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Glycoprotein nature of α_2-adrenergic receptors labeled with *p*-azido[^{3}H] clonidine in calf retina membranes

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α_2-Adrenergic receptors in calf retina membranes can be specifically labeled with the tritiated agonist *p*-azido[^{3}H]clonidine. Saturation binding in the dark occurs with high affinity (1.3 ± 0.3 nM) to a single class of sites (1122 ± 67 fmol/mg protein). Irradiation of the membrane-bound radioligand results in the labeling of a peptide band with an apparent size of 65 kDa and a characteristic pharmacological profile for an α_2-adrenergic receptor. The carbohydrate moieties of the α_2-receptor are characterized by lectin affinity chromatography and glycosidase treatment. The Nonidet P-40-solubilized, *p*-azido[^{3}H]clonidine-labeled receptors are completely retained by Con A- as well as WGA-Sepharose columns. Neuraminidase, α-mannosidase and TFMS do not affect the electrophoretic mobility of the receptor on SDS-PAGE whereas endoglycosidase F reduces the apparent size to 45 kDa.

α_2-Adrenergic receptor; *p*-Azido[^{3}H]clonidine; Lectin affinity chromatography; Glycosidase; (Calf retina)

1. INTRODUCTION

α_2-Adrenergic receptors are located at both pre- and postjunctional sites in the central nervous system and peripheral organs, as well as non-innervated tissues such as blood platelets [1]. Species and tissue differences of these receptors have already been documented with respect to their ligand-binding properties and their ability to produce different responses at the membrane level [2]. Photoaffinity labeling could constitute a powerful tool for the investigation of possible differences in the molecular properties of these receptors, since they allow apparent M_r determinations by SDS-PAGE.

Here, we characterise the glycoprotein nature of [^{3}H]PAZ-labeled α_2-adrenergic receptors in calf retina membranes by both lectin-Sepharose affinity chromatography and endo- and exoglycosidase treatments. The vertebrate retina is developed embryologically from the brain and offers a convenient model system for investigation of the physiology and biochemistry of specific neuronal systems [3]. A considerable advantage of this system also resides in the high initial α_2-receptor concentration, the preponderance of high-affinity sites for the agonist [^{3}H]PAZ (about 80% of the total population), so that no purification step is required prior to photoaffinity labeling [4].

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Abbreviations: [^{3}H]PAZ, *p*-azido[^{3}H]clonidine; PMSF, phenylmethylsulfonyl fluoride; Con A, concanavalin A; WGA, wheat germ agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Materials and membrane preparation*

[^{3}H]PAZ (41 Ci/mmol) and endoglycosidase F were supplied by New England Nuclear. Neuraminidase (from *Clostridium perfringens* type X) and α-mannosidase were from Sigma. PD-10 columns and Sepharose CL 4B were obtained from Pharmacia. All other materials were from sources described in [4].

Calf eyes were obtained from a local slaughterhouse and membranes were prepared as in [4].

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2.2. *[^{3}H]PAZ binding*

Radioligand binding was performed essentially as described [4]. Briefly, 50 μl [^{3}H]PAZ (0.1–10 nM) and 50 μl buffer A [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 100 μM PMSF] or phentolamine (10 μM) were added in dim light to a 400 μl membrane suspension (0.25–0.5 mg/ml) in buffer A. After 90 min incubation at 25°C, the samples were filtered, washed and assessed for radioactivity as in [4].

2.3. *Photoaffinity labeling*

Calf retina membranes (0.2–0.3 mg/ml) in buffer A were incubated in dim light with 4 nM [^{3}H]PAZ for 90 min at 25°C in a final volume of 10 ml. 0.1 ml of 50 mM reduced glutathione was then added to the samples as a scavenger. The samples were transferred into plastic petri dishes (10 cm diameter) and irradiated for 10 min with ultraviolet light (254 nm). The membranes were subsequently washed three times by centrifugation at 39 000 × g for 20 min and resuspended in fresh buffer A, prior to further treatment.

2.4. *Lectin affinity chromatography*

Con A was prepared according to Van Driessche et al. [5] and stored in 60% $(NH_4)_2SO_4$. WGA was purified according to Block and Burger [6].

The lectins were coupled to Sepharose CL 4B as described by Jacobs and Cuatrecasas [7]. Prior to use, lectin columns with bed volumes of about 10 ml were extensively washed with equilibration buffer [10 mM Tris-HCl (pH 7.4), 0.2% Nonidet P-40, 145 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and the following protease inhibitors: 100 μM PMSF, 100 μM benzamidine, 5 μg/ml pepstatin and 5 μg/ml leupeptin]. [^{3}H]PAZ-labeled membranes were solubilized in Nonidet P-40 buffer [0.8% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 145 mM NaCl and the above protease inhibitors] for 1 h with constant stirring at room temperature. The preparation was then centrifuged at 48 000 × g for 30 min. After application of the solubilized membranes, the lectin columns were slowly eluted with equilibration buffer and the radioactivity content of the collected fractions was assayed by scintillation counting. When their radioactivity content had returned to the baseline, the columns were subsequently eluted with equilibration buffer containing 0.3 M α-methyl-D-mannopyranoside (for Con A columns) or 0.3 M *N*-acetylglucosamine (for WGA columns). Flow-through fractions and specifically eluted fractions were pooled, desalted with a PD-10 column and lyophilized overnight.

2.5. *Deglycosylation procedure*

2.5.1. Exoglycosidase treatment

[^{3}H]PAZ-labeled membranes were suspended in 100 mM (pH 5.0) or 50 mM (pH 4.5) sodium acetate buffer supplemented with protease inhibitors for neuraminidase and α-mannosidase treatment, respectively. The reaction was started by addition of 0.5–1 U/ml neuraminidase or 3–4 U/ml α-mannosidase (final concentrations) for 6 h at 37°C. The reactions were stopped by centrifugation and subsequent suspension in SDS-PAGE sample buffer [50 mM Tris-HCl (pH 8.8), 10% SDS, 5 mM EDTA, 10 mM DTT, 10% glycerol].

2.5.2. Endoglycosidase F treatment

[^{3}H]PAZ-labeled membranes were solubilized in 100 mM sodium phosphate (pH 6.1), 50 mM EDTA, 0.8% Nonidet P-40, 0.1% SDS as described in section 2.4. The soluble fraction (1 mg protein/ml) was incubated with 10 U/ml (final concentration) endoglycosidase F in a total volume of 100 μl for 5 h at 37°C. The samples were then desalted on a PD-10 column to exchange the buffer with an aqueous solution of 0.01% SDS, and lyophilized overnight prior to SDS-PAGE.

2.5.3. TFMS treatment

The method of Edge et al. [8] was employed with some minor modifications described by El Battari et al. [9].

2.6. *SDS-PAGE*

SDS-PAGE was performed according to Laemmli [10] using 10% acrylamide slab gels. Following electrophoresis, the gels were prepared for fluorography using Enlightning (New England Nuclear) according to the manufacturer's instructions.

3. RESULTS AND DISCUSSION

The specific binding of [^{3}H]PAZ to calf retina membranes is reversible in the dark and occurs to one class of non-cooperative sites. The Scatchard plot of [^{3}H]PAZ saturation binding data is linear ($r = 0.96$, $n_H = 1.02$) with an equilibrium dissociation constant of 1.3 ± 0.3 nM ($n = 3$) and a total number of binding sites (B_{max}) of 1122 ± 67 fmol/mg protein (fig.1). The equilibrium dissociation constant is in good agreement with the value for unlabeled clonidine for the high-affinity sites, and the number of sites corresponds to the amount of sites for the radiolabeled agonist [^{3}H]UK 14304

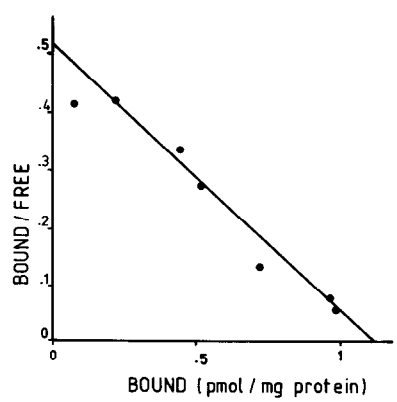

Fig.1. Scatchard plot of [^{3}H]PAZ saturation binding to calf retina membranes. Calf retina membranes were incubated in the dark with increasing concentrations of [^{3}H]PAZ (0.1–10 nM). Data shown represent specific binding as described in section 2 (duplicate determinations).

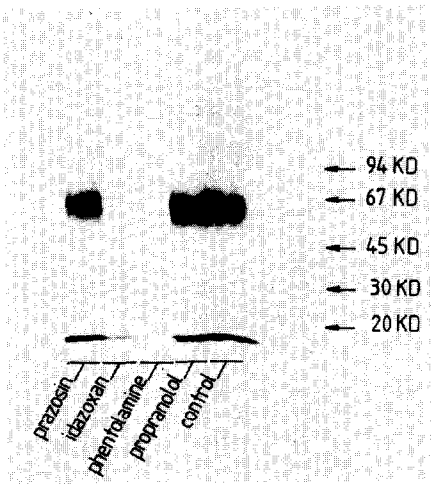

Fig.2. Autoradiograph of the pharmacological specificity of [^{3}H]PAZ photoaffinity labeling. Membranes were incubated with 4 nM [^{3}H]PAZ in the presence of 1 μM prazosin, 10 μM idazoxan, 10 μM phentolamine, 10 μM propranolol, or in the absence of competitor (control). Photolysis, SDS-PAGE and autoradiography were performed as described in section 2.

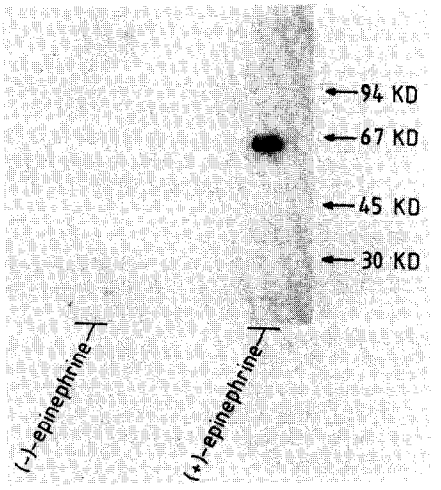

Fig.3. Stereoselective inhibition of [^{3}H]PAZ incorporation by epinephrine. Membranes were incubated with [^{3}H]PAZ in the presence of 1 μM (−)-epinephrine or 1 μM (+)-epinephrine and treated further as in fig.2.

(1.3 pmol/mg protein) in this tissue [4]. The equilibrium dissociation constant for [^{3}H]PAZ is one to two orders of magnitude lower than the recently reported values for the photoreactive antagonists [^{3}H]SKF-102229 and ^{125}I-rau-AzPC and the irreversible antagonist [^{3}H]phenoxybenzamine [11–13].

Irradiation of [^{3}H]PAZ (4 nM)-labeled calf retina membranes results in covalent binding of the radioligand. The photolyzed membranes can be solubilized and subjected to SDS-PAGE. Whereas successful affinity labeling by antagonists often requires a preliminary partial purification step for α_2-adrenergic receptors [11–13], this step is not necessary for [^{3}H]PAZ labeling in calf retina membranes. Indeed, the autoradiogram of the gel shows one broad peptide band with an apparent molecular mass 64 kDa (fig.2). The binding characteristics of the 64 kDa peptide correspond to those of an α_2-adrenergic receptor. [^{3}H]PAZ labeling is inhibited by the selective α_2-adrenergic antagonist idazoxan (10 μM) as well as by the non-α-adrenergic subtype selective antagonist phentolamine (10 μM), but not by the β-adrenergic antagonist propanolol (10 μM) and the α_1-adrenergic antagonist prazosin (1 μM) (fig.2). In addition, labeling of this peptide is inhibited in a stereoselective manner by the agonist epinephrine (fig.3).

The [^{3}H]PAZ-labeled calf retina α_2-receptors display similar molecular sizes to those recently described for photoreactive antagonists in, for example, human platelets or porcine brain [11,12]. The fact that antagonist- and agonist-labeled receptors show comparable bands on SDS-PAGE, indicates that the apparent molecular size of the functional G_i-coupled receptors is the same, or at least very similar to that of the non-coupled receptors [14]. Despite the similarities in molecular size, the pharmacological binding properties of α_2-adrenergic receptors have been reported to be heterogeneous [2]. For instance, α_2-adrenergic receptors in calf retina [4] display an antagonist potency order (idazoxan = phentolamine > rauwolscine) which is different from that observed in human platelets (rauwolscine = phentolamine > RX 781094) [2]. Accordingly, a putative classification of the α_2-receptors will have to await the comparative characterization of receptor amino acid sequences by cloning techniques.

Sugar moieties on the α_2-adrenergic receptor were first characterized by investigating their retention on immobilized Con A and WGA lectins. The affinity-labeled membranes were first solubilized in 0.8% Nonidet P-40 and subsequently

diluted to obtain a final detergent concentration of 0.2%. This reduction in detergent concentration allowed more efficient retention of the [^{3}H]PAZ-labeled peptide by the lectin-Sepharose columns. As shown in fig.4, both the Con A- (which binds specifically to mannose-containing carbohydrates) [15] and the WGA-Sepharose (which interacts with *N*-acetylglucosamine) [15] columns are able to retain the total α_2-adrenergic receptor population; i.e. the 64 kDa peptide is absent in the flow-through fractions. Subsequent elution of the columns with 0.3 M α-methyl-D-mannopyranoside (for Con A-Sepharose) or 0.3 M *N*-acetylglucosamine (WGA-Sepharose) results in the elution of the absorbed 64 kDa peptide (fig.4).

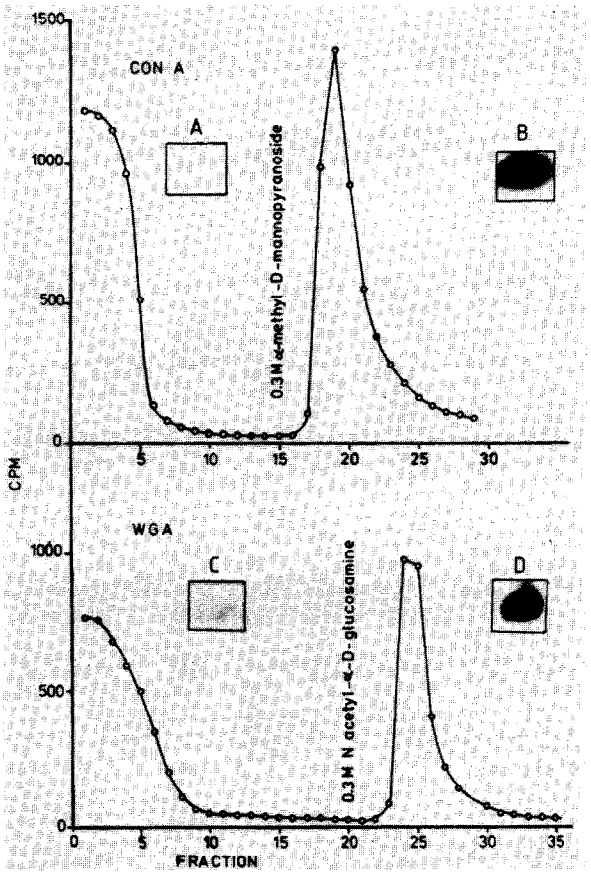

Fig.4. Lectin affinity chromatography of [^{3}H]PAZ-labeled α_2-adrenergic receptors. Membranes were labeled with [^{3}H]PAZ, solubilized with Nonidet P-40 and applied onto Con A-Sepharose and WGA-Sepharose columns as described in section 2. After the radioactivity of the flow-through had returned to the baseline level, the columns were eluted with the complementary sugars. The flow-through and specifically eluted fractions were pooled, desalted, lyophilized and subjected to SDS-PAGE. The autoradiograms of these gels are shown in the insets. Con A chromatography: A, fractions 1–8; B, fractions 16–25. WGA chromatography: C, fractions 1–8; D, fractions 22–30.

Further information on the glycoprotein nature of the α_2-adrenergic receptors was obtained by investigating the effect of exoglycosidases on their apparent molecular mass. Following [^{3}H]PAZ labeling, membranes were treated with the neuraminidase (to hydrolyse terminal sialic acid residues) and α-mannosidase (to hydrolyse terminal mannose residues) for 6 h as outlined in section 2. Both treatments do not affect the electrophoretic mobility of the receptor (fig.5). When the incubation time is increased up to 24 h there is still no alteration in electrophoretic mobility (not shown). These findings, together with the results of the lectin affinity chromatography, suggest that the carbohydrates on these receptors are all bisected biantennary complex type chains [15]. The lack of effect of neuraminidase is rather unexpected, since complex type carbohydrates usually contain terminal sialic acids and since the removal of even a single sialic acid residue already produces a relatively dramatic change in the electrophoretic mobility of glycoproteins [15]. The apparent

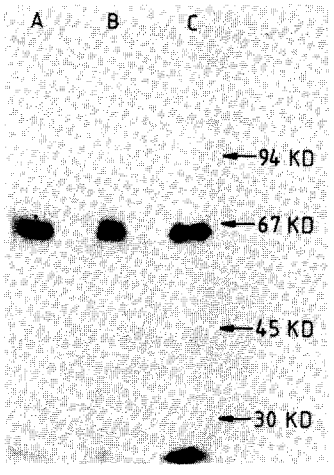

Fig.5. Exoglycosidase treatment of [^{3}H]PAZ-labeled α_2-adrenergic receptors. Membranes were labeled with [^{3}H]PAZ, washed with sodium acetate buffer and subsequently incubated with buffer only (lane A), 0.5–1 U/ml neuraminidase (lane B) or 3–4 U/ml α-mannosidase (lane C) for 5 h and the reaction was stopped by centrifugation.

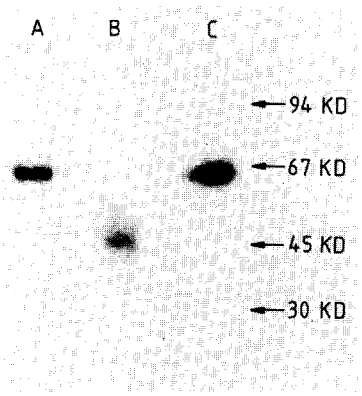

Fig.6. Effect of endoglycosidase F and TFMS on [^{3}H]PAZ-labeled α_2-adrenergic receptors. Membranes were labeled with [^{3}H]PAZ and solubilized with SDS/Nonidet P 40. The soluble fraction was incubated in buffer only (lane A), in the presence of 10 U/ml endoglycosidase F for 5 h (lane B), or lyophilized overnight and incubated with 2:1 (v/v) TFMS/anisole (lane C) as described by Edge et al. [8].

absence of terminal sialic acids might be due to a single interruption in the highly ordered enzymatic reactions required to produce a complex oligosaccharide chain with terminal sialic acid residues. Another possibility is that sialic acid is actually present, but is not susceptible to neuraminidase hydrolysis. For example, sialyl-*N*-acetylgalactosamine linkages are relatively resistant to neuraminidase [15].

[^{3}H]PAZ-labeled membranes were also treated respectively with endoglycosidase F (to remove both complex and high-mannose type carbohydrate chains which are *N*-linked to asparagine) [15] and TFMS (to remove the chains which are *O*-linked to serine or threonine) [8]. As depicted in fig.6, endoglycosidase F treatment produces a dramatic decrease in the apparent molecular mass of the α_2-adrenergic receptor, whereas TFMS has no perceptible effect. The final product of endoglycosidase F treatment is a 45 kDa peptide (fig.6). Prolongation of the incubation times does not further affect the electrophoretic mobility of the receptor on SDS-PAGE. This value is similar to that expected for the protein backbone in the human platelet receptor on the basis of its DNA sequence [16]. Further work has to be elaborated to determine if the carbohydrate chains on the α_2-receptors are necessary for binding of the hormone, transmembrane signalling or mobilization of second messengers.

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