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GUANINE NUCLEOTIDE EFFECTS ON AGONIST BINDING TO SEROTONIN 5HT$_2$ RECEPTORS IN RAT FRONTAL CORTEX

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Abstract—Specific [^{3}H]ketanserin binding to serotonin 5-HT$_2$ receptors of rat frontal cortex tissue is of high affinity, saturable and unaffected by guanine nucleotides. Antagonists displace [^{3}H]ketanserin from a single recognition site (pseudo-Hill coefficients close to unity), which is also unaffected by guanine nucleotides. Agonist displacement of either [^{3}H]ketanserin or [^{3}H]spiperone from three different membrane preparations showed pseudo-Hill coefficients less than one, and may be described in terms of two agonist binding sites with differing agonist affinities. In the presence of guanine nucleotides, overall agonist affinity was lowered slightly, with little or no change in psuedo-Hill coefficient.

Serotonin 5HT$_2$ receptors have been shown to be responsible for certain actions of serotonin, e.g. serotonin-induced behavioural excitation in rodents, vascular smooth muscle contraction, platelet activation [1, 2]. These receptors have been studied using the ligand-binding technique with a variety of antagonists (see [1, 2] for reviews) but [^{3}H]ketanserin [3] remains the most suitable radioligand owing to its high affinity and specificity.

The biochemical mechanism activated upon occupation of the serotonin 5HT$_2$ receptor is not yet fully resolved. Accumulating evidence, however, suggests that serotonin is able to stimulate inositol phospholipid metabolism in a number of tissues such as guinea-pig ileum [4], blow-fly salivary gland [5], rat brain [6, 7], rat thoracic aorta [8] and human [9, 10] and rabbit platelets [11]. In rat brain stimulation of inositol phospholipid breakdown by serotonin shows many of the characteristics of the 5HT$_2$ receptor subtype but some anomalies remain [6]. As yet only in the platelet and rat aorta has the serotonin stimulated inositol phospholipid breakdown been linked definitively to stimulation of 5HT$_2$ serotonin receptors.

If the serotonin 5HT$_2$ receptor is indeed coupled to stimulation of inositol phospholipid metabolism then by analogy with other receptor systems [12] a guanine nucleotide regulatory protein (G-protein) would be expected to be involved in coupling receptor and effector. There is conflicting evidence on this point from the effects of guanine nucleotides on agonist binding to serotonin 5HT$_2$ receptors: some authors have reported significant effects [13–16], some have not [17, 18]. In preliminary experiments we reported a weak effect of guanine nucleotides on agonist binding to serotonin 5HT$_2$ receptors in rat frontal cortex [19]. In the present paper we report a detailed study of these effects in a variety of preparations. We have used our own membrane preparation as well as the preparations used by other workers who have reported effects of guanine nucleotides [15, 16].

MATERIALS AND METHODS

Materials

All materials used were of analytical grade or of the highest purity available. [^{3}H]ketanserin (78.6–95.0 Ci/mmole) was from New England Nuclear (Boston, MA) and [^{3}H]spiperone (15.5–21.0 Ci/mmole) was from Amersham International, U.K. Gifts of drugs are gratefully acknowledged: 8-hydroxy-2-(di-*n*-propylamino)-tetralin, isomers of mianserin, and ritanserin (Beecham Pharmaceuticals, Harlow, U.K.); domperidone, ketanserin, pipamperone and spiperone (Janssen Pharmaceutica, Beerse, Belgium); prazosin (Pfizer Central Research, Sandwich, Kent, U.K.); 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (Prof. C. A. Marsden, University of Nottingham); *d*-LSD and methysergide (Sandoz Ltd., Basel, Switzerland). Serotonin, 5-methoxytryptamine, mepyramine, atropine, and guanine nucleotides were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.).

Tissue preparation

Three different procedures for preparing membranes were adopted, each utilising cerebral cortical tissue from male Hacking and Churchill CFY strain rats (150–175 g), dissected over ice.

The standard membrane preparation, membrane preparation A, was prepared by homogenising frontal cortex tissue in ice-cold 0.32 M sucrose (1:10 w/v), using a Teflon-glass homogeniser (15 strokes, 800 rpm). The homogenate was centrifuged (1500 *g*, 10 min, 4°), and the supernatant retained on ice. The pellet was re-homogenised in ice-cold 0.32 M sucrose (1:5 w/v), and centrifuged as before. The combined supernatants were centrifuged (120,000 *g*, 60 min,

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4°), the pellet washed twice (1 × 40 vol., 1 × 20 vol.) in buffer I, (KCl 5.36 mM, $CaCl_2$ 1.80 mM, NaH_2PO_4 0.91 mM, glucose 25 mM, sucrose 50 mM, HEPES 20 mM, pH 7.4) with intermediate centrifugation, and incubated for 15 min at 37° between washing steps. The final pellet obtained by centrifugation (120,000 *g*, 30 min, 4°) was resuspended in HEPES–phosphate–saline buffer [20] (buffer I with NaCl 110 mM, $MgSO_4$ 0.81 mM) containing pargyline (10 μM) and dithiothreitol (100 μM), pH 7.4, at a concentration of 3 ml/g original wet weight tissue, and stored at −80° until use. This gave a protein concentration of approximately 12 mg/ml, as measured by the method of Lowry *et al.* [21], incorporating a precipitation step using 10% trichloracetic acid.

Membrane preparation B was performed as described in [15]. Briefly, frontal cortices were homogenised in ice-cold 0.25 M sucrose (1:10 w/v) and centrifuged (1086 *g*, 10 min, 4°). The supernatant was diluted (1:40 w/v) in 50 mM Tris–HCl buffer, pH 7.4, and then centrifuged (35,000 *g*, 10 min, 4°). The pellet was resuspended in buffer and recentrifuged. The final resuspension was in 50 mM Tris–HCl, 0.5 mM EDTA, 10 mM $MgSO_4$, pH 7.4. Membranes were stored at −80° until use.

Membrane preparation C was performed as described in [16], except that HEPES was substituted for Tris. Briefly, whole cortices were homogenised in ice-cold 50 mM HEPES-buffer (1:40 w/v), pH 7.5, and centrifuged (40,000 *g*, 10 min, 4°). The pellet was resuspended in the same buffer and the process repeated. After the second wash, the homogenate was incubated at 37° for 15 min, centrifuged as above, and the pellet resuspended in 50 mM HEPES-buffer containing 4 mM $CaCl_2$, 10 μM pargyline and 5.7 mM ascorbic acid, pH 7.7. The preparation was again incubated at 37° for 15 min, and then stored at −80° until use.

Storage of the tissue at −80° did not have any apparent effect on the receptor binding properties in any of the membrane preparations.

Radioligand binding assays

Membrane preparation A. Mixed mitochondrial/microsomal membranes of rat frontal cortex (approx. 180 μg protein/ml) were incubated with [^{3}H]ketanserin (final assay concentration approximately 1 nM in 0.1% ethanol) in HEPES–phosphate–saline buffer containing pargyline (10 μM) and dithiothreitol (100 μM), and other drugs where indicated. Incubations, in a final volume of either 2 ml (filter manifold) or 1 ml (cell harvester), were carried out at 37° for 15 min. At this time, association and antagonist-induced dissociation had reached equilibrium (not shown).

In preliminary experiments, specific [^{3}H]ketanserin binding was shown to be best defined using 1.0 μM pipamperone as displacing drug. Assay termination was carried out by a rapid filtration technique using either a Millipore 12-well filter manifold (3 × 5 ml ice-cold buffer wash, pH 7.4, GF/B filter discs) or a Dynatech Automash 2000 automated cell harvester (15 ml ice-cold phosphate-buffered saline, pH 7.4, Whatman grade 934AH filter sheet). In separate experiments, these two techniques have been shown to yield qualitatively comparable results. Filtration experiments using the cell harvester technique are now preferred, as filtration of samples is performed more rapidly, and the levels of non-specific binding reduced (for example, at 0.93 nM [^{3}H]ketanserin and 142 μg protein/ml, filter binding represented 20% total (filter manifold) and 9% total (cell harvester)). Using either technique, specific [^{3}H]ketanserin binding showed an approximately linear dependence on added protein, up to 200 μg/ml.

Membrane preparation B. Assays were performed as described in [15], except that 1.0 μM pipamperone was used instead of 1.0 μM cinanserin to define non-specific binding.

Membrane preparation C. Assays at 37° and 22° were performed as described in [14], except that HEPES was substituted for Tris.

For each type of binding assay, radioactivity trapped on the filters was determined by liquid scintillation spectrometry in 3.5 ml scintillation fluid ("Optiphase X", "Scintillator 199") containing 300 μl water, at an efficiency of approximately 35–40%.

Data analysis

Data from saturation and competition binding (displacement) experiments were analysed using a non-linear least squares computer curve fitting program as described in [22]. Saturation binding data using different concentrations of radioligand (L) were analysed using equation (1) evaluating B_{max} (maximum number of receptors) and K_d (dissociation constant for radioligand)

$$B/B_{max} = \frac{L}{L + K_d} \quad (1)$$

Competition (displacement) data were analysed using equation (2) evaluating IC_{50} (concentration of displacing substance that gives half maximal occupancy) and n (pseudo-Hill coefficient). Y is the fractional occupancy of receptor sites at displacing ligand concentration (X).

$$Y = \frac{X^n}{X_n + (IC_{50})^n} \quad (2)$$

IC_{50} values obtained from competition binding experiments were corrected for receptor occupancy by radioligand using the equation described by Withy *et al.* [22], and the appropriate K_d values. For membrane preparations B and C, K_d values were taken from Refs 15 and 16, respectively.

RESULTS

Antagonist binding to serotonin $5HT_2$ *receptors*

Initially the nature of [^{3}H]ketanserin binding to the standard preparation of rat frontal cortex membranes (preparation A) was determined by competition (displacement) versus [^{3}H]ketanserin (1.0 nM approx.) with a series of serotonergic and non-serotonergic substances (Table 1). Displacement occurred to the same plateau level in each case (specific binding as defined by 1 μM pipamperone) and in all cases pseudo-Hill coefficients were very close to one so that binding of the competing substance could be described as interaction at a single

Table 1. Antagonist binding to rat frontal cortex serotonin $5HT_2$ receptors from the standard membrane preparation (A)

Antagonist	Corrected IC_{50} (nM)	n	Number of experiments
Ritanserin	1.3 ± 0.1	0.9 ± 0.1	2
Spiperone	1.6 ± 0.4	0.9 ± 0.1	4
Ketanserin	2.3 ± 0.9	0.9 ± 0.2	3
Methysergide	4.3 ± 1.3	0.8 ± 0.1	4
Pipamperone	5.0 ± 0.7	1.1 ± 0.2	4
(+) Mianserin	6.0 ± 2.9	1.0 ± 0.1	3
d-LSD	9.6 ± 3.1	0.9 ± 0.1	3
(±) Mianserin	11.3 ± 6.6	1.0 ± 0.1	5
(−) Mianserin	68.7 ± 11.9	1.2 ± 0.2	3
Mepyramine	395 ± 12	1.2 ± 0.5	2
Domperidone	3072 ± 545	0.9 ± 0.3	2
Rauwolscine	>1000	—	1
Atropine	>10,000	—	2
Prazosin	⩾10,000	—	3

Displacement of specific [^{3}H]ketanserin binding by the substances shown was carried out as described in the Materials and Methods section. Experimental data were fitted to equation (2) and values for corrected IC_{50} and the pseudo-Hill coefficient (n) determined as described. The corrected IC_{50} values are essentially identical with K_i values for the competing substances as the pseudo-Hill coefficients are very close to one. Values are expressed as mean ± SEM for three or more observations and mean ± range for two observations.

set of sites. Stereoselectivity was observed for displacement by the isomers of mianserin; mepyramine and prazosin displacement occurred with low affinity indicating the absence of H_1 histamine and α_1 adrenergic receptor interactions. Thus [^{3}H]ketanserin binding is to a homogeneous population of serotonin $5HT_2$ receptors. Saturation analysis (Fig. 1) indicated a single saturable population of high affinity sites (equation (1) was a good fit to the data in each case) (K_d 1.78 ∓ 0.05 nM, B_{max} 371 ∓ 19 fmoles/mg protein, mean ∓ SEM, 3 experiments). Association and dissociation experiments gave data in agreement (k_{ass} $2.23 \mp 1.29 \times 10^8\ M^{-1}\ min^{-1}$, k_{diss} $0.31 \mp 0.06\ min^{-1}$; mean ∓ SD (5 experiments), K_d 1.37 nM).

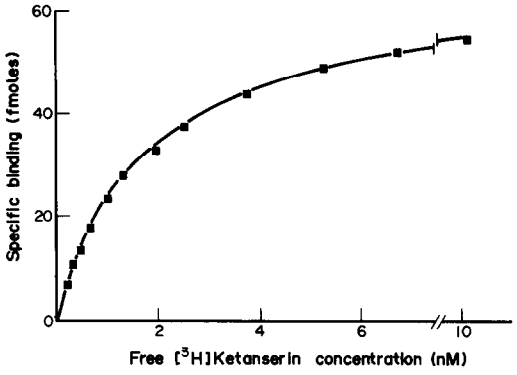

Fig. 1. Saturation analysis of specific [^{3}H]ketanserin binding (defined as that displaceable by 1 μM pipamperone) to the standard membrane preparation (A). Data from a single representative experiment are shown in which the free concentration of [^{3}H]ketanserin ranged from 0.2 nM to 10 nM. For this experiment, computer curve fitting to equation (1) gave values of $K_d = 1.7$ nM and $B_{max} =$ 357.8 fmoles/mg protein.

GTP (0.1 mM) had no effect on specific [^{3}H]ketanserin binding (+GTP (0.1 mM) K_d 1.95 ∓ 0.02 nM, B_{max} 379 ∓ 15 fmol/mg protein, mean ∓ range, two experiments) to the standard membrane preparation (preparation A) and saturation analysis of [^{3}H]ketanserin binding to preparation B indicated a similar lack of effect of GTP. Other nucleotides (GMP, GDP, AMP, ADP, ATP) also failed to give an effect. There were also no effects of GTP (0.1 mM) on antagonist binding in displacement experiments, e.g. preparation A, *d*-LSD, control K_i 9.6 ∓ 3.1 nM, +GTP 12.1 ∓ 5.1 nM, mean ∓ SEM, 3 experiments).

Agonist binding to serotonin $5HT_2$ receptors

Agonist binding was determined by displacement (competition) of specific [^{3}H]ketanserin binding in

Table 2. Agonist binding to rat cerebral cortex serotonin $5HT_2$ receptors and effect of guanine nucleotides under various assay conditions

Agonist	Membrane preparation	Control		+0.1 mM GTP		Number of experiments
		Corrected IC_{50}(nM)	n	Corrected IC_{50}(nM)	n	
Serotonin	A	2535 ± 681	0.55 ± 0.03	5333 ± 1265	0.67 ± 0.01	4, 3
	B	236 ± 67	0.57 ± 0.04	797 ± 94	0.61 ± 0.01	3
	C	3183 ± 1187	0.38 ± 0.02	2791 ± 672	0.39 ± 0.03	3
	C^1	919 ± 199	0.44 ± 0.02	1181 ± 105	0.51 ± 0.05	3
5-Methoxytryptamine	A	1367 ± 314	0.56 ± 0.04	2467 ± 530	0.67 ± 0.06	3
	B	238 ± 56	0.57 ± 0.04	1134 ± 67	0.73 ± 0.05	3
RU24969	A	2050 ± 217	0.74 ± 0.04	3300 ± 631	0.81 ± 0.03	3
8-OHDPAT	A	14590 ± 2026	0.64 ± 0.05	19920 ± 4263	0.61 ± 0.05	4, 3

Agonist binding was determined by displacement of either [^{3}H]ketanserin or [^{3}H]spiperone binding, and IC_{50} values (corrected for occupancy of receptors by [^{3}H]ligand) and pseudo-Hill coefficients (n) were determined by fitting the data to equation 2. For preparations B and C (C^1), K_d values were taken from Refs 15 and 16. Details of the conditions used for each preparation are given in the Materials and Methods section. Preparation C = [^{3}H]spiperone binding at 22° for 60 min. Preparation C^1 = [^{3}H]spiperone binding at 37° for 15 min. Data are given as mean ± SEM. RU24969, 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole. 8-OHDPAT, 8-hydroxy-2-(di-n-propylamino) tetralin.

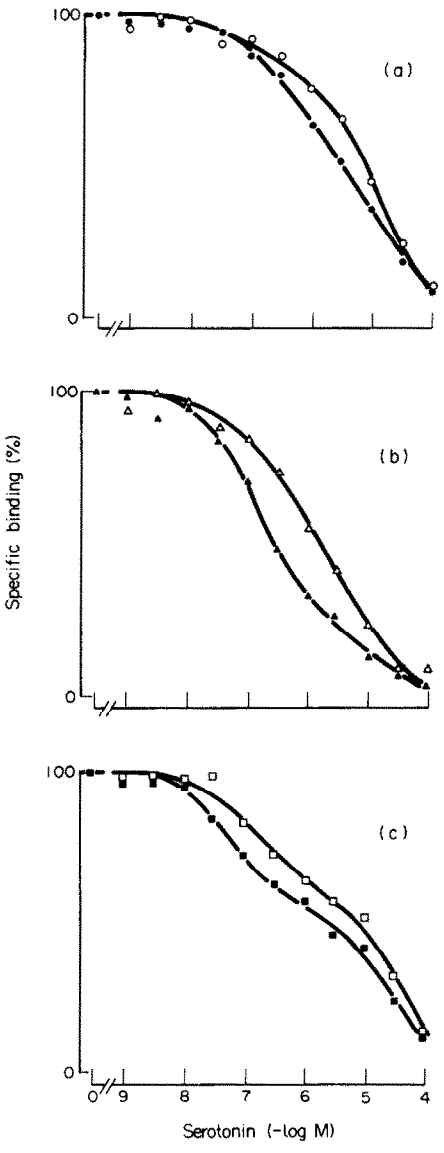

Fig. 2. Displacement by serotonin of specific [^{3}H]ketanserin binding (panels a and b) and specific [^{3}H]spiperone binding (panel c) to serotonin $5HT_2$ receptors of rat cerebral cortex tissue (membrane preparation A, B, C respectively). Membrane preparations A, B and C were prepared, and competition binding experiments carried out, as described in the Materials and Methods section. Data are means of triplicate determinations from single representative experiments replicated as in Table 2. The curves plotted are the computer best fit for a two binding site model (equation (2) of [22]). (A: control, 60%, 9 μM, 40%, 160 nM, +GTP, 83%, 7.5 μM, 17%, 39 nM; B: control, 32%, 5 μM, 68%, 76 nM, +GTP, 60%, 4 μM, 40%, 93 nM; C: control, 56%, 15 μM, 44%, 40 nM, +GTP, 61%, 21 μM, 29%, 87 nM). In each experiment, closed symbols = serotonin, open symbols = serotonin + 0.1 mM GTP. Individual errors on each point were <5% mean.

the membrane preparations (A, B) or specific [^{3}H]spiperone binding in membrane preparation C. In the standard preparation (A) binding of serotonin, 5-methoxytryptamine and the supposed $5HT_1$ serotonin receptor selective ligands 8-OHDPAT and RU24969 was studied (Table 2, Fig. 2). Displacements could not be described by a one binding site model, being characterised by pseudo-Hill coefficients less than one. Serotonin and 5-methoxytryptamine displacements in preparations B and C showed similar shallow displacement curves (pseudo-Hill coefficient less than one) and the affinities of the two agonists were in good agreement with those published in the original work [15, 16]. It is clear from the data that the affinities of agonists measured in different preparations and in the same preparation but under different conditions (compare preparation C and C^1) vary considerably. Thus agonist affinities are very dependent on the conditions used for membrane preparation and performing ligand-binding assays.

The effect of GTP (0.1 mM) on agonist displacement was determined and in each case when parallel experiments were performed with and without GTP a small effect to decrease the agonist affinity without changing the pseudo-Hill coefficient greatly was observed for all membrane preparations (Fig. 2). Similar effects were seen with the non-hydrolysable GTP analogue, GppNHp. Owing to the small size of the effect, when IC_{50} values from several experiments were averaged as in Table 2 no overall effects were seen in some cases. For the amalgamated data the effects of GTP were significant ($P < 0.01$, Students t-test, two tailed) only in preparation B. In individual experiments with the different preparations, however, an effect was always seen. Agonist displacement curves could be resolved into contributions from two classes of sites and generally the effect of GTP was to reduce the proportion of the higher affinity site with small effects on the affinities of the two sites (see legend to Fig. 2).

Effect of Mg^{2+} ions on serotonin $5HT_2$ receptor binding

Alteration of Mg^{2+} ion concentration had little effect on specific [^{3}H]ketanserin binding in the standard preparation (A) (e.g. zero Mg^{2+}, K_d 1.57 nM, B_{max} 383 fmol/mg protein; 15 mM Mg^{2+}, K_d 1.97 nM, B_{max} 416 fmol/mg). For serotonin binding to $5HT_2$ serotonin receptors increasing concentrations of magnesium ions increased the affinity of serotonin slightly (Table 3). Effects of GTP on serotonin displacements were similar under all conditions. A small decrease in affinity was observed with little effect on pseudo-Hill coefficient.

DISCUSSION

In this report we have examined the properties of serotonin $5HT_2$ receptors with particular reference to the effects of guanine nucleotides.The general ligand-binding properties were similar to many other receptors in that antagonists bound with high affinity and in a homogeneous manner consistent with interaction at a single class of sites, whereas agonist binding was more complex being characterised by pseudo-Hill coefficients less than one. This general pattern has been described for a number of receptors coupled to G-proteins, e.g. muscarinic acetylcholine [23], β-adrenergic [24], D_2 dopamine [22, 25]. Agonist affinity at the serotonin $5HT_2$ receptor was depen-

Table 3. Serotonin displacement of specific [^{3}H]ketanserin binding to preparation A, in the absence or presence of 0.1 mM GTP, at varying concentrations of Mg^{2+}-ions

	Control		+0.1 mM GTP	
$[Mg^{2+}]$(mM)	Corrected $IC_{50}(\mu M)$	n	Corrected $IC_{50}(\mu M)$	n
0	1.75 ± 0.50	0.54 ± 0.02	2.74 ± 0.89	0.59 ± 0.05
5	1.26 ± 0.13	0.54 ± 0.01	3.77 ± 0.72	0.64 ± 0.01
10	0.90 ± 0.09	0.54 ± 0.01	2.02 ± 0.28	0.65 ± 0.05
15	1.04 ± 0.12	0.57 ± 0.01	2.16 ± 0.06	0.66 ± 0.03

Serotonin displacement of [^{3}H]ketanserin binding to serotonin $5HT_2$ receptors from preparation A, at the final magnesium ion concentrations shown, was carried out as described in the Materials and Methods section. IC_{50} values (corrected for occupancy of receptors by [^{3}H]ketanserin) and pseudo-Hill coefficients (n) were determined as described in [22], using K_d values obtained for each Mg^{2+}-ion concentration. Data are mean ± SEM for three independent experiments in triplicate.

dent on the conditions used, preparations B and C giving higher agonist affinities at 37° than preparation A. This may be due to the presence of high divalent cation concentrations and low Na^+ in preparations B and C. In addition agonist affinities in preparation C were temperature dependent being greater at 37°, a similar effect has been described for D_2 dopamine receptors in anterior pituitary [26].

Guanine nucleotides did not alter antagonist affinities but there was a small but measurable effect on agonist binding. This was to decrease agonist affinity without changing the pseudo-Hill coefficient greatly. The effect was dependent on conditions being greatest in preparation B (present report and [15]). This may be due to the high Mg^{2+} concentration in this preparation and indeed in other systems increased levels of Mg^{2+} have been reported to increase agonist affinities and so could amplify the actions of guanine nucleotides [27]. Addition of increased Mg^{2+} to the standard membrane preparation (A) failed, however, to increase the size of the guanine nucleotide effect observed. Preparation B also does not include a 37° incubation during the preparation and whether this can account for its properties is not clear.

In preparation B we observed comparable effects of guanine nucleotides on agonist affinities to those reported in [15]. In the presence of guanine nucleotides, however, the agonist competition curves were still flat (pseudo-Hill coefficient <1) whereas in [15] agonist competition curves in the presence of guanine nucleotides were characterised by pseudo-Hill coefficients close to one. Preparation C gave a small effect of guanine nucleotides on agonist binding and whereas agonist affinity was temperature dependent the effect of guanine nucleotides was not dependent of the assay temperature in contradiction with the original report [16]. Preparation C is very similar to that used in [17, 18] where no guanine nucleotide effects were observed so the small size of the guanine nucleotide effect is not unexpected.

The pattern of data reported here, namely, homogeneous, guanine nucleotide insensitive antagonist binding and heterogeneous, guanine nucleotide sensitive agonist binding is typical of receptors coupled to G-proteins (see for example Ref. 25). Thus it can be concluded that the serotonin $5HT_2$ receptor is coupled to a G-protein. The serotonin $5HT_2$ receptor is notable for the small and variable size of the guanine nucleotide effect. For other receptors guanine nucleotide effects have been related to conversion of receptor–G-protein complexes (higher agonist affinity) to free receptor (lower affinity). Rigorous analysis of such data requires the use of a ternary complex model (see Ref. 25 for discussion) and in the present paper a model of two independent mass action sites has been used. In terms of this latter model the effect of guanine nucleotides in the present system is to promote only a partial interconversion of higher and lower affinity agonist sites. This may be a reflection of equilibrium constraints in the receptor–G protein interaction.

It is notable that, at least in brain and platelet, second messenger effects linked to serotonin $5HT_2$ receptors are small. The maximum stimulation of [^{3}H]inositol phosphate accumulation in [^{3}H]inositol labelled slices of rat frontal cortex with serotonin (acting at serotonin $5HT_2$ receptors), carbachol (acting at muscarinic acetylcholine receptors) and methoxamine (acting at α_1-adrenergic receptors) are in the ratio 1:4:10 (Shearman and Strange, unpublished).

Although the maximum second messenger response for a receptor is a complex function of the number of receptors, the receptor reserve, the tightness of coupling of receptor to effector etc., it is notable that serotonin $5HT_2$ receptors appear in most cases to be only weakly sensitive to guanine nucleotides and give small second messenger responses.

In summary, we have investigated the properties of $5HT_2$ serotonin receptors in rat brain and demonstrated weak coupling to guanine nucleotide regulatory proteins.

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