Solubilization and characterization of GABA_B receptor binding sites from porcine brain synaptic membranes

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1 The characteristics of membrane bound GABA_B receptors in pig brain are similar to those in rat brain as judged by *in vitro* binding experiments and sensitivity to GTP. The rank order of affinity of GABA_B receptor ligands was CGP 54626 > GABA $\sim$ (–)-baclofen >> CGP 35348 = CGP 36742 > (+)-baclofen in membranes from both species.

2 For solubilization of GABA_B receptors from pig brain, washed membranes were preincubated with 5 mM MgSO₄ and subsequently incubated with various detergents. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS) (0.5%) proved to be the most successful, solubilizing $22.7 \pm 4.7\%$ (mean $\pm$ s.e.mean, $n = 6$) of GABA_B receptors.

3 Binding of [³H]-GABA to GABA_B receptors solubilized with 0.5% CHAPS exhibited similar characteristics to the binding at membrane bound receptors since, firstly, the K_d and B_{max} values (around 30 nM and 450 fmol mg^{–1} protein, respectively) were comparable; secondly, stereospecific binding for baclofen was obtained in both forms; thirdly, the affinity for the agonists GABA and (–)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 were the same; fourthly, comparable sensitivity to Ca²⁺ (2.5 mM) was observed and finally, a similar sensitivity to GTP was apparent.

4 Saturation experiments performed with the GABA_B antagonist, [³H]-CGP 54626, indicated a higher K_d value and a lower B_{max} value for solubilized (7.7 ± 2.6 nM and 1033 ± 41 fmol mg^{–1} protein, mean $\pm$ s.e.mean, $n = 3$) than for membrane bound receptors (1.35 ± 0.08 nM, 1171 ± 20 fmol mg^{–1} protein, $n = 3$).

Keywords: γ -Aminobutyric acid; GABA_B receptor; solubilization; baclofen; CGP 35348; CGP 36742; CGP 54626

Introduction

GABA_B receptors in the mammalian brain are coupled to adenylyl cyclase, Ca²⁺ channels and K⁺ channels via G-proteins. But at present little information is available about the possible structure of the receptor. The affinity of agonists for GABA_B receptors is decreased by guanyl nucleotides, in particular guanosine 5' triphosphate (GTP, Hill *et al.*, 1984), and pertussis toxin, which ADP ribosylates the associated G-protein (Asano *et al.*, 1985; Knott *et al.*, 1993).

Kuriyama and colleagues have recently described the solubilization and purification of GABA_B receptors from bovine brain. To solubilize the receptors, they used either 0.2% deoxycholate sodium (DOC Na) plus 1 M KCl (Ohmori *et al.*, 1990) or 1.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) plus 0.2% asolectin (Ohmori & Kuriyama, 1990). Treatment with DOC Na completely eliminated the stimulating effects of calcium ions as well as the inhibitory effects of GTP on GABA_B receptor binding. Since they did not characterize their solubilized binding sites, we are currently performing experiments to characterize the sites solubilized from porcine brain.

The aim of the present study was therefore to solubilize GABA_B receptors in a GTP-sensitive form, which bind agonists and antagonists with similar affinities to membrane bound receptors. Part of our results have already been presented in abstract form to the British Pharmacological Society (Facklam & Bowery, 1993).

Methods

Tissue preparation

Whole pig brains were obtained from a local slaughterhouse, transported in ice-cold 0.32 M sucrose, dissected free from

white matter and stored at -80°C . Crude synaptic membranes were prepared from pig cerebrum according to the method of Zukin *et al.* (1974). All steps were carried out on ice, solutions were kept at 4°C . The tissue was thawed and homogenized in 0.32 M sucrose + 0.1 mM phenylmethylsulphonylfluoride (PMSF) + $20\text{ }\mu\text{g ml}^{-1}$ bacitracin (1 g tissue per 6 ml) in a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenate was centrifuged for 10 min at 1600 g, the supernatant collected and recentrifuged for 20 min at 31,000 g. The resulting pellet was lysed by resuspension in distilled water at 0.1 mM PMSF + $20\text{ }\mu\text{g ml}^{-1}$ bacitracin and the mixture centrifuged for 20 min at 12,000 g. The supernatant was used to rinse off the upper, soft layer of the pellet. The combined suspension was centrifuged for 20 min at 48,000 g. The final crude synaptic membranes were resuspended and centrifuged once again with distilled water + PMSF + bacitracin and stored at -20°C for at least 18 h.

Washing of crude synaptic membranes

Membranes were suspended in ice-cold distilled water (0.5 g original tissue per 40 ml), centrifuged for 10 min at 13,000 g and washed twice in distilled water. The pellet was then suspended in 50 mM Tris HCl buffer, pH 7.4, incubated at room temperature for 15 min, centrifuged and washed once again by resuspension, incubation and centrifugation. The washed membranes were either used directly or stored at -20°C with no loss of binding activity.

Solubilization

Washed membranes were preincubated with 50 mM Tris HCl buffer pH 7.4 + 5 mM MgSO₄ for 30 min at room temperature, subsequently incubated at a protein concentration of $2.5\text{--}3.5\text{ mg ml}^{-1}$ with various detergents in the presence of 5 mM MgSO₄ + $20\text{ }\mu\text{g ml}^{-1}$ bacitracin at 4°C for 90 min and centrifuged for 60 min at 100,000 g. The supernatant con-

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tained the solubilized fraction. Digitonin was dissolved by heating the solution in a waterbath at 90°C for 20 min.

Dialysis

If inhibition or saturation experiments were performed with the preparations solubilized with 0.5% CHAPS, they were dialysed for 3 h against 100 volumes Tris HCl buffer pH 7.4 + 5 mM MgSO₄ + 20 µg ml⁻¹ bacitracin at 4°C (molecular weight cut-off: 12,000–14,000).

[³H]-GABA_B binding

Crude, preincubated (see solubilization) or solubilized preparations (about 0.3 and 0.06 mg protein per tube, respectively) were incubated in the presence of 2.5 mM CaCl₂, 40 µM isoguvacine HCl, varying concentrations of unlabelled test compound and [³H]-GABA (5 nM final concentration) in 50 mM Tris HCl buffer, pH 7.4, for 10 min at room temperature. The final volume was 0.8 ml. Nonspecific binding was detected by addition of 100 µM (–)-baclofen. The maximum concentrations of the detergents tolerated were 0.05% for CHAPS and 0.1% for the remainder. In the case of soluble receptors γ-globulin and polyethyleneglycol 8000 were added (final concentration 0.15 w/v% and 16 w/v%, respectively, final volume 1.2 ml). The mixture was left to precipitate for 10 min at room temperature. The assay was terminated by centrifugation at 7 500 g for 3 min. The supernatant was aspirated off and the pellet quickly washed twice with 1.5 ml ice cold, distilled water.

The tissue was digested overnight in Soluene-350 solubilizer. The test tube and its contents were transferred to a scintillation vial containing 0.4 ml of 0.2 M HCl and 10 ml Optiphase. The tritium content of each sample was determined by liquid scintillation spectrometry.

[³H]-GABA_A binding

This was performed as [³H]-GABA_B binding with the following exceptions: no CaCl₂ was present; 100 µM (–)-baclofen was used to block GABA_B sites and isoguvacine was omitted generally but used to define nonspecific binding (40 µM).

Figure 1 Formulae of GABA_B antagonists from CIBA, Basel.

[³H]-CGP 54626 binding (Bittiger *et al.*, 1992)

This was performed as [³H]-GABA_B binding with the following exceptions: [³H]-CGP 54626 (final concentration: 1 nM for crude and 2 nM for solubilized preparations) was used; no isoguvacine was present; nonspecific binding was assayed in presence of 10 µM CGP 54626; incubation time was 15 min and the mixture was left to precipitate for 1 min after addition of γ-globulin and polyethyleneglycol 8000.

Protein determination

Estimates of the protein content were made by the bicinchoninic acid assay (Dunn, 1989). This assay was found not to interact with any of the detergents used.

Analysis of binding data

Nonspecific binding was subtracted from the total amount of radioactivity bound to yield the amount of ligand specifically bound. In the case of inhibition experiments, specific binding (% of control) was plotted versus the inhibitor concentration and fitted with nonlinear least squares regression analysis to the following equation:

$$y = 100/(1 + (x/IC_{50})^n)$$

where y = specific binding, x = inhibitor concentration, IC_{50} = concentration of inhibitor reducing specific binding by 50% and n = slope factor.

In the case of saturation experiments specific binding (fmol mg⁻¹ protein) was plotted versus the free concentration (nM) of the radioligand and fitted with nonlinear least squares regression analysis to the following equation:

$$y = B_{max} * x / (K_d + x)$$

where y = specific binding, x = free concentration of radioligand, B_{max} = maximum amount bound and K_d = affinity constant.

Materials

[³H]-GABA (60 Ci mmol⁻¹) was purchased from Amersham, [³H]-CGP 54626 (60 Ci mmol⁻¹), CGP 54626, CGP 36742, CGP 35348 (structural formulae, see Figure 1), (–)-baclofen and (+)-baclofen were a generous gift from CIBA, Basel, Switzerland. All other materials were obtained from commercial sources.

Results

Solubilization of GABA_B receptors

For solubilization of the receptors, washed membranes were preincubated with 5 mM MgSO₄, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 g. Specific GABA_A and GABA_B binding was determined in the pellet and the supernatant, containing the solubilized fraction (Table 1).

Digitonin (1%), sodium cholate (1%) and CHAPS (0.5%) solubilized around 20% of GABA_B receptors. DOC Na produced little binding in the supernatant whilst reducing the residual binding in the pellet to a low level. By comparison, these detergents, solubilized between 24% and 65% of GABA_A sites. From these three conditions 0.5% CHAPS was chosen for further investigation (Table 2), since it showed the most favourable ratio of GABA_B to GABA_A receptors and the highest concentration of GABA_B sites in the pellet, which might indicate that this detergent maintains the receptor G-protein complex. Adding 1 M KCl to DOC Na, sodium cholate or CHAPS did not increase the recovery of GABA_B sites in the supernatant (data not shown).

Table 1 Solubilization of GABA_A and GABA_B receptors with various detergents

	n	Protein [%]	GABA _A binding		GABA _B binding	
			pellet [%]	supernatant [%]	pellet [%]	supernatant [%]
Digitonin 0.5%	3	10.9 ± 0.6	108.3 ± 12.5	12.2 ± 6.3	31.6 ± 2.1	0
Digitonin 1.0%	4	32.3 ± 4.4	151.1 ± 22.5	52.5 ± 19	14.2 ± 2.0	19.3 ± 9.6
DOC Na 1.0%	4	54.6 ± 4.4	114.8 ± 2.2	65.5 ± 6.2	4.3 ± 1.6	8.4 ± 3.1
Sodium cholate 1.0%	4	27.5 ± 4.4	34.5 ± 0.2	42.6 ± 7.4	0.3 ± 0.3	20.7 ± 6.8
CHAPS 0.5%	6	20.5 ± 0.8	37.4 ± 5.0	24.3 ± 8.5	40.9 ± 6.2	22.7 ± 4.7

Washed membranes were preincubated with 5 mM MgSO₄, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 g. Specific GABA_A and GABA_B binding (5 nM [³H]-GABA + 100 μM (–)-baclofen or 40 μM isoguvacine, respectively) was determined in the pellet and the supernatant, containing the solubilized fraction. Values are expressed as a percentage of starting material (mean ± s.e.mean). Experiments were performed in triplicate, *n* equals number of individual experiments.

Table 2 Solubilization of GABA_A and GABA_B receptors with CHAPS under various conditions

	n	Protein [%]	GABA _A binding		GABA _B binding	
			pellet [%]	supernatant [%]	pellet [%]	supernatant [%]
CHAPS (0.5%), Mg ²⁺ absent	4	21.8 ± 0.5	28.4 ± 0.7	33.0 ± 5.9	26.2 ± 4.0	13.7 ± 7.1
CHAPS 0.5%	6	20.5 ± 0.8	37.4 ± 5.0	24.3 ± 8.5	40.9 ± 6.2	22.7 ± 4.7
CHAPS 0.75%	4	24.9 ± 0.7	53.7 ± 6.7	42.4 ± 4.8	37.3 ± 4.7	25.6 ± 3.8
CHAPS 1.0%	4	24.7 ± 1.1	54.8 ± 4.6	45.2 ± 8.4	33.0 ± 3.3	4.8 ± 3.2

Washed membranes were preincubated with 5 mM MgSO₄, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 g. Specific GABA_A and GABA_B binding (5 nM [³H]-GABA + 100 μM (–)-baclofen or 40 μM isoguvacine, respectively) was determined in the pellet and the supernatant, containing the solubilized fraction. Values are expressed as a percentage of starting material (mean ± s.e.mean). Experiments were performed in triplicates, *n* equals number of individual experiments.

Table 3 Saturation experiments to membrane bound and 0.5% CHAPS-solubilized GABA_B receptors performed with the agonist [³H]-GABA and the antagonist [³H]-CGP 54626

		n	Membrane bound receptors mean $\pm$ s.e.mean	n	Solubilized receptors mean $\pm$ s.e.mean
[³ H]-GABA	<i>K_d</i> [nM]	3	25.9 $\pm$ 1.9	6	38.6 $\pm$ 5.9
	<i>B_{max}</i> [fmol mg ⁻¹ protein]	3	454 $\pm$ 99	6	409 $\pm$ 70
[³ H]-CGP 54626	<i>K_d</i> [nM]	3	1.35 $\pm$ 0.08	3	7.7 $\pm$ 2.6
	<i>B_{max}</i> [fmol mg ⁻¹ protein]	3	1171 $\pm$ 20	3	1033 $\pm$ 41

Experiments were performed in triplicate, *n* equals number of individual experiments.

Preincubation of crude membranes in the absence of MgSO₄ decreased the recovery of GABA_B sites solubilized with 0.5% CHAPS by about 10% (Table 2). Increasing the CHAPS concentration to 0.75% did not increase the yield of GABA_B sites in the supernatant and 1% CHAPS solubilized only 5% of the GABA_B sites. Addition of 0.2% asolectin to 0.5% CHAPS did not result in a higher recovery of solubilized GABA_B sites (data not shown). Since 0.5% CHAPS showed a ratio of solubilized GABA_B to GABA_A sites of about 1, compared to 0.6 with 0.75% CHAPS, 0.5% (= 8.1 mM CHAPS) was chosen to solubilize GABA_B receptors.

To support the view, that the GABA_B sites were truly solubilized, the solubilized material was subjected to additional centrifugation for 2 h at 100,000 g and then subsequently to filtration through filters of a pore size of 0.22 μm (Millipore, type GVWP). Neither treatment decreased specific GABA_B binding.

Characterization of GABA_B sites solubilized with 0.5% CHAPS

The characteristics of solubilized, dialysed GABA_B sites were compared to membrane-bound receptors. Saturation experiments (Table 3, Figure 2) were performed with the agonist

[³H]-GABA and the antagonist [³H]-CGP 54626. With [³H]-GABA the *K_d* values for membrane bound and solubilized receptors were approximately 30 nM and the *B_{max}* values about 450 fmol mg⁻¹ protein. With [³H]-CGP 54626 the *K_d* value was 5 times higher for solubilized receptors and the *B_{max}* value was significantly lower (*P* < 0.05, Student's unpaired, two-tailed *t* test).

Inhibition experiments against [³H]-GABA (Table 4, Figure 3) were carried out with the agonist (–)-baclofen, (+)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 (Figure 1) revealing comparable affinities for membrane bound and solubilized GABA_B sites. Total binding was around 7000 d.p.m. for crude and 2000 d.p.m. for solubilized preparations. The high nonspecific binding of 70% of total binding observed for solubilized sites, compared to 50% for membrane-bound receptors, probably stems from the fact that some free unbound radioligand is precipitated by the addition of γ-globulin and polyethyleneglycol 8000.

Detection of specific GABA_B binding was assayed in the presence of Ca²⁺ or Mg²⁺ (Bowery *et al.*, 1983). Normally, GABA_B binding was assayed in the presence of 2.5 mM Ca²⁺. If no Ca²⁺ was added, specific [³H]-GABA_B binding was decreased to 53.5 ± 4.0% (mean ± s.e.mean, *n* = 5) of the value observed in presence of 2.5 mM Ca²⁺ for membrane

bound receptors and to $43.8 \pm 9.7\%$ ($n = 6$) for solubilized receptors. If crude membranes were either preincubated in the absence of MgSO_4 , or if 5 mM EDTA was added to the binding assay, no specific binding was detected.

Agonist binding to GABA_B receptors is GTP-sensitive, i.e., affinity decreases in presence of guanyl nucleotides (Hill *et al.*, 1984). Specific [^3H]- GABA_B binding was measured in the

Figure 2 Saturation experiments shown as Scatchard plots of a representative of 3–6 experiments performed in triplicate of the agonist [^3H]-GABA (a) and the antagonist [^3H]-CGP 54626 (b) to membrane bound ($\bullet$) and solubilized ($\circ$) GABA_B receptors.

Table 4 Displacement of 5 nM [^3H]-GABA binding to membrane bound and 0.5% CHAPS-solubilized GABA_B receptors

		Membrane-bound receptors	Solubilized receptors
(–)-Baclofen	IC_{50} [μM]	0.048 ± 0.002	0.024 ± 0.004
	slope factor	0.94 ± 0.03	0.86 ± 0.13
(+)-Baclofen	IC_{50} [μM]	> 30	> 30
CGP 35348	IC_{50} [μM]	15.4 ± 1.6	25.6 ± 5.0
	slope factor	0.86 ± 0.07	0.85 ± 0.13
CGP 36742	IC_{50} [μM]	14.2 ± 1.8	8.2 ± 2.3
	slope factor	0.73 ± 0.06	0.69 ± 0.13
CGP 54626	IC_{50} [μM]	0.0024 ± 0.0003	0.0018 ± 0.0005
	slope factor	1.08 ± 0.14	0.72 ± 0.13

Values $\pm$ s.d. of curve fitted to mean data of 3–5 experiments, performed in triplicate.

Figure 3 Displacement of 5 nM [^3H]-GABA binding to membrane bound (a) and solubilized GABA_B receptors (b). Symbols represent means $\pm$ s.e. mean of 3–6 experiments performed in triplicate. Lines are best theoretical fits to data as determined by nonlinear least squares regression analysis: ($\bullet$) (–)-baclofen; ($\blacksquare$) (+)-baclofen; ($\blacktriangle$) CGP 35348; ($\blacklozenge$) CGP 36742; (∇) CGP 54626.

Figure 4 Displacement of 5 nM [^3H]-GABA binding to membrane bound ($\bullet$) and solubilized GABA_B receptors ($\circ$) by GTP. Symbols represent means $\pm$ s.e. mean of 4 experiments performed in triplicate. Lines are best theoretical fits to data as determined by nonlinear least squares regression analysis.

presence of increasing concentrations of GTP (Figure 4). For membrane-bound receptors the IC₅₀ value of the curve fitted to the main data of 4 experiments was $0.21 \pm 0.03 \mu\text{M}$ (mean $\pm$ s.e.mean) and the slope factor 0.74 ± 0.06 . The corresponding values for solubilized GABA_B sites were $0.38 \pm 0.10 \mu\text{M}$ and 1.20 ± 0.32 , respectively.

Specific [³H]-CGP 54626 binding to membrane bound receptors showed no GTP-sensitivity up to $100 \mu\text{M}$. Specific binding to solubilized receptors, however, was increased from 100% to $160.2 \pm 8.9\%$ with $100 \mu\text{M}$ GTP (mean $\pm$ s.e.mean, $n = 5$; $P < 0.005$, one sample, Student's two tailed t test) and to $137.2 \pm 17.6\%$ with $10 \mu\text{M}$.

Discussion

Solubilization of GABA_B receptors

The detergents chosen, digitonin, sodium cholate, DOC Na and CHAPS, were mainly those, which have been used successfully in solubilizing other G-protein coupled receptors (Poyner, 1990). For 1% digitonin and 1% DOC Na the sum of the GABA_A sites found in the pellet and the supernatant, expressed as a percentage of starting material, was higher than 150%. This might be explained by an increase in the B_{max} value or the affinity for GABA. Indeed Enna & Snyder (1977) found that treatment of rat crude brain membranes with 0.05% Triton X-100 increased specific [³H]-GABA_A binding about fivefold; 0.03% DOC Na, however, reduced [³H]-GABA_A binding.

A difficulty, experienced with the solubilization of G-protein coupled receptors, e.g. α_2 -adrenoceptors (Smith & Limbird, 1981) or D₂ dopamine receptors (Kilpatrick & Caron, 1983), is that the soluble receptors are insensitive to GTP and bind only antagonists, but not agonists with an affinity similar to membrane bound receptors. This could be explained by dissociation of the G-protein from its receptor during the solubilization procedure. We found for all the detergents and conditions tested that the sum of the GABA_B sites in the pellet and the supernatant, assayed with the agonist [³H]-GABA, was clearly lower than 100%. This could be due to dissociation of the G-protein. The only situations, where this sum was observed to be greater than 60%, were with 0.5% and 0.75% CHAPS. This compared with 35% or lower in the other cases. The zwitterionic detergent CHAPS has proved to be successful in solubilizing α_2 -adrenoceptors (Sladeczek *et al.*, 1984; Matsui *et al.*, 1985), D₂ dopamine (Kuno *et al.*, 1983) and opioid receptors (Chow & Zukin, 1983) in a form, which is GTP-sensitive and binds antagonists and agonists with an affinity similar to membrane-bound receptors. If α_2 -adrenoceptors (Smith & Limbird, 1981) and D₂ dopamine receptors (Kilpatrick & Caron, 1983) were solubilized with digitonin, the receptors were insensitive to GTP and agonists exhibited a lower affinity than for membrane bound receptors. In our study the GABA_B receptors solubilized with 0.5% CHAPS were GTP sensitive and bound antagonists and agonists with affinities comparable to these obtained with membrane bound sites.

CHAPS (1.0%) solubilized only 5% GABA_B sites in contrast to 25% with 0.5% and 0.75% CHAPS. This marked concentration-dependence is in line with previous reports by Guicheney *et al.* (1983), Kuno *et al.* (1983) and Simonds *et al.* (1980), where the highest CHAPS concentration tolerated was 10 mM (= 0.61%).

Compared to the high yield observed for GABA_A sites, a recovery of 25% for GABA_B sites is rather low. This figure, however, matches with the reports of Kuno *et al.* (1983) who solubilized 26% of D₂ dopamine receptors with 10 mM CHAPS + 0.72 M NaCl and Simonds *et al.* (1980) who obtained 20% of opioid binding sites after using 10 mM CHAPS.

Kuriyama and colleagues (Ohmori & Kuriyama, 1990) reported, in contrast to our findings, a yield of 30–40% of

GABA_B sites with 1.2% CHAPS and 0.2% asolectin. However, the authors did not indicate the protein concentration they used and if it was higher than in the present study, this might provide an explanation for the different results.

Characterization of GABA_B sites solubilized with 0.5% CHAPS

Membrane-bound GABA_B receptors in pig brain were compared to those in rat brain. For [³H]-GABA, K_d values between 20 and 80 nM and B_{max} values between 600 and 1200 fmol mg⁻¹ protein have been described in rat brain membranes (Bowery *et al.*, 1983; Cross & Horton, 1987; Szekely *et al.*, 1987). Thus, our values in pig brain (26 nM and 454 fmol mg⁻¹ protein respectively) are at the lower range of the ones reported in rat brain. Bittiger *et al.* (1992) found for [³H]-CGP 54626 a K_d of 1.5 nM and a B_{max} of 3700 fmol mg⁻¹ protein, the K_d being the same as in pig brain with a three fold higher B_{max} value. The ratio of the B_{max} value obtained with the antagonist to the value obtained with an agonist was in both cases 2.6.

The IC₅₀ values for (–)-baclofen, (+)-baclofen, CGP 35348, CGP 36742 and CGP 54626 in pig brain were, within a factor of three, comparable to those in rat brain (Bittiger *et al.*, 1990; Bowery *et al.*, 1983; Froestl *et al.*, 1992; Bowery, 1993).

An important determinant of binding characteristics and affinity is the removal of endogenous GABA. Crude membranes were thoroughly washed until no more GABA_B binding inhibitory activity could be detected in the supernatant after incubation and centrifugation with buffer solution. It was also important to establish whether the characteristics of the solubilized, dialysed GABA_B sites were comparable to those of the membrane-bound receptors. Inhibition experiments against [³H]-GABA showed in both cases stereospecific binding for baclofen with similar affinities for the agonist (–)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626. Saturation experiments with the agonist [³H]-GABA revealed no statistical differences (Student's two-tailed, unpaired t test) in the K_d and B_{max} values. However, if the antagonist [³H]-CGP 54626 was used, the K_d value for solubilized receptors was 5 times higher and the B_{max} value 10% lower than for membrane bound receptors. As expected the maximal amount of receptors labelled with the antagonist was 2–3 times higher than with the agonist for membrane bound and solubilized sites. For membrane bound receptors the K_i value of CGP 54626, calculated according to Cheng & Prusoff (1973) from the IC₅₀ value and the K_d value were comparable (2.0 nM and 1.4 nM respectively). For solubilized sites, however, the K_i value was 1.6 nM and the K_d 7.7 nM.

To measure the amount of radioligand bound, soluble receptors have to be precipitated and this was performed by the addition of γ -globulin and polyethyleneglycol 8000 which increased the total incubation volume from 0.8 to 1.2 ml. Bound radioligand was then separated from unbound by centrifugation. Hence dissociation of the radioligand cannot be excluded. Indeed, if the solubilized receptors were left to precipitate for 1 min specific binding of [³H]-CGP 54626 was somewhat higher than after 10 min, for [³H]-GABA no difference was observed. It might be that dissociation accounts for the slightly, however statistically significant, lower B_{max} value labelled with [³H]-CGP 54626 in solubilized than in membrane-bound receptors.

Binding of the agonist [³H]-GABA to both membrane-bound and solubilized receptors were sensitive to GTP, the IC₅₀ values being around $0.3 \mu\text{M}$. Hill *et al.* (1984) reported an IC₅₀ value for GTP of $0.7 \mu\text{M}$ if the assay was performed in Krebs-Henseleit buffer and $16 \mu\text{M}$ in Tris HCl + 2.5 mM Ca²⁺. GTP failed to affect the binding of the antagonist [³H]-CGP 54626 to membrane bound receptors, however it increased specific binding to solubilized receptors by 60% at $100 \mu\text{M}$. Schütz & Freissmuth (1992) proposed that guanine nucleotides initiate a conversion of a low-affinity antagonist/

high-affinity agonist state into a high-affinity antagonist/low-affinity agonist state. Thus guanine nucleotides not only decrease the affinity of agonists but in addition increase the affinity of antagonists with negative intrinsic efficacy (= inverse agonists). The latter was shown to be the case for muscarinic receptors (Hulme *et al.*, 1981) and A₁ adenosine receptors (Ramkumar & Stiles, 1988), the EC₅₀ value for GTP being in both cases around 2 µM. Since in the present study a comparatively high GTP concentration was needed to increase specific [³H]-CGP 54626 binding, the observed effect is probably nonspecific.

In summary, GABA_B receptors from pig brain can be solubilized by 0.5% CHAPS. Solubilized GABA_B receptors

appear to be comparable to membrane-bound-receptors, since they show stereospecific binding for baclofen, they exhibit similar affinities for the agonists GABA and (–)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 and they exhibit comparable sensitivities to Ca²⁺ and GTP.

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