Differential photoaffinity labelling of serotonin-S_2 receptors and histamine-H_1 receptors using 7-azidoketanserin

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Received 17 January 1985

7-Azidoketanserin, a potent photoaffinity probe for serotonin-S_2 receptors was shown to irreversibly photoinactivate histamine-H_1 receptors as well. The photolabelling of H_1-receptors could be prevented by several selective histamine-H_1 antagonists. In guinea pig cerebellum, a brain area that is highly enriched in H_1-receptors, photolabelling could be directed exclusively to these receptors by adding a high concentration of pipamperone, which selectively blocks serotonin-S_2 receptors. In rat pre-frontal corex, a region that is enriched in serotonin-S_2 receptors, pyrilamine was used to block H_1-receptors, thereby directing the photolabelling exclusively to S_2-receptors.

7-Azidoketanserin *Photoaffinity labeling* *Serotonin-S_2 receptor* *Histamine-H_1 receptor* *[^{3}H]Spiperone* *[^{3}H]Pyrilamine*

1. INTRODUCTION

Serotonin-S_2 receptors were identified in receptor binding studies using [^{3}H]spiperone or [^{3}H]ketanserin as the ligand [1,2]. Their physiological role in the antagonism of several serotonergic effects as measured both in vitro and in vivo has been thoroughly investigated [1,3].

Histamine-H_1 receptors were first identified in in vitro binding studies using the guinea pig ileum [4]. Later on, histamine-H_1 receptors were demonstrated in the brain and in several peripheral tissues using [^{3}H]pyrilamine, a selective H_1-antagonist [5,6]. In the periphery, several roles for H_1-receptors have been identified. In the brain, however, the precise role of H_1-receptors is not clear although they seem to be involved in sedation, one of the most prominent effects of histamine antagonists [7].

The regional distribution of serotonin-S_2 and histamine-H_1 receptors markedly differs in the rat and guinea pig brain. The guinea pig cerebellum is highly enriched in histamine receptors but is practically devoid of serotonin receptors. By contrast, in the rat pre-frontal cortex a large number of serotonin receptors, but only few histamine receptors, are found [2,8].

To get a better understanding of the molecular mechanisms of serotonin and histamine receptors, purification and molecular characterization of the receptor protein is a prerequisite. Photoaffinity labelling should provide new possibilities to achieve this goal.

Recently we reported the use of a new photoaffinity probe, 7-azidoketanserin (7-azido-3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4-(1*H*,3*H*)-quinazolinedione) for irreversible labelling of brain serotonin-S_2 receptors [9]. We now provide evidence that this new photoaffinity ligand can be used to irreversibly label brain histamine-H_1 receptors as well. Differential labelling of either of both receptor systems can be achieved using a combination of different tissues and selective displacers.

2. MATERIALS AND METHODS

2.1. *Drugs and chemicals*

7-Azidoketanserin was synthesized from the corresponding amino-derivative of ketanserin by

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00145793/85/$3.30

diazotation and subsequent reaction with sodium azide. The complete synthesis and characterization of this new compound will be described elsewhere (unpublished). The structure of the compound is depicted in fig.1.

[^{3}H]Spiperone (23.4 Ci/mmol) and [^{3}H]pyrilamine (24.1 Ci/mmol) were supplied by New England Nuclear (FRG). Drugs were kindly provided by their own companies. Histamine and serotonin were from Janssen Chimica, bufotenine was from Serva (Heidelberg, FRG). All common reagents were of the highest purity available and were obtained from different suppliers.

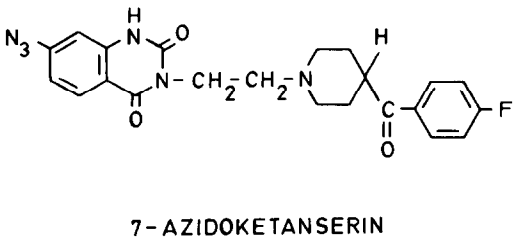
2.2. *Tissue preparation*

Female pirbright guinea pigs and female Wistar rats were killed by decapitation and their brains were rapidly removed. Pre-frontal cortex and cerebellum were dissected and immediately homogenized in 10 vols of 0.25 M sucrose. Subsequently an (M + L + P)-fraction was prepared as in [2]. The final washed pellets were suspended either in buffer A (50 mM Tris-HCl, pH 7.7) or in buffer B (50 mM Tris-HCl, pH 7.6, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline) at a final dilution of 1/100, w/v (original wet weight of tissue per volume).

2.3. *Binding assays*

For the assay of histamine-H_1 receptors, aliquots (2 ml) of the membrane preparations in buffer B were incubate with 1 nM [^{3}H]lpyrilamine for 30 min at 25°C in the presence or absence of other drugs. Free and bound ligand were separated by rapid filtration through glass-fibre filters (Whatman GF/B) using a 40-well filtration manifold (Multividor, Janssen Scientific Instruments Division). Non-specific binding was determined in the presence of 1 μM astemizole [10]. Following the binding assays, filters were equilibrated with 8 ml Insta-Gel II (Packard) and counted in a Packard Tri-Carb 460 liquid scintillation counter.

Serotonin-S_2 receptors were assayed with [^{3}H]spiperone. Aliquots (2 ml) of the membrane suspension in buffer B were incubated with 2 nM of the [^{3}H]ligand for 15 min at 37°C. Filtration and counting were performed as described above. Non-specific binding was determined using 2 μM methysergide [2].



7-AZIDOKETANSERIN

Fig.1. Chemical structure of 7-azidoketanserin.

2.4. *Photolabelling*

Aliquots of the membrane preparations in buffer A were preincubated for 15 min at 37°C with different concentrations of 7-azidoketanserin in the presence or absence of other drugs. The samples were subsequently irradiated for 10 min with long-wavelength UV light (366 mm) in quartz test tubes at an average distance of 4 cm from a Camag Universal UV lamp (Camag, Muttenz, Switzerland). Following photolysis, the samples were filtered under vacuum through glass-fibre filters (Whatman GF/B). To remove free and reversibly bound ligands, filters, kept on the filtration manifold with the vacuum released, were incubated with 5 ml of buffer B at 37°C for 5 min after which the buffer was removed by vacuum application. This wash procedure was repeated four times in total. Then free receptor sites were determined using a new filter assay procedure [11] with modifications. In short, filters positioned on the filtration manifold were incubated for 5 min with 400 μl buffer B containing either 2 nM [^{3}H]spiperone (for S_2-receptor assay) at 37°C or 4 nM [^{3}H]pyrilamine (for H_1-receptor assay) at 25°C. Incubation was stopped by vacuum application and filters were rinsed twice with 5 ml of ice-cold buffer A. For the determination of non-specific binding, filters were incubated with the labelled ligand in the presence of 2 μM methysergide or 1 μM astemizole for serotonin and histamine receptor determinations, respectively. The tissue, adsorbed to the filter, was found to retain its original receptor binding properties and serotonin-S_2 or histamine-H_1 receptors were specifically labelled by the above-described filter assay procedure (unpublished).

3. RESULTS AND DISCUSSION

The reversible binding of 7-azidoketanserin to histamine-H_1 receptors from guinea pig cerebellum was tested in competition binding experiments using [^{3}H]pyrilamine. The compound showed an IC_{50} value of 1.16 ± 0.04 nM (mean ± SE from 3 experiments performed in triplicate). This value is much lower than that of the parent compound ketanserin, which has an IC_{50} value of 18.6 nM. As previously reported, 7-azidoketanserin reveals an IC_{50} value of 2.9 nM for inhibition of [^{3}H]spiperone binding to serotonin-S_2 receptors from rat pre-frontal cortex [9]. This makes the new azido compound almost equipotent in both receptor systems.

Fig.2 shows the results of experiments in which membrane preparations of guinea pig cerebellum were preincubated with different concentrations of 7-azidoketanserin and subsequently irradiated with UV light during 10 min. The amount of free

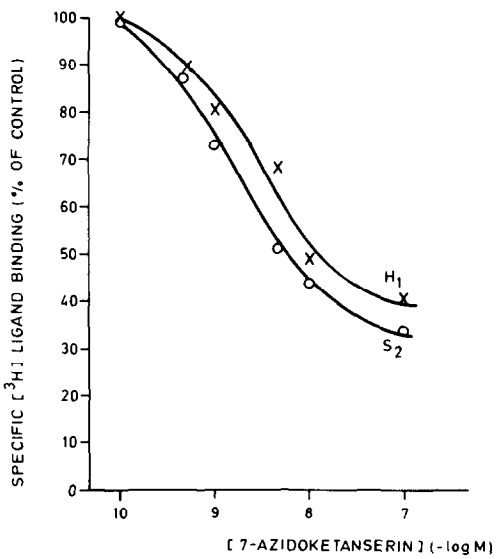


Fig.2. Effect of the concentration of 7-azidoketanserin on photolabelling of serotonin-S_2 and histamine-H_1 receptors. Membrane preparations from rat pre-frontal cortex (S_2-receptors) or guinea pig cerebellum (H_1-receptors) were preincubated with the indicated concentration of 7-azidoketanserin and subsequently photolysed for 10 min. Following photolysis the membranes were washed and remaining specific receptor binding was determined with [^{3}H]spiperone or [^{3}H]pyrilamine, respectively.

histamine-H_1 receptors decreased in a concentration-dependent manner with an apparent IC_{50} value of 12 nM. This is about two times higher than the value obtained for photolabelling of serotonin-S_2 receptors in rat pre-frontal cortex (IC_{50} = 5.60 nM) (fig.2).

At all concentrations tested, the azide was shown to dissociate completely from the receptor when irradiation was omitted. At the highest concentration used (10^{-7} M) about 60 to 70% of specific binding to both histamine and serotonin receptors was irreversibly blocked. The fact that not all the receptors are photolabelled can be explained in several different ways; possibly the azide functional group is not in a favourable position to undergo a covalent binding with the receptor protein or the azide molecules bound to these receptors are shielded for the UV light and can therefore not be photolyzed.

In experiments, similar to those described above, 7-azidoketanserin was also tested for photoinactivation of α_1-adrenergic receptors from rat forebrain. In this receptor model irreversible inactivation was seen only at higher micromolar concentrations. Therefore, at nanomolar concentrations, used to photoinactivate serotonin-S_2 and histamine-H_1 receptors, no interference by binding to α_1-adrenergic receptors is to be expected.

To make sure that excess azide could eventually be washed out before photolysis, experiments were performed in which membranes were preincubated with 10 nM 7-azidoketanserin, subsequently washed by centrifugation and then submitted to UV irradiation and binding assay. In these experiments, the same extent of photoinactivation was obtained as in the experiments described above.

Fig.3 shows the time-dependence of the photolysis reaction of 7-azidoketanserin on both serotonin and histamine receptors. Membrane preparations were preincubated with 10^{-7} M 7-azidoketanserin and subsequently photolyzed during different periods of time. Irreversible blocking of both receptors was rapid, reaching a plateau value after about 5 min. When membranes were preincubated with the solvent alone and then irradiated, no decrease of specific binding was seen, indicating that UV irradiation alone did not affect the receptor systems.

Several compounds were tested for their ability

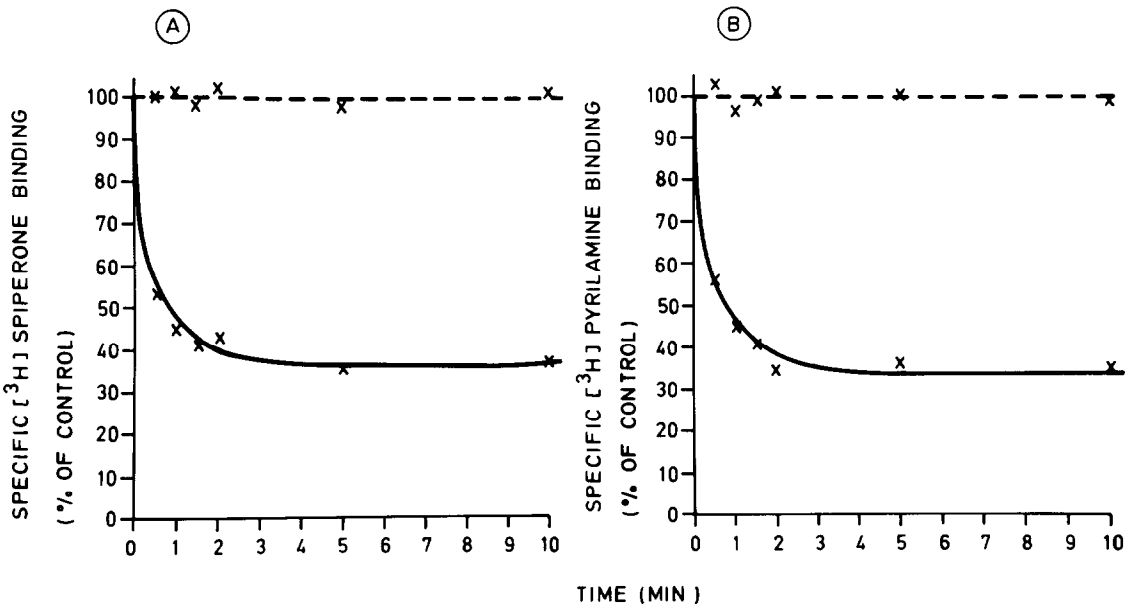


Fig.3. Effect of the time of irradiation on photolabelling of serotonin-S_2 and histamine-H_1 receptors. Membrane preparations of rat pre-frontal cortex (S_2-receptors) (A) or guinea pig cerebellum (H_1-receptors) (B) were preincubated with 10^{-7} M 7-azidoketanserin (——) or the solvent (– – –) and subsequently irradiated for the indicated periods of time. Following photolysis, the membranes were washed and remaining specific receptor binding was determined with [^{3}H]spiperone or [^{3}H]pyrilamine, respectively.

to inhibit photolabelling of histamine-H_1 receptors in guinea pig cerebellum. Aliquots of the membrane suspension were incubated with 10 nM 7-azidoketanserin and increasing concentrations of the compounds. Following irradiation for 10 min they were washed and assayed for [^{3}H]pyrilamine binding. Table 1 summarizes the results expressed as IC_{50} values for inhibition of photolabelling. They are compared to the IC_{50} values, obtained for inhibition of photolabelling of serotonin-S_2 receptors from rat pre-frontal cortex. Potent histamine antagonists such as chlorpheniramine and pyrilamine used at nanomolar concentrations were able to protect H_1-receptors against photolabelling. Histamine itself showed inhibition at high micromolar concentrations which corresponds to the potency of this neurotransmitter for binding to histamine-H_1 receptors. Serotonin antagonists (pipamperone and droperidol) and agonists (bufotenine and serotonin) did not show any inhibitory effect; neither did haloperidol (a dopamine antagonist), 2-(*N*,*N*-dipropyl)-amino-5,6-dihydroxytetralin (a dopamine agonist) nor WB 4101 (an α_1-adrenergic antagonist).

Apart from histamine-H_1 receptors, the guinea pig cerebellum also contains a small amount of [^{3}H]spiperone binding sites (fig.4A). These sites could be blocked completely by the addition of 10^{-7} M pipamperone, a selective S_2-antagonist. At this concentration, pipamperone had no effect at all on both reversible [^{3}H]pyrilamine binding [12] and photolabelling of the histamine receptors. In the same way, [^{3}H]pyrilamine binding sites in rat pre-frontal cortex could be blocked completely with 10^{-7} M pyrilamine (fig.4B) and this had no effect on reversible [^{3}H]spiperone binding [2] and photolabelling of serotonin receptors. This provides a way for differential photolabelling of either of both receptor types.

The foregoing results demonstrate that: (i) 7-azidoketanserin has an equally high affinity for binding to histamine-H_1 receptors and serotonin-S_2 receptors; (ii) after preincubation with 7-azidoketanserin and UV irradiation, H_1-receptors become irreversibly blocked; (iii) this irreversible binding can be inhibited by histamine antagonists and histamine itself but not by compounds with other pharmacological activities; (iv) the

Table 1

Inhibition by various compounds of photolabelling of histamine-H_1 receptors and serotonin-S_2 receptors with 7-azidoketanserin

Compound	IC_{50} (nM)	
	Histamine-H_1[a] receptors	Serotonin-S_2[b] receptors
Chlorpheniramine	4.0	>1000
Pyrilamine	19.9	>1000
Pipamperone	>1000	12.6
Droperidol	>1000	25.1
Ketanserin	31.6	11.2
Histamine	8900	>10000
Bufotenine	>10000	1122
Serotonin	>10000	10000
Haloperidol	>1000	>1000
2-(*N,N*-Dipropyl)-amino-5,6-di-hydroxytetralin	>10000	>10000
WB 4101	>1000	>1000

[a] Aliquots of guinea pig cerebellum membrane preprations were incubated with 10 nM 7-azidoketanserin in the presence of increasing concentrations of the test compounds. Following photolysis for 10 min, the samples were washed and assayed for [^{3}H]pyrilamine binding as described in the text. The IC_{50} value is the concentration of the compound, needed to reduce photolabelling to 50%. Percentage photolabelling was calculated as: $(B_1 - B_2)/(B_3 - B_2)$ where B_1 = binding after photolysis in the presence of azide and the test compound; B_2 = binding after photolysis in the presence of azide alone; and B_3 = binding after photolysis in the presence of the test compound alone

[b] Values taken from [9]

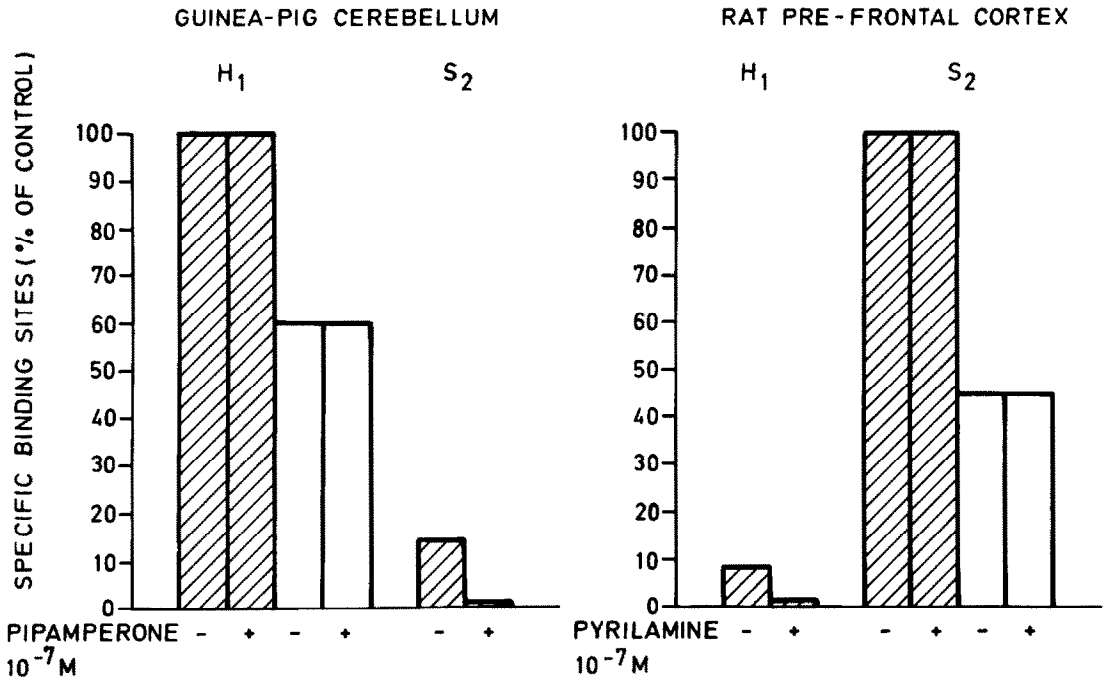


Fig.4. Differential photolabelling of serotonin-S_2 receptors and histamine-H_1 receptors. (A) Guinea pig cerebellum: [^{3}H]pyrilamine binding (shaded areas) and photolabelling in the presence of 10^{-8} M 7-azidoketanserin (blank areas) of H_1-receptors and [^{3}H]spiperone binding to S_2-receptors in the presence (+) or the absence (−) of 10^{-7} M pipamperone (the total amount of S_2-sites was calculated as a percentage of the total mount of H_1-sites). (B) Rat pre-frontal cortex: [^{3}H]spiperone binding (shaded areas) and photolabelling in the presence of 10^{-8} M 7-azidoketanserin (blank areas) of S_2-receptors and [^{3}H]pyrilamine binding to H_1-receptors from rat pre-frontal cortex in the presence (+) or the absence (−) of 10^{-7} M pyrilamine (the total amount of H_1-sites was calculated as a percentage of the total amount of S_2-sites).

photolabelling reaction with 7-azidoketanserin can be directed exclusively to either H_1- or S_2-receptors using a highly enriched tissue (guinea pig cerebellum for H_1-receptors; rat pre-frontal cortex fr S_2-receptors) and by blocking remaining unwanted receptors with a high concentration of either pipamperone or pyrilamine. Therefore, radioactively labelled 7-azidoketanserin, which is presently being synthesized, should provide a powerful tool for the elucidation of the molecular structure of both serotonin-S_2 and histamine-H_1 receptors.

ACKNOWLEDGEMENTS

We thank Jos Van Hertrooy for his skilful technical assistance. Part of this work was supported by a grant from IWONL.

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