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Identification of the serotonin-S_2 receptor ligand binding site by photoaffinity labelling with 7-azido-8-[^{125}I]ketanserin ([^{125}I]AZIK)

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7-Azido-8-[^{125}I]ketanserin ([^{125}I]AZIK) was characterized as a potent photoaffinity probe for serotonin-S_2 receptors. In reversible binding experiments, [^{125}I]AZIK bound with high affinity ($K_d = 0.69$ nM) to rat frontal cortex membranes. When incubation with [^{125}I]AZIK was followed by UV irradiation, the binding was found to be irreversible. Protection experiments with various drugs demonstrated the serotonin-S_2 nature of the photoaffinity labelling. SDS-polyacrylamide gel electrophoresis of the photolabelled membranes allowed one to identify the serotonin-S_2 receptor ligand binding site as a single polypeptide with a molecular mass of approx. 67 500 Da. [^{125}I]AZIK will be a valuable tool for the elucidation of the serotonin-S_2 receptor structure.

Serotonin-S_2 receptor; Photoaffinity labeling; 7-Azido-8-ketanserin; SDS electrophoresis

1. INTRODUCTION

Several serotonin-binding sites have been identified in various brain areas using in vitro radioligand binding assays. Amongst these, the serotonin-S_2 receptor is pharmacologically the best characterized. The receptor shows high affinity for several serotonin antagonists and a low affinity for serotonin. Numerous functional correlates were established between the potency of antagonists to inhibit serotonin-mediated responses in pharmacological tests and their potency to inhibit radioligand binding to serotonin-S_2 receptors [1].

Serotonin-S_2 receptors were first identified in vitro in rat frontal cortex tissue with [^{3}H]spiperone [2]. Later on, [^{3}H]ketanserin was introduced as the first selective serotonin-S_2 receptor ligand [3]. Recently, a derivative of ketanserin, 7-[^{3}H]aminoketanserin, was characterized as a potent radioligand with very low non-specific binding [4] and an iodinated analogue, 7-amino-8-[^{125}I]ketanserin ([^{125}I]AMIK), was introduced as the first selective serotonin-S_2 radioligand with high specific radioactivity [5].

The isolation, purification and molecular characterization of serotonin-S_2 receptors have only very recently been tackled. Solubilization of biologically active serotonin-S_2 receptors was achieved using lysophosphatidylcholine as detergent [6]; however, solubilization yields were very low. Optimal solubilization was obtained using a mixture of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and salt [4,7]. These solubilized receptors could also be reconstituted into phospholipid vesicles [8].

A further step towards the elucidation of the serotonin-S_2 receptor structure was made possible by the introduction of the potent photoaffinity probe 7-azidoketanserin [9]. This new compound was shown to irreversibly inhibit the binding to serotonin-S_2 and histamine-H_1 receptors following UV (ultraviolet) irradiation. Differential pho-

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toinactivation of either receptor could be achieved using a combination of selective brain tissues and specific displacers [10].

In the present report we describe the binding characteristics of a radioiodinated derivative of 7-azidoketanserin (7-azido-8-[^{125}I]ketanserin ([^{125}I]AZIK)) and its use in the identification of the serotonin-S_2 receptor ligand binding site by photoaffinity labelling and subsequent SDS-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Drugs and chemicals*

7-azido-8-[^{125}I]ketanserin (7-azido-3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-8-[^{125}I]iodo-2,4-(1*H*,3*H*)quinazolinedione; ~2175 Ci/mmol) was custom synthesized and HPLC-purified by Amersham (Amersham, England). Acrylamide, SDS and Coomassie blue R 250 were supplied by LKB (Bromma, Sweden); serotonin, imidazole, trichloroacetic acid, periodic acid, sulfosalicylic acid, *N,N'*-diallyltartardiamide, bacitracin and benzamidine by Janssen Chimica (Beerse, Belgium); EDTA, bovine serum albumin (BSA) and soybean trypsin inhibitor by Sigma (St. Louis, USA) and bufotenine and phenylmethylsulfonyl fluoride by Serva (Heidelberg, FRG). Protein molecular mass standards were obtained from BioRad (Richmond, USA). All drugs were kindly provided by their companies of origin.

2.2. *Tissue preparation*

Female Wistar rats were killed by decapitation and their brains were removed from the skull. Prefrontal cortex was rapidly dissected and immediately homogenized at a dilution of 1:10 (w/v) (original wet wt of tissue per volume) in 0.25 M sucrose. A total mitochondrial plus microsomal (M + L + P)-fraction was prepared by differential centrifugation as described [3]. The final pellet was suspended in 50 mM Tris-HCl, pH 7.7, at the indicated dilutions. When experiments were performed in the presence of protease inhibitors, the buffers contained 5 mM EDTA, 0.05 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml bacitracin and 0.01 mg/ml soybean trypsin inhibitor.

2.3. *Reversible binding assay*

Aliquots (500 μl) of an M + L + P-fraction (dilution 1:500, w/v) were incubated in the dark at 37°C for 15 min with [^{125}I]AZIK and unlabelled drugs or their solvent in a total volume of 550 μl. Incubation was stopped by addition of 3 ml ice-cold Tris-HCl, pH 7.7, containing 120 mM sodium chloride and followed by rapid filtration over Whatman GF/C glass fibre filters using a Multividor 40-well filtration manifold (Janssen Scientific Instruments Division, Beerse, Belgium). Filters were rinsed twice with 5 ml ice-cold buffer and were then counted in an LKB 1271 gamma counter.

2.4. *Photolabelling*

1 ml aliquots of an M + L + P-fraction (dilution 1:100, w/v) were incubated in the dark at 37°C for 15 min with [^{125}I]AZIK and unlabelled drugs or their solvent. The samples were then cooled to 4°C and centrifuged at 39100 × *g* (r_{max}) for 10 min. The pellets were rehomogenized using a glass-teflon homogenizer in 50 mM Tris-HCl, pH 7.7, containing 10 mg/ml BSA at a final dilution of 1:300 (w/v). This suspension was irradiated at 4°C for 3 min with long-wavelength UV-light (366 nm) in quartz test tubes at an average distance of 4 cm from a Camag Universal UV-lamp (Camag, Muttenz, Switzerland). During irradiation the samples were continuously mixed using a multiaxle rotating mixer. Following irradiation the samples were filtered through Whatman GF/C glass fibre filters. To remove unbound and reversibly bound radioligand, filters were incubated at 37°C for 5 min with 10 ml of 50 mM Tris-HCl, pH 7.7, containing 120 mM sodium chloride. This procedure was repeated four times in total after which the filters were counted as described above.

2.5. *SDS-polyacrylamide gel electrophoresis*

1 ml aliquots of an M + L + P-fraction (dilution 1:100, w/v) were photolabelled as described above. Following UV irradiation, the suspensions were diluted up to 1:1000 (w/v) with 50 mM Tris-HCl, pH 7.7, and incubated at 37°C for 15 min with 1 μM pipamperone to remove reversibly bound radioligand and then cooled to 4°C. The suspensions were centrifuged at 39100 × *g* (r_{max}) for 10 min; the pellets were washed with the same

volume of Tris-buffer and again centrifuged. The washed pellets were then transferred to Eppendorf microtubes with 1 ml of Tris-buffer and centrifuged at 13000 rpm for 10 min. The pellets were solubilized in 50 μl SDS sample buffer (10 mM imidazole-phosphate, pH 7, 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol) and denatured by boiling for 3 min.

10 μl aliquots were loaded on 2 mm thick 7.5% acrylamide slab gels (7.5% (w/v) acrylamide, 0.3% (w/v) *N,N'*-diallyltartardiamide, 0.1% (w/v) SDS and 50 mM imidazole-phosphate, pH 7) together with protein molecular mass markers. Electrophoresis with a continuous buffer system was performed at 4°C for 105 min with a constant current of 100 mA using an LKB 2117 Multiphor and an LKB 2197 power supply. At the end of the run, the gel was fixed in a solution of 11.4% (w/v) trichloroacetic acid, 3.4% (w/v) sulfosalicylic acid and 30% (v/v) methanol for 1 h at room temperature. The protein markers were stained with 0.25% (w/v) Coomassie blue R 250 in methanol/water/acetic acid (46:46:8, v/v) for 2 h and destained in ethanol/water/acetic acid (30:60:10, v/v). The parts of the gel, containing the labelled samples were soaked in a preserving solution (ethanol/water/acetic acid/glycerol, 30:50:10:10, v/v) for 1 h, dried and exposed at −70°C to Kodak X-omatic film using Kodak X-omatic regular intensifying screens. In some experiments the gels were cut into 2 mm slices and counted for radioactivity.

3. RESULTS

3.1. *Reversible binding of [^{125}I]AZIK*

Fig.1 shows a typical saturation curve for reversible [^{125}I]AZIK binding to an M + L + P-fraction from rat frontal cortex. The binding was saturable in the nanomolar range. Non-specific binding was about 50% of total binding at routine concentrations (0.3 nM) increasing to more than 60% at full saturation. Scatchard analysis of the specific binding data showed a straight line; from four independent experiments, an equilibrium dissociation constant (K_d) of 0.69 ± 0.21 nM and a maximal number of binding sites (B_{max}) of 21.0 ± 3.5 fmol/mg tissue could be derived. Several compounds were tested for their potency to inhibit reversible [^{125}I]AZIK binding. For all the compounds, monophasic inhibition curves were obtained. The derived IC_{50} values are summarized in table 1. Serotonin antagonists (ketanserin, pipamperone and methysergide) inhibited [^{125}I]AZIK binding at nanomolar concentrations; serotonin agonists (serotonin and bufotenine) were active at micromolar concentrations. Compounds with other pharmacological profiles (prazosin, α_1-adrenergic antagonist; pyrilamine, histamine-H_1 antagonist; and domperidone, dopamine-D_2 antagonist) were poorly or not active.

3.2. *Photolabelling with [^{125}I]AZIK*

As shown in fig.2, the amount of specific photolabelling increased with the [^{125}I]AZIK con-

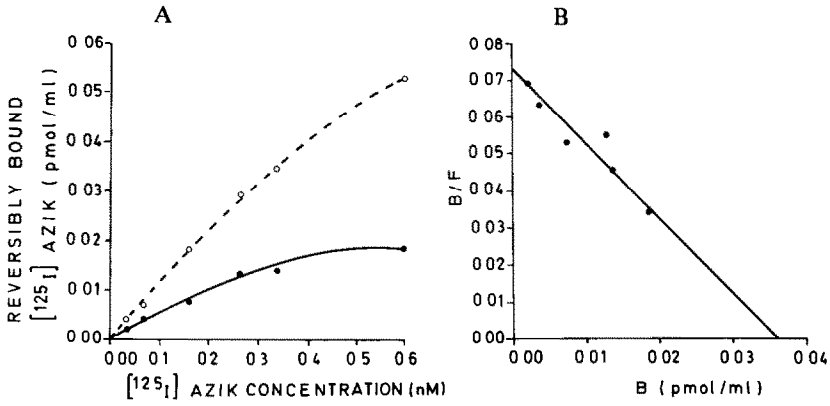

Fig.1. (A) Typical saturation curves of reversible total (○---○) and specific (●——●) [^{125}I]AZIK binding to an M + L + P-fraction of rat frontal cortex. (B) Scatchard analysis of specific binding. Binding was performed as described in section 2. Specific binding was calculated as the difference between total binding and binding in the presence of a thousandfold excess of pipamperone.

Table 1

Competition of various compounds in reversible [^{125}I]AZIK binding and potencies of these compounds for protection against photolabelling with [^{125}I]AZIK in a membrane preparation of rat frontal cortex

Compound	$-\log IC_{50}$ (M)	
	Reversible binding	Photolabelling
Ketanserin	8.90 ± 0.07	8.77 ± 0.58
Pipamperone	8.52 ± 0.18	8.68 ± 0.11
Methysergide	8.36 ± 0.16	8.76 ± 0.06
Bufotenine	6.22 ± 0.25	6.74 ± 0.19
Serotonin	5.75 ± 0.14	5.77 ± 0.03
Domperidone	7.02 ± 0.03	6.58 ± 0.05
Prazosin	6.24 ± 0.40	5.10 ± 0.28
Pyrilamine	5.85 ± 0.07	6.20 ± 0.14

Reversible binding assays and photolabelling were performed as described in section 2 using 0.3 nM final [^{125}I]AZIK concentration. Values were obtained from two independent experiments and are expressed as mean ± SD

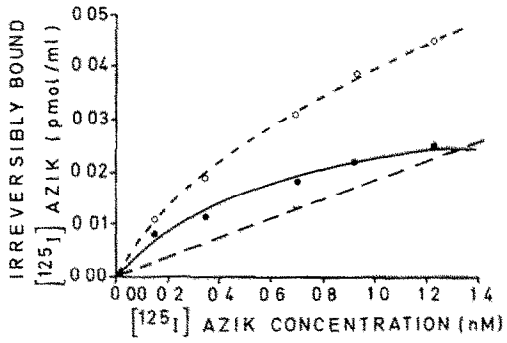

Fig.2. Photolabelling of rat frontal cortex membranes as a function of [^{125}I]AZIK concentration (○---○, total photolabelling; △---△, non-specific photolabelling; ●——●, specific photolabelling). Photolabelling was performed as described in section 2 using a thousandfold excess of pipamperone to define specific photolabelling.

centration, reaching a maximum at 1.2 nM radioligand concentration. To obtain the optimal ratio between specific and total photolabelling it was necessary to remove the excess of free azide

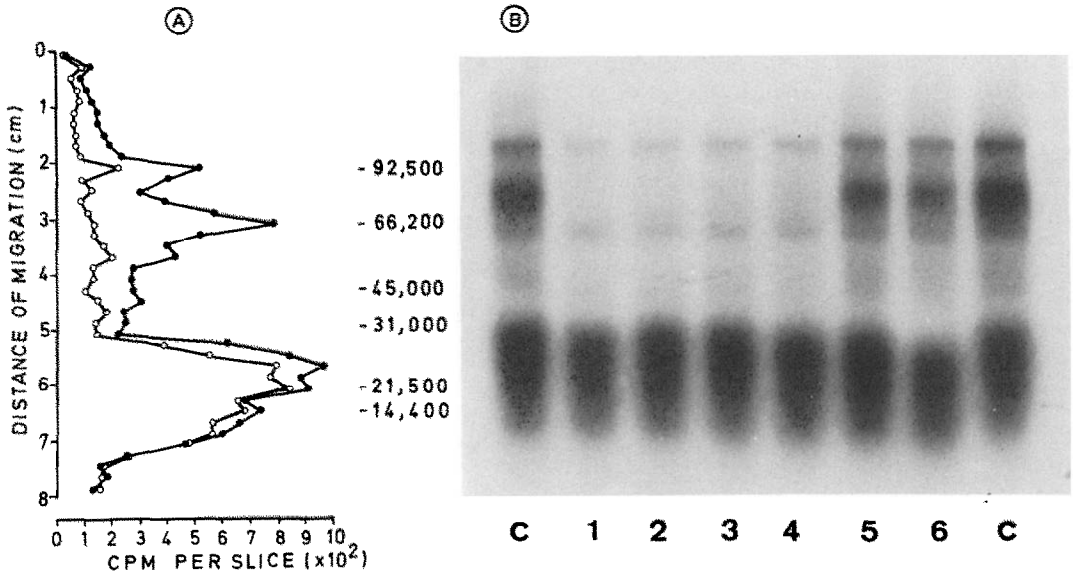

Fig.3. SDS-polyacrylamide gel electrophoresis of photolabelled rat frontal cortex membranes. Membranes were photolabelled with 0.5 nM [^{125}I]AZIK in the presence of a protease inhibitor mixture. (A) Part of the gel was sliced and counted: (●) control, (○) in the presence of 0.1 μM pipamperone. (B) Autoradiography of the gel after photolabelling in the presence of different compounds: c, control; 1, 0.1 μM pipamperone; 2, 0.1 μM methysergide; 3, 10 μM bufotenine; 4, 10 μM serotonin; 5, 0.1 μM pyrilamine; 6, 0.1 μM prazosin. Protein molecular mass markers (Da): phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400).

after the incubation, prior to UV irradiation. A further increase could be obtained from the inclusion of BSA as a free radical scavenger during photolysis. Using these optimal conditions in routine experiments (0.3 nM [^{125}I]AZIK), specific photolabelling reached about 70% of total photolabelling.

Several compounds were tested for their potency to protect the receptor against photolabelling with [^{125}I]AZIK. They were included at increasing concentrations during the incubation preceding the centrifugation step. The derived IC_{50} values are shown in table 1 and corresponded to those obtained for the reversible binding of [^{125}I]AZIK.

3.3. *SDS electrophoresis*

Aliquots of an M + L + P-fraction of rat frontal cortex (including protease inhibitors) were photolabelled using 0.5 nM [^{125}I]AZIK and subsequently subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. Following electrophoresis, part of the gel was used for autoradiography and the other part was sliced and counted for radioactivity. The results of a typical experiment are shown in fig.3. Specific serotonin-S_2 receptor photolabelling was concentrated in one major band corresponding to a molecular mass of 67595 ± 1324 Da (mean ± SD, 7 independent determinations). Non-specific photolabelling was recovered in a broad band in the lower molecular mass region of the gel and in three faint bands with molecular masses of approx. 95, 52 and 34 kDa. Essentially the same results were obtained when protease inhibitors were omitted from the incubation mixtures or when 10% instead of 7.5% gels were used (not shown). Fig.3B shows the results of an experiment in which several compounds were included in the incubation, before photolysis. Radioactivity in the 67500 Da band could only be displaced by serotonin-S_2 antagonists (methysergide and pipamperone) and serotonin agonists (serotonin and bufotenine); the other compounds were poorly or not active.

4. DISCUSSION

Since [^{125}I]AMIK (an iodinated derivative of 7-aminoketanserin) was found to be a potent, high-affinity ligand for the serotonin-S_2 receptor [5], it was speculated that an iodinated analogue of 7-azidoketanserin (a compound, previously shown to irreversibly block the serotonin-S_2 receptor after UV photolysis [9]) might be useful in photoaffinity labelling experiments. In the present report, [^{125}I]AZIK was characterized as a high affinity ligand (K_d = 0.69 nM), capable of binding irreversibly to serotonin-S_2 receptors following UV irradiation.

The extent of specific photoaffinity labelling was dependent on azide concentration. At saturation, about 25% of the reversible specific binding sites were photolabelled. This compares favourably to the yields obtained with photoaffinity systems for other neurotransmitter receptors (e.g. [11,12]). Part of the specific binding may have been lost by dissociation of the ligand during centrifugation, prior to UV irradiation. This experimental step was however necessary in order to keep non-specific photolabelling at a minimum.

Analysis of the photolabelled membranes by SDS electrophoresis showed specific incorporation of [^{125}I]AZIK into one band with a molecular mass of 67500 Da. Displacement of radioactivity from this band revealed a clear serotonin-S_2 profile. Since no additional specifically labelled bands were found (either in the presence or absence of protease inhibitors) it may be concluded that this band corresponds to the serotonin-S_2 receptor ligand binding site. Whether or not this binding site is part of a larger protein complex remains to be elucidated. Previous molecular mass estimates by gradient sedimentation of solubilized serotonin-S_2 receptors gave a value of approx. 80 kDa [7]. Unfortunately in these experiments the contribution of the bound detergent could not be determined. Anyhow since solubilization was performed using a non-denaturing detergent (CHAPS/salt), and the value obtained in the present report was close to the one, it might tentatively be concluded that the serotonin-S_2 receptor consists of only one polypeptide. Recently, a study using radiation inactivation revealed a molecular mass of around 60 kDa for the serotonin-S_2 receptor ligand binding site [13]. Based on the monoexponential decay curves they found in their experiments, these authors too concluded that the serotonin-S_2 binding site probably does not involve a high molecular mass complex.

In summary, we demonstrated that [^{125}I]AZIK is a very potent photoaffinity probe for the serotonin-S_2 receptor. Since photoaffinity labelling

can overcome a lot of detection problems during purification and characterization of a receptor protein, [^{125}I]AZIK will be a valuable tool in the search for the molecular structure of the serotonin-S_2 receptor.

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