Biochemical Pharmacology, Vol. 39, No. 1, pp. 7–18, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

PHYSICAL EVIDENCE OF THE COUPLING OF SOLUBILIZED 5-HT$_{1A}$ BINDING SITES WITH G REGULATORY PROTEINS

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(*Received* 28 *April* 1989; *accepted* 12 *July* 1989)

Abstract—Previous investigations (El Mestikawy *et al.*, *J Neurochem* **51**: 1031–1040, 1988) have shown that 5-HT$_{1A}$ binding sites (R[5-HT$_{1A}$]) solubilized by CHAPS from rat hippocampal membranes can be modulated by guanine nucleotides, as expected from their solubilization together with associated G regulatory proteins (G). Studies of the hydrodynamic properties of solubilized R[5-HT$_{1A}$] have been presently carried out in order to assess in a more direct way the presence of R[5-HT$_{1A}$]–G complexes in the soluble extract. Under control conditions, the sedimentation of a CHAPS extract from hippocampal membranes through a 5–30% sucrose gradient (200,000 *g*, 17 hr, 4°) gave two maxima of [^{3}H]8-OH-DPAT binding activity corresponding to sedimentation coefficients of 8.0 S and 10.0 S, respectively. Running the gradient in the presence of 1 μM GTP revealed a significant reduction of the 10.0 S peak, as expected from the loss of material (probably a G protein) normally associated with R[5-HT$_{1A}$]. Conversely, attempts to prevent the dissociation of R[5-HT$_{1A}$]–G by treatment of CHAPS soluble hippocampal extracts with the cross-linking reagent disuccinimidyl suberate (0.1 mM) resulted in a significant increase (+70%) in [^{3}H]8-OH-DPAT binding activity associated with the appearance of a new sedimenting material with a higher coefficient (16.5 S). Furthermore, [^{3}H]8-OH-DPAT binding became almost completely insensitive to guanine nucleotides as expected from the irreversible coupling by disuccinimidyl suberate of R[5-HT$_{1A}$] with G protein(s). WGA-agarose chromatography of CHAPS soluble hippocampal extract supplemented with GTP allowed the physical separation of R[5-HT$_{1A}$] from the bulk of G proteins, and a concomitant decrease of [^{3}H]8-OH-DPAT high affinity binding capacity. Partial recovery of the latter could be achieved by reconstituting R[5-HT$_{1A}$]–G complexes upon the addition of a mixture of pure bovine Gi + Go to G-deprived soluble extracts. Finally *in vivo* treatment with Pertussis toxin (5 μg intracerebroventricularly, 48 hr before killing) resulted in a significant reduction of the specific binding of [^{3}H]8-OH-DPAT (−36%) to hippocampal membranes and corresponding CHAPS soluble extracts, and a marked decrease in the inhibitory effect of GppNHp. Accordingly the G protein associated with R[5-HT$_{1A}$] belongs probably to the Gi or Go families.

Although the pharmacology of serotonin (5-hydroxytryptamine, 5-HT)‡ receptor binding sites has been well characterized over the past fifteen years [1, 2], it is only recently that the transduction mechanisms associated with some of these receptors has been completely demonstrated.

The situation has become clear for 5-HT$_2$ and 5-HT$_{1C}$ receptor sites since several groups [3–5] contributed to demonstrating that these receptors are functionally coupled to phospholipase C in the cerebral cortex and the choroid plexus, respectively. Similarly, it is now well established that 5-HT$_{1B}$ and 5-HT$_{1D}$ sites are negatively coupled to adenylate cyclase in various brain regions [6, 7], and are probably species variants of the same class of receptors. In contrast, in the case of 5-HT$_3$ receptors, the effector system is still poorly known, but may involve a cation channel [8, 9].

For 5-HT$_{1A}$ receptor subsites, the situation still remains controversial, although the inhibitory influence of guanine nucleotides on the specific binding of 5-HT$_{1A}$ agonists [10] *indirectly* supports their functional coupling with G protein-dependent transducing mechanisms [11]. It has thus been reported that central 5-HT$_{1A}$ receptors may be coupled with K^+ channels [12], phospholipase C [13] and either negatively [13–15] or positively [16] with adenylate cyclase. In all cases G proteins appeared to be involved in the coupling mechanism [12–16] as expected from recent cloning and sequencing studies showing that the 5-HT$_{1A}$ receptor belongs to the G protein-coupled receptor family [17, 18]. However the nature of endogenous G protein(s) functionally associated with 5-HT$_{1A}$ receptors is yet unknown.

Interestingly, GTP modulation of agonist binding onto 5-HT$_{1A}$ sites has been found not only in brain membranes but also in their soluble extracts after a

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‡ Abbreviations: i.c.v., intracerebroventricularly; i.p., intraperitoneally; PT, Pertussis toxin; BSA, bovine serum albumin; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; 5-HT, 5-hydroxytryptamine; NAG, *N*-acetylglucosamine; OβG, octyl-β-D-glucoside; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; GTP, guanosine-5′-triphosphate; GppNHp, guanylyl-imidodiphosphate; PEG, polyethylene glycol; PEI, polyethylenimine; DSS, disuccinimidyl suberate; DMSO, dimethyl sulfoxide; BHR, Bolton–Hunter reagent; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylene diamine tetraacetic acid; WGA, wheat germ agglutinin; R[5-HT$_{1A}$], 5-HT$_{1A}$ receptor binding subunit.

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treatment with the zwitterionic detergent CHAPS [19]. Since this observation suggests that solubilized 5-HT_{1A} sites may still be associated with G proteins, a series of experiments has been presently performed in an attempt to demonstrate *directly* this point. For this purpose various physico-chemical procedures suitable to soluble preparations were applied to CHAPS soluble extracts from rat hippocampal membranes: sucrose gradient sedimentation under various conditions, protein cross-linking by a bifunctional reagent, partial purification on a wheat germ agglutinin–agarose column [20]. In addition, we also investigated the effects of *in vivo* treatment with the Gi/Go inactivating toxin, Pertussis toxin [21], on 5-HT_{1A} binding sites, and the possible reconstitution of high affinity 5-HT_{1A} binding sites in phospholipid vesicles enriched in pure Gi/Go proteins. The aim of these latter experiments was to contribute, at least partly, to the identification of endogenous G protein(s) probably associated with 5-HT_{1A} receptors in brain membranes.

MATERIALS AND METHODS

Pertussis toxin injections

Adult male Sprague–Dawley rats (250–300 g) were used throughout. Pertussis toxin (PT) was infused into both lateral ventricles following a procedure adapted from Nomura *et al.* [22]. In brief, the animals were anesthetized with pentobarbital (40 mg/kg i.p.) and placed in a stereotaxic frame with the head in a horizontal position. A midsagittal incision was made from eyes to ears and the bregma was exposed. Small holes were made bilaterally at points 1.7 mm lateral to midline and 1.0 mm posterior to the bregma. PT solution (2.5 μg/5 μl of saline) was injected bilaterally at a depth of 4.0 mm from the surface of the brain. Control animals were injected with 5 μl of saline, bilaterally. At the dose used (5 μg/rat), about 30% of animals treated with PT died within 3 days. Because of this toxicity, we usually killed PT-treated and control animals 48 hr after injection.

Membrane preparation

Animals were killed by decapitation, their brains removed and dissected at 0–4° [23]. Tissues were usually stored at −80° for 1–3 weeks before use. Frozen tissues were homogenized in 40 volumes (v/w) of 0.05 M Tris–HCl, containing 10 mM Phenylmethylsulfonylfluoride (PMSF), pH 7.4, with a Polytron PT10 O.D. disrupter. Homogenates were centrifuged at 40,000 *g* for 20 min at 4°, and the pellets were washed twice by resuspension in 40 volumes of the same buffer and centrifugation. The sedimented material was then gently homogenized in the same volume of buffer and incubated for 10 min at 37° to remove endogenous 5-HT [24]. After centrifugation as before, the membranes were washed three more times, and finally suspended in 2.5 volumes of 0.05 M Tris–HCl supplemented with 2 mM PMSF, pH 7.4, before storage at −80°.

Solubilization procedure

Hippocampal membranes were solubilized by 10 mM CHAPS essentially as described by El Mestikawy *et al.* [19]. The membrane suspension (approx. 20 mg protein/ml) was thawed at 4°, mixed with 0.1 vol. (v/v) of 0.1 M CHAPS in 0.05 M Tris–HCl, pH 7.4, briefly sonicated (20 W, 5 sec) and left at 4° for 60 min. After incubation, the mixture was centrifuged at 100,000 *g* for 60 min at 4°. The clear supernatant (approx. 6–7 mg protein/ml) was collected and filtered through Millex GV 0.22 μm filters (Millipore) before its use as the source of solubilized 5-HT_{1A} binding sites.

Binding assays

[^{3}H]8-*OH-DPAT.* Membranes: Aliquots (10–25 μl corresponding to 0.2–0.5 mg of protein) of membrane suspension were mixed with 0.05 M Tris–HCl, pH 7.4, containing 0.90 nM [^{3}H]8-OH-DPAT with or without 0.1 mM GppNHp, in a total volume of 0.5 ml. Samples were incubated for 60 min at 15° [19], then filtered through Whatman GF/B filters under vacuum. Filters were washed three times with 3 ml of ice-cold Tris–HCl buffer, dried, and immersed in 4 ml of Aquasol® (New England Nuclear, Boston, MA) for radioactivity counting. Non specific binding was determined from similar samples supplemented with 10 μM of unlabelled 5-HT.

Soluble preparations: Aliquots (20–100 μl, corresponding to 0.1–0.4 mg of protein) of various solubilized fractions were mixed with 0.05 M Tris–HCl, pH 7.4, containing 2 mM CHAPS, various concentrations of [^{3}H]8-OH-DPAT and drugs in a total volume of 0.5 ml. Incubation, filtration, washing and counting were as described for membranes except that GF/B filters were pretreated with 0.25% polyethylenimine (PEI) as described by Bruns *et al.* [25]. Non specific binding was also measured in the presence of 10 μM unlabelled 5-HT.

[^{3}H]*GppNHp.* Aliquots (20–30 μl, corresponding to 0.10–0.15 mg of protein) of various solubilized fractions were mixed with 0.05 M Tris–HCl, pH 7.4, containing 2 mM CHAPS and 24 nM of [^{3}H]GppNHp in a total volume of 0.5 ml. Samples were incubated at 4° for 15 min [26], then 0.2 ml of 0.5% (w/v) γ-globulin and 0.5 ml of 30% (w/v) polyethylene glycol (PEG) were added and the mixture was allowed to precipitate for 5 min at 4°. Samples were filtered through Whatman GF/B filters and the filters were washed twice with 3 ml of 0.05 M Tris–HCl buffer containing 10% PEG. Filters were dried and counted as above. Non specific binding was determined in the presence of 100 μM cold GTP.

Sucrose gradient centrifugation

Usually 0.3 ml of CHAPS soluble extract from hippocampal membranes were layered on top of linear 5–30% sucrose gradients (12.5 ml) in 0.05 M Tris–HCl, pH 7.4, containing 2 mM CHAPS and other compounds (where indicated in the section of Results). The loaded gradients were spun at 200,000 *g* for 17 hr at 4° in a Beckman SW41Ti rotor. Fractions of 0.25 ml were collected at the bottom of each tube, and 50–100 μl aliquots (for [^{3}H]8-OH-DPAT) or 20–30 μl aliquots (for [^{3}H]GppNHp) were assayed for binding experiments according to the procedures described above. Calibration of the gradients was achieved with thyroglobulin (18.4 S), catalase (11.4 S), alcohol dehydrogenase (7.4 S)

β-amylase and BSA (4.2 S) from Sigma Chemical Co.

Coupling with disuccinimidyl suberate

Disuccinimidyl suberate was solubilized in a small volume of dimethyl-sulfoxide (DMSO), and then added at 0.1 mM final concentration in a diluted CHAPS extract from hippocampal membranes (0.2–0.4 mg protein/ml in 0.05 M Tris–HCl, pH 7.4, containing 2 mM CHAPS). Control samples were supplemented with the same volume of DMSO alone (1% v/v final concentration). After incubation for 1 hr at 15°, samples were concentrated/dialyzed using a MicroProDiCon apparatus (model MPDC-310, Bio Molecular Dynamics, Beaverton, Oregon) against 1.5 l of Tris–HCl buffer containing 2 mM CHAPS (2 changes) to a final volume of 100 μl. The samples were then diluted with the same buffer to the appropriate volume for binding assays or sucrose gradient centrifugation. Some experiments were performed using 10 mM DTT as scavenger after the incubation period. The results were always identical to those obtained when DTT was omitted. In fact, we assumed that Tris buffer acted as a scavenger readily eliminating unreacted DSS molecules during incubation time.

Separation of R[5-HT_{1A}] from G proteins

Among the various procedures already tested for the purification of R[5-HT_{1A}] [20], wheat germ agglutinin agarose (WGA–agarose) chromatography allowed the separation of R[5-HT_{1A}] from G regulatory proteins, and was thus selected for the present study. Five ml of WGA–agarose were mixed with 5 ml of CHAPS soluble hippocampal extract which was preincubated with 0.1 mM GTP for 30 min at 15°. Binding of R[5-HT_{1A}] to WGA–agarose was allowed to proceed at 4° for 2 hr using a batch procedure. The mixture was poured into a column and washed with 20 ml of 0.05 M Tris–HCl, pH 7.4, containing 0.1 mM GTP and then with 100 ml of Tris buffer alone at a flow rate of 10 ml/hr. Elution was performed with 0.25 M *N*-acetylglucosamine in Tris buffer. Using this procedure, specific [^{3}H]8-OH-DPAT binding was hardly detected in the eluted fractions, in contrast to the high binding activity observed when GTP had been omitted for the preincubation and washing steps [19, 20]. All eluted fractions that normally contained [^{3}H]8-OH-DPAT binding activity when GTP was omitted, were pooled and concentrated/dialyzed with a MicroProDiCon apparatus to a final volume of 100 μl, and then diluted to the appropriate volume for reconstitution experiments.

Reconstitution of active 5-HT_{1A} binding sites

We used a simplified adaptation of the procedure originally described by Cerione *et al.* [27] for the reconstitution of β_2-adrenergic receptors. Briefly, 300 μl of G protein-free material eluted from WGA-agarose and then concentrated/dialyzed were supplemented with 0.5 mg/ml azolectin and 0.9% octyl-β-D-glucoside (OβG), and mixed with 100 μl of a solution of purified G proteins (1 mg protein/ml) to a final volume of 460 μl. Prior to use, azolectin was sonicated for 10 min in a water bath sonicator. Purified G proteins consisted of a mixture of Gi + Go extracted from bovine brain using the three step procedure (DEAE–Sephacel, Ultrogel AcA 34, heptylamine-Sepharose) of Sternweis and Robishaw [28]. As they were stored (−80°) in 0.02 M Tris–HCl plus 1 mM EDTA, 1 mM DTT, 0.8% cholate and 0.2 M NaCl, pH 7.4, this buffer alone instead of the G protein solution was added to the WGA-agarose eluate in control experiments (see Results). After a 60 min incubation at 4°, the supplemented WGA-agarose eluate was applied onto a 1 ml Extracti-Gel column equilibrated in 0.05 M Tris–HCl, pH 7.4, and the column was eluted with 1.5 ml of the same buffer. [^{3}H]8-OH-DPAT binding was assayed directly on 100 μl aliquots of the eluate.

Protein determination

Proteins were estimated using the Folin phenol procedure [29] with BSA as the standard.

Chemicals

[^{3}H]8-OH-DPAT (110 Ci/mmol) was from the Service des Molécules Marquées (CEA, 91191 Gif sur Yvette, France) and [^{3}H]GppNHp (23 Ci/mmol) was from Amersham International (Buckinghamshire, U.K.). Other compounds were: CHAPS (Serva, Heidelberg, F.R.G.), serotonin creatinine sulfate (Merck, Darmstadt, F.R.G.), ipsapirone (Troponwerke, Cologne, F.R.G.) methiothepin (Hoffmann-la-Roche, Basel, Switzerland), WGA–agarose (Pharmacia, Uppsala, Sweden), disuccinimidyl suberate and Extracti-Gel (Pierce), 3-(4-hydroxyphenyl)propionic acid, *N*-hydroxysuccinimide ester (Bolton–Hunter reagent, Aldrich Chemical Co., Strasbourg, France), GTP and GppNHp (Boehringer, Mannheim, F.R.G.). Pertussis toxin, polyethylenimine, PMSF, γ-globulin, polyethylene glycol, sucrose, DTT, *N*-acetylglucosamine, azolectin, octyl-β-D-glucoside and cholate were from Sigma. All other compounds were the purest commercially available (Merck, Prolabo).

RESULTS

Effects of GppNHp on [^{3}H]8-OH-DPAT specific binding to membranes and soluble extracts from control or PT-treated rats

Two days after the i.c.v. administration of 5 μg PT, a significant decrease of [^{3}H]8-OH-DPAT specific binding to 5-HT_{1A} sites was found in membranes from the hippocampus, septum and cerebral cortex, but not from the hypothalamus (Table 1). Attempts to enhance the amplitude of this effect by using higher doses of PT or allowing longer periods of recovery after the treatment were not possible because of the relatively high toxicity of the toxin (about 30% mortality within 72 hr after the i.c.v. administration of 5 μg PT).

When binding assays were performed in the presence of 0.1 mM GppNHp, lower levels of [^{3}H]8-OH-DPAT specific binding were found with membranes from both control and PT-treated rats (Table 1). However the negative effect of the nucleotide was relatively more pronounced on control membranes,

Table 1. Effects of *in vivo* treatment with Pertussis toxin on [^{3}H]8-OH-DPAT specific binding with or without GppNHp to membranes from various brain regions

Brain structure	Addition	[^{3}H]8-OH-DPAT specifically bound (fmol/mg prot.) Control	Pertussis toxin	Δ%
Hippocampus	None	132.7 ± 6.1	84.3* ± 2.6	−36
	GppNHp	32.6 ± 2.0	32.0 ± 1.8	−2
Septum	None	101.9 ± 3.0	70.4* ± 2.5	−31
	GppNHp	27.8 ± 1.1	24.5 ± 0.6	−12
Cerebral cortex	None	46.9 ± 1.6	38.5* ± 1.3	−18
	GppNHp	11.9 ± 0.5	10.9 ± 0.4	−8
Hypothalamus	None	48.6 ± 1.4	49.7 ± 1.2	+2
	GppNHp	15.1 ± 0.4	16.5 ± 0.3	+9

Pertussis toxin (5 μg/rat) was injected i.c.v. 48 hr before killing. Binding assays were carried out on membranes with 0.90 nM [^{3}H]8-OH-DPAT in the presence or the absence of 0.1 mM GppNHp. Each value of [^{3}H]8-OH-DPAT specific binding (in fmol/mg prot.) is the mean ± SE of triplicate determinations in three separate experiments. Δ% are the percent changes due to Pertussis toxin treatment.

* $P < 0.05$ when compared to respective control values (Student's *t*-test).

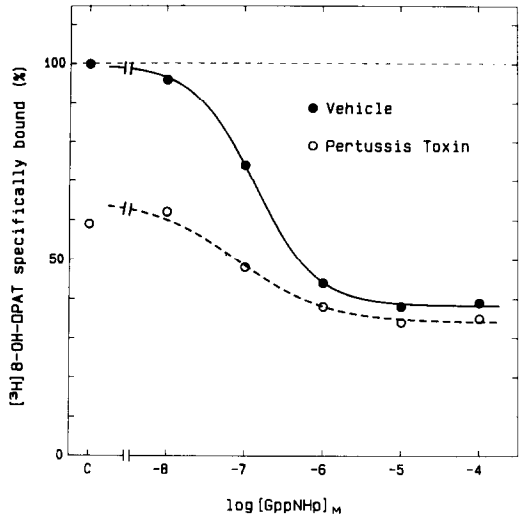

Fig. 1. Effects of GppNHp on [^{3}H]8-OH-DPAT specific binding to CHAPS soluble extracts from hippocampal membranes of control or Pertussis toxin-treated rats. Pertussis toxin (5 μg/rat) or the vehicle was injected i.c.v. 48 hr before killing. Binding assays were carried out on 50 μl aliquots of respective CHAPS soluble extracts, with 1.0 nM of [^{3}H]8-OH-DPAT and various concentrations of GppNHp (abscissa). The specific binding of [^{3}H]8-OH-DPAT is expressed as percentage of that found with soluble extracts from control rats, in the absence of GppNHp. Each point is the mean of three independent determinations (100% = 109 ± 5 fmol/mg protein).

so that [^{3}H]8-OH-DPAT specific binding which persisted in the presence of 0.1 mM GppNHp was no longer different in membranes from control or PT-treated rats (Table 1).

Binding assays carried out with CHAPS soluble extracts from hippocampal membranes also revealed a significant decrease in [^{3}H]8-OH-DPAT specific binding due to the *in vivo* treatment with PT or the *in vitro* addition of increasing concentrations of GppNHp (Fig. 1). Interestingly the reduction due to the nucleotide was not additive with that due to the toxin since [^{3}H]8-OH-DPAT specific binding which persisted in the presence of saturating concentrations of GppNHp (10 μM–0.1 mM) was not lower in soluble extracts from PT-treated rats than in those from control animals (Fig. 1).

Scatchard analyses of [^{3}H]8-OH-DPAT specific binding to soluble extracts from hippocampal membranes of control rats revealed that the reduction due to 0.1 mM GppNHp was associated with a marked decrease in Bmax (Fig. 2).

Sucrose gradient sedimentation of soluble 5-HT$_{1A}$ binding sites

When the 100,000 *g* supernatant from CHAPS-treated hippocampal membranes was submitted to sucrose gradient sedimentation in a linear gradient (5–30% sucrose), [^{3}H]8-OH-DPAT specific binding activity sedimented as a broad peak with two maxima I, II, corresponding to sedimentation coefficients of 8.0 S and 10.0 S, respectively (Fig. 3). Usually 75–85% of total [^{3}H]8-OH-DPAT specific binding activity in the sample layered onto the gradient was recovered in the sedimented fractions. When 10 μM Mn^{2+} (+2 nM 8-OH-DPAT) were included in the gradient, the recovery was lower (55–65%, probably because of the competition by added 8-OH-DPAT still present in binding assays, see Materials and Methods), but the resolution of the two maxima at 8.0 S and 10.0 S was markedly increased (Fig. 3). Preincubation of the CHAPS soluble extract with 1 μM GTP (+2 nM 8-OH-DPAT) for 30 min at 15°, and subsequent sedimentation in a sucrose gradient (5–30%) supplemented with the same concentration of the nucleotide, also altered the sedimentation profile of [^{3}H]8-OH-DPAT binding activity (Fig. 3): sedimentation coefficients were not changed but the peak at 10.0 S was dramatically reduced. Absolute levels of [^{3}H]8-OH-DPAT binding activity were also less in experiments with 1 μM GTP since the nucleotide and 8-OH-DPAT added in the gradient were diluted but still present in the binding assay mixture (see Materials and Methods). As illustrated in Fig.

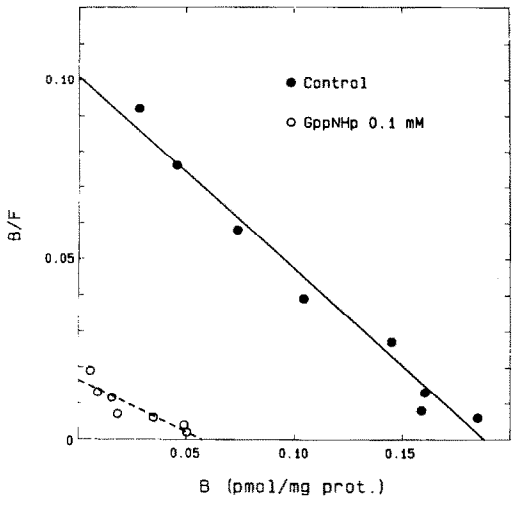

Fig. 2. Scatchard plots of [^{3}H]8-OH-DPAT specific binding to CHAPS soluble extracts from rat hippocampal membranes. Effects of GppNHp. Binding assays were performed on 50 μl aliquots of a CHAPS extract from control rats, with 0.1–10 nM [^{3}H]8-OH-DPAT, and in the presence or the absence of 0.1 mM GppNHp. Each point is the mean of triplicate determinations. B = [^{3}H]8-OH-DPAT specifically bound in pmol/mg prot. B/F = [^{3}H]8-OH-DPAT specifically bound/[^{3}H]8-OH-DPAT remaining free, in fmol per sample. Calculations of K_d and B_{max} were as in Ref. 19. Control: K_d = 0.54 nM; B_{max} = 182 fmol/mg prot. GppNHp: K_d = 1.05 nM; B_{max} = 57 fmol/mg prot. Similar data were obtained in two separate experiments.

3 the direct inhibitory effect of GTP was particularly obvious when 10 μM, instead of 1 μM, of the nucleotide was added to the gradient. [^{3}H]8-OH-DPAT binding activity was then markedly decreased, but still associated mainly with the peak at 8.0 S. Experiments with GppNHp instead of GTP gave identical results (not shown).

Effects of disuccinimidyl suberate (DSS) and Bolton–Hunter reagent (BHR) on soluble 5-HT_{1A} binding sites

Preincubation for 1 hr at 15° of CHAPS soluble extract from hippocampal membranes with DSS yielded to a concentration-dependent increase of [^{3}H]8-OH-DPAT specific binding, up to +90% with 0.3–1.0 mM of the bifunctional reagent (Fig. 4). Higher concentrations could not be used because of the poor solubility of DSS. Time course studies revealed that the effect of DSS (0.1 mM) levelled off within 1 hr at 15° (not shown).

To demonstrate that the increase in [^{3}H]8-OH-DPAT specific binding could really be attributed to the cross-linking properties of DSS, further experiments were performed with the closest related molecule commercially available, but with only one identical functionality: 3-(4-hydroxyphenyl)-propionic acid, *N*-hydroxysuccinimide ester (Bolton–Hunter reagent, BHR). In contrast to DSS, BHR did not increase [^{3}H]8-OH-DPAT specific binding, and even a decrease in 5-HT_{1A} binding activity was found in CHAPS soluble extracts which were preincubated for 1 hr at 15° with 1.0–3.0 mM of the monofunctional reagent (Fig. 4).

Scatchard analyses of [^{3}H]8-OH-DPAT specific binding revealed that DSS (0.1 mM) treatment increased the B_{max} of 5-HT_{1A} binding sites, with no change in their affinity (Fig. 5). In contrast, preincubation of CHAPS soluble extracts in the presence of 2 mM BHR produced a decrease in both the affinity and B_{max} of the 5-HT_{1A} binding sites (Fig. 5).

Characteristics of 5-HT_{1A} binding sites in soluble hippocampal extracts treated with DSS

In addition to increasing [^{3}H]8-OH-DPAT specific binding, DSS treatment (0.1 mM for 1 hr at 15°) markedly reduced the capacity of 5-HT_{1A} sites to be modulated by GppNHp (Fig. 6). With 0.1 mM of the guanine nucleotide, the resulting decrease in [^{3}H]8-OH-DPAT specific binding was of −19% in DSS-treated extracts, whereas it reached −67% in control extracts (Fig. 6). As shown in Fig. 6, BHR treatment also prevented the inhibitory effect of GppNHp on [^{3}H]8-OH-DPAT specific binding still detected in soluble hippocampal extracts.

Instead of adding GppNHp to CHAPS soluble extracts after DSS treatment, the reverse protocol was used in another series of experiments, i.e. soluble extracts were first supplemented with 0.1 mM of the guanine nucleotide before the preincubation with 0.1 mM DSS. Under such conditions, the bifunctional reagent did not increase [^{3}H]8-OH-DPAT specific binding, and even a lower 5-HT_{1A} binding activity (−52%) was found in soluble extracts exposed to GppNHp then DSS, than in controls. Furthermore [^{3}H]8-OH-DPAT specific binding which persisted in soluble extracts treated by GppNHp then DSS was no longer sensitive to further addition of the guanine nucleotide in the assay mixture. Scatchard analyses of [^{3}H]8-OH-DPAT specific binding to soluble extracts extensively dialysed after pretreatment with GppNHp then DSS revealed a significant decrease in B_{max} with no change in affinity as compared to respective values with soluble extracts exposed to GppNHp alone (Table 2). Conversely, pretreatment with DSS then GppNHp produced a significant increase in B_{max} (Table 2).

Sucrose gradient sedimentation of DSS-pretreated CHAPS soluble extract revealed the presence of a new peak (III) of [^{3}H]8-OH-DPAT specific binding activity, with high sedimentation coefficient: 16.5 S (Fig. 7), in addition to those (I, II) already found with control extracts (see Fig. 3).

To determine whether the 16.5 S material really corresponded to 5-HT_{1A} binding sites, several sucrose gradients loaded with control or DSS-pretreated CHAPS soluble extracts were run in parallel, and fractions 10 to 19 of DSS gradients and 23 to 34 of both types of gradients (see Figs 3 and 7) were pooled for the pharmacological characterization of [^{3}H]8-OH-DPAT specific binding. Displacement studies indicated that the 16.5 S material exhibited the same high affinity as the 8.0–10.0 S material for the 5-HT_{1A} agonist ipsapirone, and the same moderate affinity for the 5-HT antagonist methiothepin (Table 3).

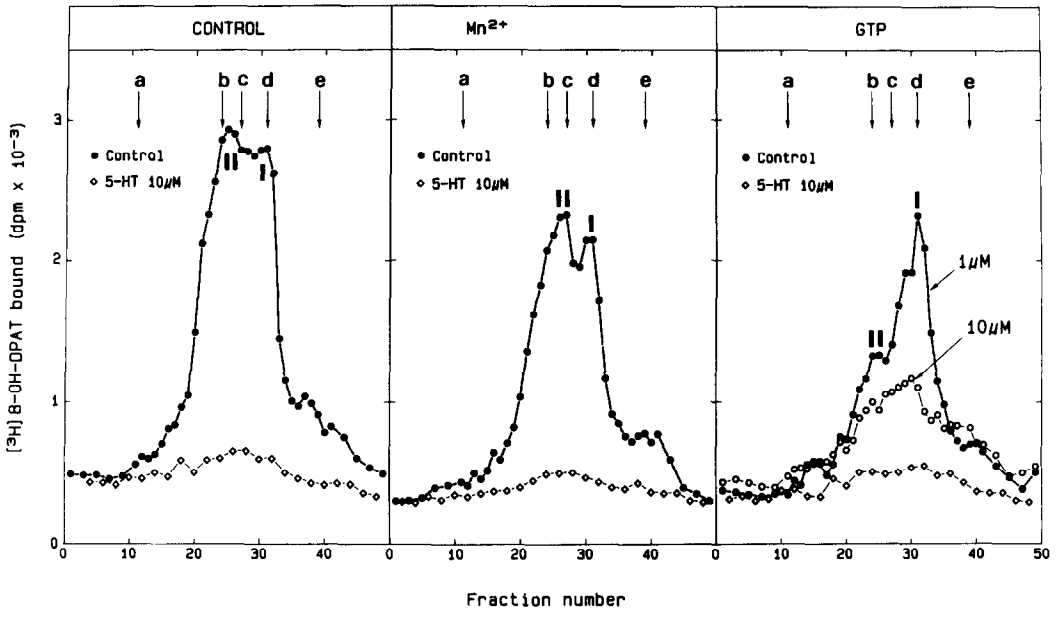

Fig. 3. Sucrose gradient sedimentation of soluble 5-HT_{1A} binding sites under control conditions, and in the presence of Mn^{2+} or GTP. Soluble extracts from CHAPS-treated membranes were preincubated for 30 min at 15° in the absence (control, left panel) or the presence of 2 nM 8-OH-DPAT plus either 10 μM Mn^{2+} (middle panel) or 1–10 μM GTP (right panel). Aliquots (0.3 ml) of each extract were then layered on top of sucrose gradients (5–30% sucrose) containing the same additives as those for the preincubation. Gradients were spun at 200,000 *g* for 17 hr at 4°, and 250 μl fractions were collected from the bottom of each tube. Aliquots (0.1 ml) were used for the measurement of [^{3}H]8-OH-DPAT (1.0 nM) binding in the presence (◇) or the absence (●, ○) of 10 μM 5-HT. Each point is the mean of duplicate determinations of [^{3}H]8-OH-DPAT bound (in dpm × 10^{-3}) per sample. Separate gradients run in parallel were used for determination of total and non specific binding. Calibration of the gradients with (a) thyroglobulin; (b) catalase; (c) alcohol dehydrogenase; (d) β-amylase; and (e) BSA, allowed the estimation of the sedimentation coefficients of peaks I and II: 8.0 S and 10.0 S, respectively. The same experiments were repeated three times with similar results.

Effects of DSS treatment on the sedimentation profile of soluble [^{3}H]*GppNHp binding sites in sucrose gradient*

As shown in Fig. 8, maximal [^{3}H]GppNHp specific binding activity sedimented around 6.0 S in the sucrose gradient, but significant binding activity was also found in all collected fractions after sedimentation of a CHAPS soluble extract from hippocampal membranes. DSS treatment of the extract produced only a discrete decrease (−10%) in [^{3}H]GppNHp specific binding activity and slight changes in its distribution along the sucrose gradient. Thus, a small reduction of activity sedimenting around 6.0 S and some shift of the remaining activity towards higher sedimentation coefficients were found with DSS-treated as compared to control CHAPS soluble extracts (Fig. 8).

Reconstitution of active 5-HT_{1A} binding sites

When CHAPS soluble extracts from hippocampal membranes were chromatographed on WGA-agarose, [^{3}H]8-OH-DPAT binding could usually be recovered by elution with *N*-acetylglucosamine with a yield of *ca.* 55% [19]. The addition of 0.1 mM GTP to the CHAPS soluble extract prior to WGA–agarose chromatography resulted in a dramatic loss of [^{3}H]8-OH-DPAT specific binding activity since the yield fell to 5.3%, in spite of extensive dialysis to remove any trace of GTP in the eluate (Table 4). Furthermore the eluted material exhibited no significant [^{3}H]GppNHp specific binding activity (not shown).

The addition of azolectin plus Oβ*G* to the WGA eluate did not change its [^{3}H]8-OH-DPAT binding activity unless exogenous G (Gi + Go) regulatory proteins were present in the reconstituting mixture (Table 4). Thus a three-fold increase in [^{3}H]8-OH-DPAT specific binding activity was noted when the eluted material together with G regulatory proteins were included in phospholipid vesicles (Table 4). Furthermore the resulting [^{3}H]8-OH-DPAT specific binding was sensitive to guanyl nucleotides as 0.1 mM GppNHp reduced its value down to that of the eluted material incorporated into phospholipid vesicles in the absence of G regulatory proteins. In contrast, the latter binding activity remained unaltered when 0.1 mM GppNHp was added to the assay mixture (Table 4).

DISCUSSION

Indirect evidence of the involvement of G regulatory proteins in the transduction processes associated with central 5-HT receptors first derived from

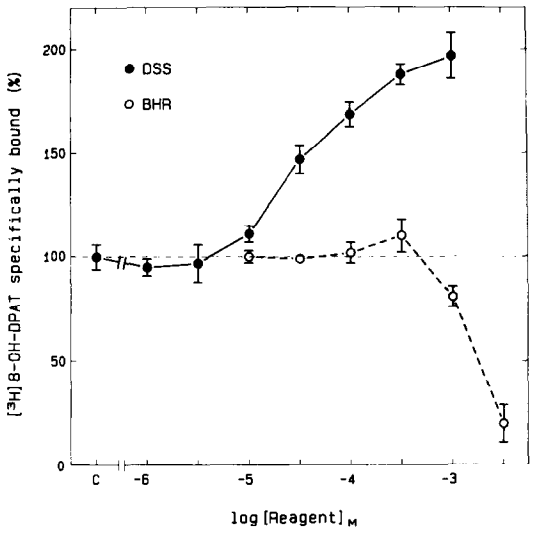

Fig. 4. Effects of pretreatment by disuccinimidyl suberate or Bolton–Hunter reagent on [^{3}H]8-OH-DPAT specific binding to soluble CHAPS hippocampal extracts. Soluble extracts were pretreated by various concentrations (abscissa) of DSS (●) or BHR (○) for 60 min at 15°, then dialysed and concentrated as described in Materials and Methods. Binding assays were performed with 1.0 nM [^{3}H]8-OH-DPAT, and data are expressed in percentage of the specific binding to soluble CHAPS extract preincubated for 60 min at 15° in the absence of DSS or BHR (C on abscissa: 100% = 92.3 ± 5.2 fmol/mg prot.). Each point is the mean ± SE of triplicate determinations in four independent experiments.

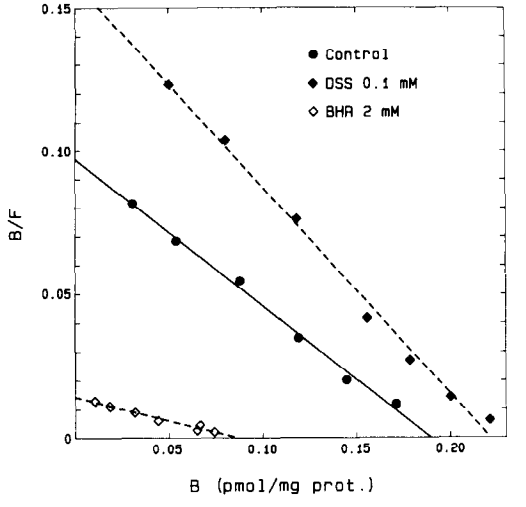

Fig. 5. Scatchard plots of [^{3}H]8-OH-DPAT specific binding to soluble CHAPS hippocampal extracts treated or not by disuccinimidyl suberate or Bolton–Hunter reagent. Soluble extracts were pretreated with 0.1 mM DSS (◆), 2 mM BHR (◇) or none (●) as in the legend to Fig. 4. Binding assays were performed with 0.1–10 nM [^{3}H]8-OH-DPAT. Each point is the mean of triplicate determinations in a typical experiment. B = [^{3}H]8-OH-DPAT specifically bound in pmol/mg prot. B/F = [^{3}H]8-OH-DPAT specifically bound/[^{3}H]8-OH-DPAT remaining free, in fmol per sample. Control: K_d = 0.53 nM; B_{max} = 185 fmol/mg prot. DSS: K_d = 0.42 nM; B_{max} = 224 fmol/mg prot. BHR: K_d = 1.67 nM; B_{max} = 87 fmol/mg prot. Similar data were obtained in two separate experiments.

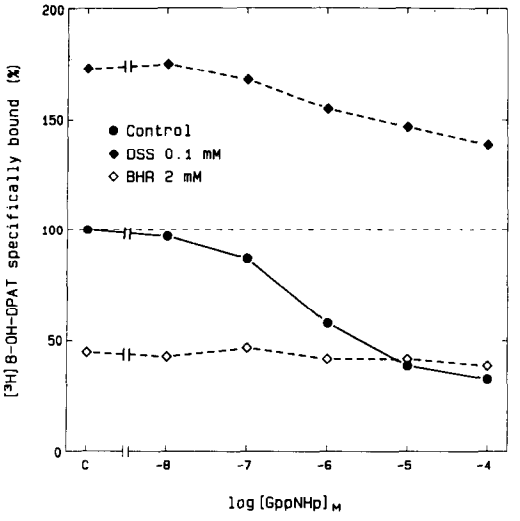

Fig. 6. Effects of GppNHp on [^{3}H]8-OH-DPAT specific binding to soluble CHAPS hippocampal extracts treated or not by disuccinimidyl suberate or Bolton–Hunter reagent. CHAPS soluble extracts were pretreated with 0.1 mM DSS (◆), 2 mM BHR (◇) or none (●) as in the legend to Fig. 4, and binding assays were performed with 1.0 nM [^{3}H]8-OH-DPAT, in the presence of various concentrations of GppNHp (abscissa). [^{3}H]8-OH-DPAT specific binding is expressed in percentage of that found in the absence of GppNHp (C on abscissa) with CHAPS extracts preincubated for 60 min at 15° without DSS or BHR. Each point is the mean of triplicate determinations in four independent experiments.

binding studies showing a negative influence of guanine nucleotides upon agonist binding to 5-HT$_1$ and 5-HT$_2$ sites in brain membranes [30]. In the case of 5-HT$_{1A}$ receptors, further evidence came recently with the use of PT which ADP-ribosylates and inactivates the α-subunit of at least two classes of G regulatory proteins, Go and Gi [28, 31]. Indeed inactivation of Go and/or Gi by PT has been shown to prevent the various cellular events (adenylate cyclase inhibition, K^+ channel opening) normally triggered by 5-HT$_{1A}$ agonists [12, 32–34].

However, neither the effect of PT on the binding of agonists to 5-HT$_{1A}$ sites, nor more direct investigations on the interaction of these sites with G regulatory proteins by physical and chemical methods have been reported so far. Attempts to perform experiments relevant to both of these points have been made in the present study.

In vivo PT treatment induced a marked decrease in [^{3}H]8-OH-DPAT specific binding to membranes from various brain regions, particularly the binding component sensitive to guanine nucleotides. Indeed [^{3}H]8-OH-DPAT binding which persisted in the presence of a saturating concentration (0.1 mM) of GppNHp was not affected by *in vivo* PT treatment. Similar observations were made for other receptor types [35–38], strongly suggesting that like the latter, 5-HT$_{1A}$ binding sites exist under two forms in brain membranes, only one being coupled to a G regulatory protein (probably Gi or Go). At least in the

Table 2. Effects of pretreatment with GppNHp and/or DSS on the characteristics of [^{3}H]8-OH-DPAT specific binding to CHAPS soluble hippocampal extract

Treatment	[^{3}H]8-OH-DPAT specific binding K_d (nM)	B_{max} (fmol/mg prot.)
(1) Control, GppNHp	0.65 ± 0.16	133.7 ± 16.4
(2) DSS, GppNHp	0.52 ± 0.14	203.4* ± 18.2
(3) GppNHp, DSS	0.69 ± 0.19	64.2* ± 20.2

The same batch of CHAPS soluble extract from hippocampal membranes was divided into three fractions which were respectively treated as follows: (1) Control, GppNHp: preincubation for 30 min at 15°, and then incubation for 60 min at 15° after the addition of DMSO (1% final concentration); 0.1 mM GppNHp was added just before dialysis/concentration (see below); (2) DSS, GppNHp: preincubation for 30 min at 15°, and then incubation for 60 min at 15° in the presence of 0.1 mM DSS (added with DMSO, 1% final concentration); 0.1 mM GppNHp was added as in (1); (3) GppNHp, DSS: preincubation for 30 min at 15° with 0.1 mM GppNHp, and then incubation for 60 min at 15° in the presence of 0.1 mM DSS + DMSO as in (2). Samples were dialysed/concentrated against 0.05 M Tris–HCl, pH 7.4, containing 0.1% CHAPS, using a MicroProDiCon apparatus, and used for binding assays with 0.85–5.20 nM [^{3}H]8-OH-DPAT. K_d (in nM) and B_{max} (in fmol/mg prot.) values are the means ± SE of three separate determinations.

* $P < 0.05$ when compared to B_{max} of "control, GppNHp" extracts (Student's t-test).

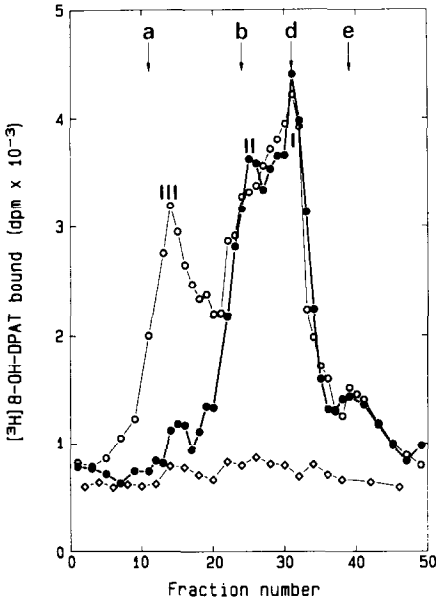

Fig. 7. Sucrose gradient sedimentation of soluble 5-HT$_{1A}$-binding sites after pretreatment with (○) or without (●) disuccinimidyl suberate. Soluble CHAPS extract from hippocampus was treated with 0.1 mM DSS or none as described in the legend to Fig. 4. After dialysis/concentration, 300 μl aliquots were layered on top of sucrose gradients (5–30% sucrose), which were then centrifuged at 200,000 g for 17 hr at 4°. Aliquots (0.1 ml) of the same fractions (250 μl) from the two identical gradients (for each condition) were used for the measurement of [^{3}H]8-OH-DPAT binding in the absence (○, ●) or the presence (◇) of 10 μM 5-HT (similar levels of non specific binding were found with control and DSS-treated extracts). Each point is the mean of duplicate determinations of [^{3}H]8-OH-DPAT bound (in dpm × 10^{-3}) per sample. (a) Thyroglobulin; (b) catalase; (d) β-amylase; and (e) BSA. Calibration of the gradients allowed the estimation of the sedimentation coefficient of peak III; 16.5 S, generated by DSS treatment. The same experiment has been repeated three times with similar results.

rat hippocampus, concentration-dependent inhibition of [^{3}H]8-OH-DPAT binding by GppNHp levelled off at −65–70%, suggesting that the respective proportions of G-coupled and -uncoupled R[5-HT$_{1A}$] in this region were 65–70% and 30–35%, respectively.

The recent availability of a solubilization procedure for 5-HT$_{1A}$ binding sites [19] allowed us to examine directly the possible existence of both G-coupled and uncoupled R[5-HT$_{1A}$] using a sucrose gradient sedimentation procedure. In fact, CHAPS soluble extracts from hippocampal membranes did contain two forms of [^{3}H]8-OH-DPAT binding activity associated with material sedimenting at 8.0 S and 10.0 S. Sedimentation in the presence of 1 μM GTP resulted in the selective reduction of [^{3}H]8-OH-DPAT binding activity at 10.0 S, suggesting that this material corresponded to the 5-HT$_{1A}$ site coupled to a G regulatory protein, and the 8.0 S material to the uncoupled binding site. However, attempts to stabilize the coupling of 5-HT$_{1A}$ sites to G by adding Mn^{2+} (as shown for other receptors, see Ref. 39) did not reduce the [^{3}H]8-OH-DPAT binding activity of the 8.0 S material for the benefit of the 10.0 S material, but only improved the resolution of the two peaks. These data would suggest that not all 8.0 S form is freely convertible to 10.0 S form by association with G protein, i.e. that at least part of the 8.0 S form corresponds to R[5-HT$_{1A}$] permanently uncoupled to a G protein. Indeed Scatchard analyses revealed that maximal inhibition by guanine nucleotides allowed the persistence of high affinity [^{3}H]8-OH-DPAT binding sites, which might correspond to such uncoupled R[5-HT$_{1A}$]$_H$. In contrast, dissociation of R[5-HT$_{1A}$]–G due to guanine nucleotides probably produced free R[5-HT$_{1A}$]$_L$ with very low affinity for the radioactive agonist, and

Table 3. Characteristics of the inhibition by ipsapirone and methiothepin of [^{3}H]8-OH-DPAT specific binding to material sedimenting in various fractions of sucrose gradient—effects of DSS treatment

Material	IC_{50} (nM) Ipsapirone	Methiothepin
CHAPS soluble extract	4.3 ± 1.2	456 ± 124
Sucrose gradient, fractions 23–34	3.2 ± 0.9	115 ± 75
DSS + sucrose gradient, fractions 10–19	3.6 ± 1.1	225 ± 59
DSS + sucrose gradient, fractions 23–34	4.4 ± 1.4	142 ± 56

Sucrose gradient sedimentation was performed using either control CHAPS soluble extract from hippocampal membranes, or the same extract pretreated with 0.1 mM DSS as described in Materials and Methods. The fractions with [^{3}H]8-OH-DPAT specific binding activity (fractions 23–34 for the control extract, and 10–19 and 23–34 for the DSS-treated extract, see Figs 3 and 7) were pooled and used for binding assays (0.8 nM [^{3}H]8-OH-DPAT) in the absence or the presence of various concentrations (0.1 nM–10 μM) of ipsapirone or methiothepin. IC_{50} values (in nM) were calculated from logit–log inhibition plots. Each value is the mean ± SE of at least three independent determinations.

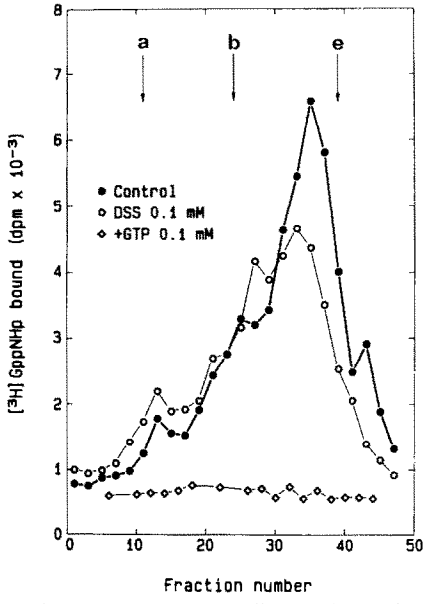

Fig. 8. Sucrose gradient sedimentation of soluble [^{3}H]GppNHp binding sites after pretreatment with (○) or without (●) disuccinimidyl suberate. DSS treatment of soluble CHAPS extracts and sucrose gradient sedimentation were as described in the legend to Fig. 7. Binding assays were performed on 30 μl aliquots of each collected fraction, with 24 nM [^{3}H]GppNHp, plus (◇) or minus (○, ●) 0.1 mM GTP (similar levels of non specific binding were found with control and DSS-treated extracts). Each point is the mean of triplicate determinations of [^{3}H]GppNHp bound (in dpm × 10^{-3}) per sample. (a) Thyroglobulin; (b) catalase and (e) BSA. The same experiment has been repeated three times with similar results.

therefore no longer detectable by binding assay (as shown by the marked reduction in B_{max} due to GppNHp, see Fig. 2).

The apparent molecular weights of the 8.0 S and 10.0 S materials with [^{3}H]8-OH-DPAT binding activity could not be determined (from the measurement of Stokes radius) because solubilized 5-HT_{1A} binding sites aggregate and eluate as a broad peak in gel filtration chromatography, even in the presence of detergents (not shown). However, an approximation of the apparent MWs of the CHAPS micelles carrying 5-HT_{1A} sites could be made from the sedimentation of selected markers in the sucrose gradient: 150 kD and 200 kD for the 8.0 S and 10.0 S peaks, respectively. The difference in sedimentation coefficients ($\Delta S = 2$) of the two forms might well correspond to the G protein still attached to 5-HT_{1A} binding sites only under the 10.0 S form, since previous studies on other receptor types have shown that the dissociation of G resulted in similar decreases in their sedimentation coefficients: 2.0 S for α-adrenergic receptors [40], VIP receptors [41] and mu-opioid receptors [42], and 1.8 S for M_2-muscarinic receptors [43].

If soluble R[5-HT_{1A}] is functionally coupled to G, it should be possible to stabilize this coupling by cross-linking the two proteins with a bifunctional reagent. Several lines of evidence converge for proposing that this is the case when CHAPS soluble extracts from hippocampal membranes were treated with the bifunctional reagent DSS:

(1) DSS treatment enhanced [^{3}H]8-OH-DPAT specific binding as expected from an increased proportion of 5-HT_{1A} sites under the higher affinity state, i.e. coupled to G protein. Indeed DSS induced a significant increase in the B_{max} of high affinity 5-HT_{1A} sites. By contrast treatment with the monofunctional analogue BHR induced a marked reduction of [^{3}H]8-OH-DPAT high affinity binding (probably due to chemical alteration of R[5-HT_{1A}] and/or G);

(2) DSS treatment markedly reduced the negative modulation of [^{3}H]8-OH-DPAT binding by guanine nucleotides, without affecting markedly [^{3}H]-GppNHp specific binding. Therefore prevention by DSS of guanine nucleotide modulation could not be ascribed to some alteration of GppNHp-binding onto G regulatory proteins, but probably resulted from the failure of guanine nucleotides to dissociate the R[5-HT_{1A}]–G irreversible complex;

(3) Prior dissociation of R[5-HT_{1A}]–G by GppNHp

Table 4. Functional reconstitution of 5-HT_{1A} receptor binding sites with G regulatory proteins

Material Addition	[^{3}H]8-OH-DPAT specifically bound (fmoles) None	GppNHp	%
CHAPS soluble extract	2280 ± 46	1149 ± 21	−50
WGA agarose eluate			
—Dialysis, concentration			
+ azolectin, OβG	121 ± 4	113 ± 6	−7
—Reconstitution, no G	105 ± 5	105 ± 6	0
—Reconstitution + Gi, Go	325 ± 8	112 ± 4	−66

CHAPS soluble extract (5 ml) from hippocampal membranes was chromatographed on WGA–agarose in the presence of 0.1 mM GTP as described in Materials and Methods. G protein-free material was eluted with 0.25 M *N*-acetylglucosamine (10 ml) and dialyzed/concentrated to 0.1 ml. After redilution to 0.8 ml, 0.3 ml aliquots were supplemented with 0.5 mg/ml azolectin and 0.9% OβG, with or without 0.1 ml of a mixture of unresolved G proteins (Go + Gi) in their storage buffer or 0.1 ml of the storage buffer alone (no G) in a final volume of 0.46 ml. After incubation for 1 hr at 4°, the mixtures were applied to Extracti-Gel columns and eluted with 1.5 ml of buffer. Binding assays were performed on 100 μl aliquots of each eluate, with 1.0 nM [^{3}H]8-OH-DPAT and in the absence or the presence of 0.1 mM GppNHp. Data are the means ± SE (triplicate determinations in two separate experiments) of total [^{3}H]8-OH-DPAT specific binding capacity (in fmoles) of each material. The per cent reduction due to 0.1 mM GppNHp is indicated in parentheses.

not only prevented the enhancing effect of subsequent DSS treatment on [^{3}H]8-OH-DPAT specific binding, but induced a marked reduction of the density of high affinity 5-HT_{1A} sites. These observations suggest that the stimulatory effect of DSS treatment resulted from the stabilization of pre-existing R[5-HT_{1A}]–G complexes. In the absence of such complexes, DSS might chemically alter R[5-HT_{1A}] or/and G which have been dissociated by GppNHp, then making reassociation no longer possible. Indeed, [^{3}H]8-OH-DPAT binding which persisted after treatment with GppNHp then DSS was insensitive to guanine nucleotide modulation, as expected from the existence of only uncoupled R[5-HT_{1A}]$_H$ in the extracts.

Surprisingly, sucrose gradient sedimentation of DSS-treated CHAPS extracts from hippocampal membranes did not reveal an increase of the 10.0 S sedimenting material as expected from the cross-linking of R[5-HT_{1A}] with G. Instead, the appearance of a new material with high sedimentation coefficient (16.5 S) was observed (Fig. 7). The precise nature of this material is not yet clear, although it had the same pharmacological profile as native 5-HT_{1A} sites (Table 3). Dimerization or cross-linking of R[5-HT_{1A}] and G with an effector system (adenylate cyclase, K^+ channel, etc) and/or regulatory proteins might be responsible for the appearance of this high molecular weight material. Indeed, radiation-inactivation studies have shown that the signalling complex of some receptors coupled to adenylate cyclase through a G regulatory protein can be even larger than the three components put together [44, 45].

In contrast to DSS treatment to permanently coupled R[5-HT_{1A}] with G, WGA–agarose chromatography of CHAPS soluble extract supplemented with GTP allowed the physical separation of these proteins, with a resulting pronounced decrease in the [^{3}H]8-OH-DPAT binding activity of the eluate. Reconstitution of R[5-HT_{1A}]–G complexes in phospholipid vesicles made in the presence of a mixture of Gi and Go produced a marked increase in [^{3}H]8-OH-DPAT binding activity, which then became sensitive to guanine nucleotides. Experiments with brain membranes [46] pretreated with *N*-ethylmaleimide also revealed that high affinity 5-HT_{1A} sites could be recovered by reconstitution with Gi and/or Go. Altogether, these observations strongly suggest that Gi and/or Go are the G regulatory proteins physiologically coupled with functional 5-HT_{1A} sites in the rat brain, as already inferred from binding studies after *in vivo* PT treatment (see above). However, on account of the possible positive coupling of 5-HT_{1A} receptors with adenylate cyclase [16] and/or phospholipase C [13], reconstitution experiments will have to be attempted also with Gs, Gp, etc, before any firm conclusion can be drawn regarding the exact nature of the endogenous G protein(s) functionally coupled with 5-HT_{1A} receptors in brain membranes.

In conclusion, *in vivo* as well as *in vitro* biochemical and physical approaches, all converge for demonstrating directly the interaction of 5-HT_{1A} binding sites with G regulatory proteins in brain membranes. In addition to R[5-HT_{1A}] functionally coupled to G, the present data also support the existence of free R[5-HT_{1A}]$_H$ with high affinity for [^{3}H]8-OH-DPAT but insensitive to modulation by guanine nucleotides. The relationships between these postulated three states of the 5-HT_{1A} receptor: the coupled R[5-HT_{1A}]–G, the dissociated form R[5-HT_{1A}]$_L$ with very low affinity for [^{3}H]8-OH-DPAT and the uncoupled form R[5-HT_{1A}]$_H$ should deserve further investigations.

Acknowledgements—This research has been supported by grants from INSERM and BAYER-PHARMA. We are

grateful to Dr J. Adrien for expert advice in intracerebroventricular injection experiments.

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