}H]phenoxybenzamine (15.1 Ci/mmole) was prepared at the Radiochemical Centre (Amersham, U.K.) as previously described [5]. The purity of the radiolabelled material was routinely assessed by thin-layer chromatography on silica-gel plates, using ethanol as eluting buffer.

Liver plasma membranes. Liver plasma membranes were prepared from female albino Wistar rats (100–200 g body wt) according to the procedure devised by Lesko *et al.* [7] except that the two phase aqueous polymer system was prepared according to Pochet *et al.* [8]. Proteins were measured according to the Lowry procedure [9] using bovine serum albumin as standard.

SDS-gel electrophoresis. The discontinuous buffer system of Laemmli [10] was used for SDS-gel electrophoresis analysis of proteins in rod gels (5 mm i.d. × 12 cm). The stacking gel contained 0.125 M Tris–HCl buffer, pH 6.8, 0.1% SDS (w/v) and 5% acrylamide (w/v), with 15% DATD/T (w/v) as crosslinker. Separation gels contained 0.375 M Tris–HCl, pH 8.8, 0.1% SDS (w/v), 13.75% (w/v) acrylamide and 9.1% DATD/T (w/v). Bromophenol blue was used as tracking dye and 50 μg of protein were layered per gel. Electrophoresis was typically carried out at 4 mA per tube for 3–4 hr at room temperature. Gels were stained in Coomassie brillant blue G250 according to Laemmli [10].

Extraction of radioactivity from DATD-cross linked gels. Gels were frozen and sliced (50 slices per 10 cm length). Each slice was placed in a scintillation vial and solubilized in 1 ml of 2% sodium periodate [11] for 2 hr at room temperature with shaking. Ten millilitres of Ready Solve EP (Beckmann) were added to each vial and the radioactivity counted in an Intertechnique SL 30 liquid scintillation counter at an efficiency of 30%. The fraction of the radioactivity eluted from the gel averaged 90% of the amount applied. (Free [^{3}H]POB which migrates in front of the tracking dye is not represented in the figures.)

Solubilization of the hepatic [^{3}H]phenoxybenzamine binding protein. Rat liver plasma membranes (1 mg/ml) were incubated with 8–10 nM [^{3}H]POB (100,000 cpm) for 3 min at 30° in 50 mM Tris–HCl, pH 7.2, containing 10 mM $MgCl_2$. The reaction was stopped by dilution in 200 ml of ice cold buffer and unbound [^{3}H]POB was removed by centrifugation at 13,000 *g* for 30 min. The pellet was resuspended in half the initial volume of distilled water in order to obtain a solution at 2 mg protein/ml. At this stage 40,000 cpm of [^{3}H]POB were bound per mg of rat liver plasma membrane protein, 45–50% (i.e. 20,000 cpm) representing specific binding (1700 fmoles/mg protein). The membranes were solubilized by addition of an equal volume of 500 mM Tris–HCl buffer, pH 6.8, containing 20% glycerol, 10% β-mercaptoethanol and 4% SDS, unless otherwise indicated, and homogeneized in a Dounce homogenizer. The samples were then agitated for 90 min at 25° prior to centrifugation at 105,000 *g* for 20 min in a 60 Ti rotor (Beckmann). The supernatant was used as the source of [^{3}H]POB binding protein. Competition experiments were carried out as previously reported [5].

Results

The hepatic α-adrenergic receptor was irreversibly labelled with [^{3}H]POB and solubilized with 2% SDS for 90 min. Following electrophoresis on SDS polyacrylamide gels, four radiolabelled peaks were obtained. In order to assess the pharmacological specificity of the peaks obtained, rat liver plasma membranes were incubated in the absence or presence of increasing concentrations of various α-adrenergic agents (0.01–100 μM) and then with [^{3}H]POB as described under Materials and Methods. In Fig. 1 are shown gel electrophoretic patterns of membranes incubated either in the absence or presence of 1 or 100 μM (−)epinephrine. Only the radioactivity in the peak migrating in the 45 K molecular weight region was specifically displaced by increasing concentrations of epinephrine. The radioactivity associated with this peak represents approximately

* The abbreviations used are: SDS, sodium dodecyl sulfate; [^{3}H]POB, tritiated phenoxybenzamine; DATD, *N*-*N*′-diallytartardiamide.

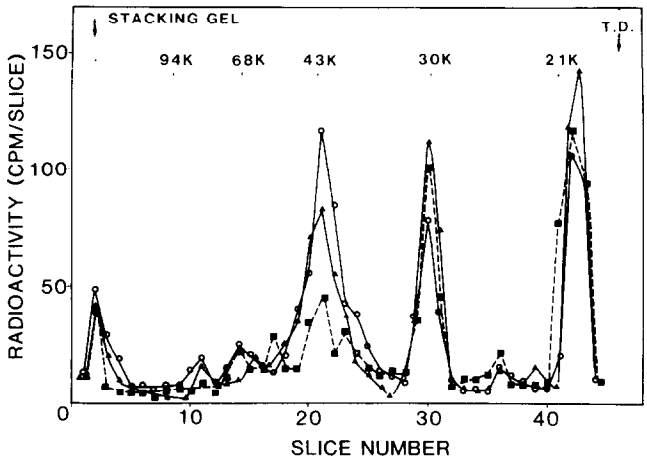

Fig. 1. SDS polyacrylamide gel electrophoretic pattern of rat liver plasma membranes labelled with [^{3}H]POB in the absence or in the presence of epinephrine. Rat liver plasma membranes were incubated with [^{3}H]POB and in the absence (○) or presence of 1 μM (▲) or 100 μM (■) epinephrine. The incubations solubilization and electrophoresis were done as described under Materials and Methods. At the end of the run, the gels were sliced and solubilized in periodic acid (see Materials and Methods) before counting the radioactivity. This experiment is representative of three different experiments; the top of the gel is on the left and bottom of the gel is at the right. T.D. = tracking dye. The molecular weight standards are indicated on the figure. The electrophoretic pattern was identical for membranes prepared according to Neville's procedure [25] (data not shown).

50% of the total membrane bound radioactivity. The radioactivity present in the lower molecular weight species was not altered. A small amount of radioactivity located in the stacking gel was also displaced by increasing concentrations of epinephrine, which might represent a small degree of receptor aggregation.

The same procedure was carried out in the presence of increasing amounts of the α-adrenergic antagonist phentolamine (from 0.01 to 100 μM). Figure 2 shows three electrophoretic patterns of the solubilized membranes labelled with [^{3}H]POB in the absence or presence of 0.1 or 1 μM phentolamine. An identical specific labelling pattern was observed with only the peak of radioactivity located in the 45 K molecular weight region specifically displaced as well as the peak located in the stacking gel. Similarly, low concentrations (1–0.01 μM) of prazosin and yohimbine selectively displaced only the peak of radioactivity migrating in the 45 K molecular weight region (data not shown). Furthermore, while 10 nM prazosin decreased the radioactivity under the 45 K peak by 53%, the same concentration of yohimbine produced only a 13% decrease. More than 10 μM yohimbine was required to displace 40% of the radioactivity in the 45 K peak. These results taken together indicate that the 45 K polypeptide corresponds to the α_1-adrenergic receptor binding site.

The exact molecular weights of the radiolabelled peaks were calculated after calibration of the gel system using several homogenous protein standards (Fig. 3). A molecular weight of 44,800 ± 1400 was determined for the species exhibiting α-adrenergic specific binding. The two other peaks corresponded to molecular weights of 22,100 ± 2600 and 17,900 ± 1200. When prelabelled membranes were

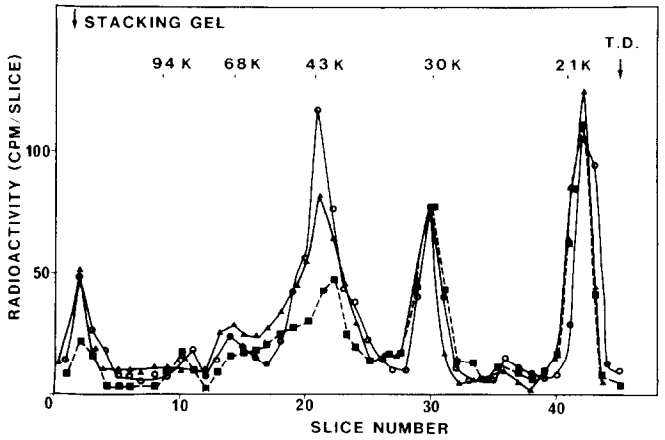

Fig. 2. SDS polyacrylamide gel electrophoretic pattern of rat liver plasma membranes labelled with [^{3}H]POB in the absence and in the presence of phentolamine. Rat liver plasma membranes were incubated with [^{3}H]POB and in the absence (○) or presence of 0.1 μM (▲) or 1 μM (■) phentolamine. The experiment was carried out as depicted in the legend of Fig. 1.

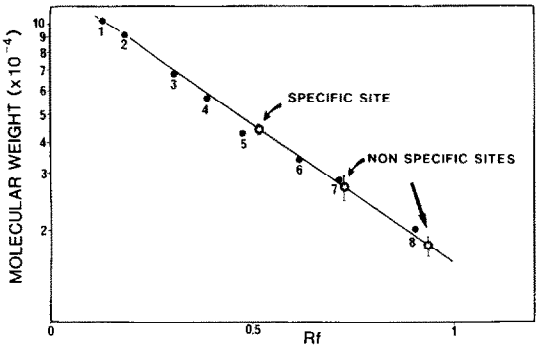

Fig. 3. Molecular weight determination of the α-adrenergic binding site. The molecular weight standards used were (1) β-galactosidase, 116,500; (2) phosphorylase B, 94,000; (3) bovine serum albumin, 68,000; (4) catalase, 57,000; (5) ovalbumin, 43,000; (6) lactate dehydrogenase, 35,000; (7) carbonic anhydrase, 30,000; (8) soybean trypsin inhibitor, 21,000. The position of the peaks are indicated on the standard curve ± S.E.M. calculated from nine experiments.

solubilized at 100° instead of 25°, a strikingly different SDS-electrophoretic pattern was observed. There was an almost total disappearance of radioactivity in the 45 K molecular weight region with a corresponding increase in the stacking gel. This peak of radioactivity present in the stacking gel was displaced by 10 μM phentolamine and corresponded to the α-adrenergic binding site. The aggregation was partly overcome by boiling the membranes in the absence of β-mercaptoethanol, or totally avoided by solubilizing the membranes for 90 min at 25°, which was the procedure routinely adopted in this study*. The other peaks of radioactivity were not modified by this treatment.

Discussion

This work represents the first determination of the subunit molecular weight for the α-adrenergic receptor of rat liver plasma membrane. By covalently labelling the α-adrenergic receptor with the specific irreversible antagonist [^{3}H]POB and characterization on SDS-gel electrophoresis, we have determined a mol. wt of 44,800 ± 1400 for the protein containing the hormone binding site. Although we did not investigate the nature of the 28,100 and 17,900 peaks of radioactivity (Fig. 1) it is clear from the inhibition experiments that they do not exhibit an α-adrenergic order of potency. In a preceding study we solubilized the hepatic α-adrenergic receptor with the non-ionic detergent Lubrol PX [5]. This procedure allowed the solubilization of the receptor in its 'native' form, and a mol. wt of 96,000 was obtained by gel filtration, and sucrose density gradient centrifugation in D_2O and H_2O after subtraction of bound detergent. One can therefore hypothesise that the hepatic α-adrenergic receptor is composed of at least two components. These could be organized in at least two different ways. The receptor may exist as a dimer composed of two equivalent 45 K subunits. Alternatively the binding site may be coupled to a protein component possibly involved in the transduction of the hormonal information or in tethering the binding site to the membrane. There is at present insufficient information available to choose between these two hypotheses.

* The α-adrenergic specific binding solubilized at 100° for 3 min did not enter DATD cross-linked gels of porosity as low as 7%, which corresponds to an exclusion limit of around mol. wt 300,000. Moreover, neither increasing the ionic strength of the solubilization medium, nor carboxyamido-methylation of the extract improved the penetration of the radioactivity within the gel.

Differences in molecular weight between native and denatured forms of a receptor have also been reported for the β-adrenergic receptor. While the native form of the receptor exhibits a molecular weight around 130,000 [12]–150,000 [1], subunits of lower molecular weight (30,000–50,000) have been observed under denaturing conditions [2, 13–6]. Thus it is difficult to ascertain whether the molecular weights determined under non-denaturing conditions truly correspond to the molecular weight of the subunit containing the receptor binding site. Various components have been implicated in the transfer of hormone signals across the plasma membrane. Binding of β-adrenergic agonists induces an increase in the apparent size of the β-adrenergic receptor that appears to correspond to a receptor interaction with another macromolecule [17]. A similar observation has been made for the α_2-adrenergic receptor of human platelet using the agonist [^{3}H]epinephrine and the antagonist [^{3}H]yohimbine. In both cases it was postulated that the GTP binding subunit remains attached to the receptor when solubilized in the presence of an agonist [18, 19]. Moreover it has been postulated for the insulin receptor that the receptor structure may be divalent for the binding hormone [20, 21].

During the course of this study, we also observed an anomalous behaviour of the α-adrenergic receptor polypeptide on gel electrophoresis under denaturing conditions. Boiling the protein samples for 3 min in 2% SDS and 5% β-mercaptoethanol resulted in an aggregation of the α-adrenergic receptor labelled with [^{3}H]POB. A similar result has been described for the acetylcholine receptor [22], the β-adrenergic receptor of frog erythrocyte [16], or the muscarinic receptor of intestinal smooth muscle [23]. This phenomenon can be overcome by solubilizing the membrane for 90 min at 25° [22, 24]. Due to these mild solubilization conditions (25° instead of 100°), we cannot eliminate a possible proteolytic conversion of a high molecular weight species into a smaller one. However, we obtained the same electrophoretic pattern when membranes were prepared in the presence of 1 mM PMSF added at all steps of the preparation.

Thus in this study, we solubilized the [^{3}H]POB labelled membranes for 90 min at 20° in 2% sodium dodecyl sulfate and 5% β-mercaptoethanol. Polyacrylamide gel electrophoresis results in three peaks of radioactivity. (Mr = 44,800 ± 1400, 28,000 ± 2600 and 17,900 ± 1200). Only the radioactivity present in the peak around 45 K is specifically displaced by incubation of the membrane with increasing concentrations of phentolamine (0.01–10 μM) and epinephrine (0.1–100 μM) prior to the labelling with tritiated phenoxybenzamine. These data indicate that the subunit which possesses the catecholamine binding site of the hepatic α_1-adrenergic receptor has a molecular weight of 44,800 ± 1400. Comparison of the current results obtained using SDS with previous findings obtained with Lubrol PX, indicates that the hepatic α-adrenergic receptor is likely to be multimeric.

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Uptake of α-difluoromethylornithine by mouse fibroblasts

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α-Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of mammalian L-ornithine decarboxylase [1]. DFMO appears to be highly specific and does not inhibit other enzymes of ornithine metabolism [2]. Covalent binding of DFMO to ornithine decarboxylase occurs when the enzyme is inactivated and both binding and inhibition are prevented by the presence of L-ornithine [3]. DFMO may prove to be a useful pharmacological agent since it inhibits the growth of certain tumors [4, 5] and parasitic protozoa [6]. It is a powerful antiproliferative agent in cell culture and depletes the cellular concentration of putrescine and spermidine [7, 8]. The inhibition of cell growth in culture and of parasites *in vivo* can be abolished by provision of exogenous polyamines suggesting that the antiproliferative effect is due to polyamine depletion [6–8]. In the present work, we have examined the uptake of DFMO into cells and its fate within the cell. The rate of uptake and the mechanism by which this is achieved are of obvious importance in the pharmacology of DFMO, particularly since DFMO is a basic amino acid analogue, and it is well known that active transport systems exist for both polyamines [9, 10] and basic amino acids [11]. If DFMO were also a substrate for these transport mechanisms, it would provide another point at which its action could be antagonized by ornithine or polyamines, but the results obtained indicate that this is not the case and that DFMO appears to enter the cell by a non-active process.

Materials and methods

Materials. DL-[5-^{14}C]DFMO (60 mCi/mmole) was obtained from Amersham/Searle, Arlington Heights, IL. DL-[U-^{14}C]Lysine (305 mCi/mmole) and L-[1-^{14}C]ornithine (59 mCi/mmole) were obtained from the New England Nuclear Corp., Boston, MA. All polyamines and amino acids were purchased from the Sigma Chemical Co., St. Louis, MO. Tissue culture dishes were purchased from Falcon Plastics, Inc., Cockeysville, MD. *n*-Butyl *p*-hydroxybenzoate was purchased from Eastman-Kodak, Rochester, NY. All sera and tissue culture media were purchased from Flow Laboratories, McLean, VA. All other chemicals were of reagent grade. Scintillation mixture (ACS-II) was from Amersham/Searle.

Cell culture. Stock and experimental cultures of SV-40 virus transformed mouse embryo fibroblasts were grown in Dulbecco's modified Eagle's medium with 3% horse serum, 2% fetal calf serum, 36 mM $NaHCO_3$, penicillin (0.09 units/ml), streptomycin (0.09 mg/ml), and 2 μM *n*-butyl *p*-hydroxybenzoate. All cultures were grown in a humidified atmosphere of 10% CO_2 at 37°. Stock cultures were subcultured every 3 days by trypsinization with a 0.25% (w/v) solution and replated in fresh medium at 3×10^5 cells/10 cm diameter tissue culture dish. Experimental cultures were seeded at 5×10^4 cells/3.5 cm diameter tissue culture dish. Proliferation was allowed to occur for 48 hr after which the medium was removed and the cell