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Effect of GABA and photoaffinity labelling on the affinity of drugs for benzodiazepine receptors in membranes of the cerebral cortex of five-day-old rats

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Recently, a number of new compounds, usually with a structure unrelated to that of benzodiazepines, like the β-carbolines [1], Ro 15-1788 [2] or CGS 8216 [3], have been discovered which seem to exert their pharmacological effects by an interaction with benzodiazepine receptors which differs from that of classical benzodiazepines [1]. Although these novel drugs do not exhibit a pharmacologically relevant selectivity for subtypes of benzodiazepine receptors, it appears possible that their mode of interaction with these subtypes of receptors differs with respect to their (partial) agonistic or (partial) inverse agonistic effects.

Currently two *in vitro* methods can be used to indicate whether a ligand for benzodiazepine receptors has agonist respectively inverse agonist properties, namely the investigation of affinity changes induced by GABA [4, 5] or by photoaffinity labelling [6] of one of the presumed four benzodiazepine binding sites of the GABA/benzodiazepine receptor complex [7].

We have used these two methods in order to investigate whether ligands for benzodiazepine receptors differ in their mode of interaction with subtypes of benzodiazepine receptors. For this, we used membranes derived from the cerebral cortex of five-day-old rats. In these membranes, like in those from hippocampus of animals of the same age, the type I benzodiazepine receptor, which contains the P_{51} photolabelled peptide, constitutes only approximately one third of total binding capacity [8–10] (W. Sieghart, personal communication). The remaining two other thirds of the binding sites consist of receptors which contain the P_{53}, P_{55} and P_{59} photolabelled peptides [9]. We therefore investigated membranes from the cerebral cortex of five-day-old rats as a tissue enriched in other benzodiazepine receptors than type I and compared the results with those from the cerebellum of adult rats [6]. It has previously been shown that the benzodiazepine receptor for the latter tissue contains >90% type I receptors respectively the P_{51} photolabelled peptide [10].

Methods and materials

Membranes from cerebral cortex of five-day-old Wistar rats were prepared at 0–4° in 50 mM Tris–citrate buffer, pH 7.1 [6]. The membranes were washed 5 times and stored frozen. Photoaffinity labelling with washed cortex membranes was performed essentially as already described [6]. Membranes were preincubated at 0° with 20 nM flunitrazepam 20 min and then irradiated for 60 min with long wave length (366 nm) u.v. light. Membranes were then washed twice in order to remove non-incorporated ligand. For binding assays [^{3}H]-flunitrazepam (72.4 Ci/mmole), [^{3}H]-βCCE (83.7 Ci/mmole) and [^{3}H]-Ro 15-1788 (87.5 Ci/mmole) were used. Control and photoaffinity labelled membranes, equivalent to 10 mg wet weight of tissue, were incubated at 0° in 1 ml 50 mM Tris–citrate buffer pH 7.1, which contained 200 mM NaCl, the drugs

Table 1. Saturation analysis of [^{3}H]-flunitrazepam, [^{3}H]-Ro 15-1788 and [^{3}H]-βCCE binding to the cerebral cortex of five-day-old rats

Ligand	(N)	K_d (nM)	B_{max} (pmoles/mg protein)
[^{3}H]-Flunitrazepam	(5)	1.38 ± 0.06	1.06 ± 0.12
[^{3}H]-Ro 15-1788	(6)	0.76 ± 0.12	0.91 ± 0.21
[^{3}H]-βCCE	(8)	0.69 ± 0.08	0.43 ± 0.19

Membranes were incubated with 0.25–2 nM [^{3}H] ligands at 0° in 50 mM Tris–citrate buffer pH 7.1 which contained 200 mM NaCl. Incubations were performed until equilibrium was obtained ([^{3}H]-flunitrazepam, 90 min; [^{3}H]-Ro 15-1788, 60 min and [^{3}H]-βCCE, 30 min). K_d and B_{max} values were determined by linear regression analysis and are mean values ± S.D.

to be tested and the radioligand. [^{3}H]-βCCE binding assays were terminated after 30 min incubation, [^{3}H]-Ro 15-1788 binding assays after 60 min and [^{3}H]-flunitrazepam binding assays after 90 min. Bound and free ligands were separated by rapid filtration through Whatman GF/B glass fibre filters and washed 2 times with 5 ml ice cold buffer. Radioactivity on the filters was determined by conventional scintillation counting. Binding assays were usually performed at least in triplicate. Unspecific binding was determined using 2 μM methyl-β-carboline-3-carboxylate [βCCM].

[^{3}H]-Ethyl-β-carboline-3-carboxylate ([^{3}H]-βCCE, 83.7 Ci/mmole) and [^{3}H]-Ro 15-1788 (87.5 Ci/mmole) and [^{3}H] flunitrazepam (72.4 Ci/mmole) were obtained from New England Nuclear (Boston, MA), Drugs and Chemicals were obtained from commercial sources or as indicated below. Flunitrazepam, diazepam and Ro 15-1788 were a gift from H. Möhler (Hofmann La-Roche, Basle, Switzerland); methyl-β-carboline-3-carboxylate (βCCM) and ethyl-β-carboline-3-carboxylate (βCCE) were provided by Claus Braestrup (A/S Ferrosan, Soeborg, Denmark), PK 9084, Zopiclone and CL 218-872 were donated by Pharmuka, Gennevilliers (France), Rhone-Poulenc, Vitry-sur-Seine (France) and American Cyanamide, Pearl River (USA) respectively.

Results

Binding properties of membranes derived from the cerebral cortex of five-day-old rats. Saturation equilibrium binding data are shown in Table 1 and indicate that in membranes from the cerebral cortex of five-day-old rats [^{3}H]-flunitrazepam and [^{3}H] Ro-15-1788 label approximately the same number of binding sites. [^{3}H]-βCCE labels less sites (Table 1) than the other ligands. Since in our assay conditions (0.25–2 nM ligand and a filtration separation technique) only high affinity (states of) [^{3}H]-βCCE binding sites are labelled, this observation is in support of the concept that membranes from five-day-old cerebral cortex of rats contain significant amounts of benzodiazepine receptors other than type I [8–10].

In contrast to membranes of adult rat cerebral cortex [11] where GABA did not perturb [^{3}H]-βCCE binding, we found in membranes from the cerebral cortex of five-day-old rats a consistent and significant reduction of [^{3}H]-βCCE binding by 10 μM GABA to 61.1 ± 8.3% (mean ± S.D.; N = 33) of the control incubations.

This GABA induced decrease of [^{3}H]-βCCE binding was maintained when membranes were used for the binding experiments which had before been photoaffinity labelled with flunitrazepam. Thus, in such photolabelled membranes [^{3}H]-βCCE binding in the presence of 10 μM GABA was 75.5 ± 6.1 (mean ± S.D.; N = 33) of the respective controls. This indicates that photoaffinity labelling of benzodiazepine binding sites does not impair the interaction between GABA recognition sites and the remaining benzodiazepine binding sites of the GABA/benzodiazepine receptor complex.

The interaction of drugs with benzodiazepine receptors in the cerebral cortex of five-day-old rats. We investigated the apparent IC_{50} values of a number of drugs in the absence or presence of 10 μM GABA in untreated membranes and in membranes which had been photoaffinity labelled with flunitrazepam and subsequently washed to remove non-incorporated flunitrazepam. Two different ligands were used namely [^{3}H]-βCCE which interacts with higher affinity with type I benzodiazapine receptors [8, 12] and [^{3}H]-Ro 15-1788 which has no selectivity [2]. The results with [^{3}H]-Ro 15-1788 are shown in Table 2 and can be summarized as follows: (i) GABA enhances the apparent affinity of drugs with benzodiazepine like effects and decreases that of the partial inverse agonists CGS 8216, βCCE and βCCM; (ii) the magnitude of the GABA induced affinity changes is similar also in membranes which had before been photoaffinity labelled with flunitrazepam; (iii) the affinity of benzodiazepines for their binding sites is greatly diminished in such photoaffinity labelled membranes.

The same investigations were also performed with [^{3}H]-βCCE as ligand and the results are shown in Table 3. They indicate that the binding sites which are populated by [^{3}H]-βCCE exhibit similar sensitivity to modulation by GABA and photoaffinity labelling as those which are labelled by [^{3}H]-Ro 15-1788. The GABA factors for CL 218-872 with [^{3}H]-Ro 15-1788 (2.1) appears to be in line with a suggested partial agonistic profile of this drug whereas the GABA factor of CL 218-872 with [^{3}H]-βCCE as ligand (3.7) is comparable to that of benzodiazepines.

Discussion

In the present study we investigated the interaction of drugs with benzodiazepine receptors in membranes from the cerebral cortex of five-day-old rats. In this brain area, at this age, the type I benzodiazepine receptors [13], which contain the P_{51} photolabelled peptide [10], constitute only the minority (<30%) of the total benzodiazepine receptor population [14] (W. Sieghart, personal communication). Other types of benzodiazepine receptors, which contain the P_{53}, P_{55} and P_{59} photolabelled peptides [10], constitute the majority of benzodiazepine receptors. Due to technical difficulties the latter binding sites have not yet been well characterized pharmacologically although initial studies revealed distinct differences when compared to benzodiazepine receptors which contain the P_{51} labelled peptide [9]. Another brain area where these drugs have already been investigated is the cerebellum of adult rats [6], where benzodiazepine receptors (type I receptors) which contain

Table 2. Effect of GABA (10 μM) and photoaffinity labelling on the affinity of drugs (expressed as IC_{50} values) for [^{3}H] Ro 15-1788 binding sites in membranes from the cerebral cortex of five-day-old rats

	Untreated membranes			Membranes photoaffinity labelled with flunitrazepam			Photoaffinity labelling induced affinity shift	
	IC_{50} (nM)		GABA	IC_{50} (nM)		GABA		
	−GABA	+GABA	factor	−GABA	+GABA	factor	−GABA	+GABA
Diazepam	12 ± 2	3.6 ± 0.5	3.3	1567 ± 665	460 ± 156	3.4	128 ± 44	125 ± 28
Flunitrazepam	1.5 ± 0.1	0.5 ± 0.1	3.0	82 ± 14	29 ± 1	2.8	56 ± 7	65 ± 13
Zopiclone	112 ± 34	87 ± 25	1.3	260 ± 69	237 ± 71	1.1	2.4 ± 0.6	2.7 ± 0.6
CL 218–872	500 ± 0	233 ± 42	2.1	883 ± 161	593 ± 98	1.5	1.8 ± 0.3	2.6 ± 0.8
PK 9084	407 ± 23	280 ± 35	1.4	1600 ± 100	1033 ± 58	1.6	4.0 ± 0.6	3.6 ± 0.4
CGS 8216	0.1 ± 0.01	0.14 ± 0.01	0.7	0.17 ± 0.02	0.23 ± 0.01	0.7	1.6 ± 0.2	1.7 ± 0.2
βCCE	2.6 ± 0.3	3.8 ± 0.8	0.7	3.0 ± 0.2	4.4 ± 0.3	0.7	1.2 ± 0.2	1.3 ± 0.3
βCCM	2.0 ± 0.2	4.4 ± 0.9	0.4	2.9 ± 0.1	5.3 ± 0.6	0.5	1.5 ± 0.2	1.2 ± 0.1

IC_{50} values (nM) are mean values (±S.D.) of at least three determinations. Binding experiments were performed, as described in methods, with 0.25 nM ^{3}H-Ro 15-1788 in the presence of 200 mM NaCl. The GABA factor was calculated from (IC_{50} − GABA)/(IC_{50} + GABA) and the photoaffinity labelling induced affinity shift was calculated from (IC_{50} in photoaffinity labelled membranes)/IC_{50} in untreated membranes).

Table 3. Effect of GABA (10 μM) and photoaffinity labelling on the affinity of drugs (expressed as IC_{50} values) for [^{3}H]-βCCE binding sites in membranes from the cerebral cortex of five-day-old rats

	Untreated membranes			Membranes photoaffinity labelled with flunitrazepam			Photoaffinity labelling induced affinity shift	
	IC_{50} (nM)		GABA	IC_{50} (nM)		GABA		
	−GABA	+GABA	factor	−GABA	+GABA	factor	−GABA	+GABA
Diazapam	20.8 ± 4.0	7.8 ± 1.6	2.7	3590 ± 613	865 ± 78	4.1	174 ± 24	111 ± 37
Flunitrazepam	5.4 ± 1.1	2.4 ± 0.6	2.2	311 ± 136	132 ± 75	2.4	59 ± 28	55 ± 24
Zopiclone	158 ± 17	82 ± 20	1.9	360 ± 53	187 ± 25	1.9	2.3 ± 0.2	2.3 ± 0.3
CL 218–872	567 ± 29	153 ± 25	3.7	1550 ± 278	640 ± 197	2.4	2.7 ± 0.4	4.2 ± 1.1
PK 9084	460 ± 101	270 ± 17	1.7	2250 ± 260	1340 ± 212	1.7	5.2 ± 1.7	5.0 ± 1.0
Ro 15-1788	1.6 ± 0.4	1.8 ± 0.5	0.9	26 ± 0.3	2.0 ± 0.4	1.3	1.6 ± 0.4	1.2 ± 0.1
CGS 8216	0.13 ± 0.04	0.16 ± 0.08	0.8	0.17 ± 0.02	0.17 ± 0.05	1.0	1.3 ± 0.2	1.1 ± 0.4
βCCM	0.90 ± 0.05	1.5 ± 0.0	0.6	1.1 ± 0.1	1.8 ± 0.2	0.6	1.2 ± 0.1	1.2 ± 0.1

IC_{50} values (nM) are mean values (±S.D.) of at least three determinations. Binding experiments were performed, as described in Materials and Methods, with 0.25 nM ^{3}H-βCCE in the presence of 200 mM NaCl. GABA factor and photoaffinity labelling induced affinity shift were calculated as described in Table 2.

the P_{51} photolabelled peptide, constitute the majority (≥90%) of all benzodiazepine binding sites [8, 9]. A comparison of the results with membranes from these two brain areas should therefore permit to reveal whether there are marked differences of the affinity and of the mode of interaction of drugs with subpopulations of benzodiazepine receptors. The present results do not point to such major differences in the mode of interaction of drugs with benzodiazepine receptor subtypes. In a recent report by Braestrup *et al.* [15], several ligands for benzodiazepine receptors have also been investigated in membranes from the forebrain of six-day-old rats and similar GABA induced affinity changes have been observed. Thus it appears that the investigated drugs interact with subpopulations of benzodiazepine receptors in a similar mode with respect to their agonist or inverse agonist effects.

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