Characterization of two classes of benzodiazepine binding sites in S*chistosoma mansoni*

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SUMMARY

As we have recently shown that GABA should be considered a putative neurotransmitter in *Schistosoma mansoni*, the present work aimed to search for $GABA_A$ receptors in adult worms using [^{3}H]-flunitrazepam to label the allosteric benzodiazepine binding site which is classically present on $GABA_A$ receptor complexes. We detected a large population ($B_{max} = 8{\cdot}25 \pm 1{\cdot}1$ pmol . mg protein^{-1}) of high affinity ($K_d = 33{\cdot}6 \pm 1{\cdot}5$ nM) binding sites for flunitrazepam. These sites harboured a singular pharmacological modulation that does not fit well with a mammalian central benzodiazepine receptor, mainly due to a very high affinity for Ro5-4864 and a very low affinity for clonazepam. We also detected a second population of benzodiazepine binding sites labelled with high affinity ($IC_{50} = 85$ nM) by [^{3}H]-PK11195, a selective ligand of the mammalian peripheral benzodiazepine receptor. In conclusion, this work describes the pharmacological properties of a large population of central-like benzodiazepine receptors supporting their study as putative new targets for the development of anti-parasitic agents. We also describe, for the first time, the presence of peripheral benzodiazepine receptors in this parasite.

Key words: *Schistosoma mansoni*, benzodiazepine, flunitrazepam, PK11195, GABA, platyhelminth.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the major neurotransmitter responsible for synaptic inhibition in the mammalian central nervous system where it mediates a rapid inhibitory transmission by activating ionotropic receptors ($GABA_A$ and $GABA_C$) coupled to chloride channels (Barnard *et al*. 1998). In invertebrates, GABA has been reported to be present in the nervous system of several phyla (Walker *et al*. 1996). Contrasting with the existence of numerous studies in nematodes (Johnson and Stretton, 1987; Martin, 1987; McIntire *et al*. 1993; Walker *et al*. 1996), the physiological actions of GABA in platyhelminths remains relatively poorly explored, with limited reports of GABA-immunoreactivity in the central and peripheral nervous system of several species, including the cestode *Moniezia expansa*, planarian *Girardia tigrina* and trematode *Fasciola hepatica* (Eriksson and Panula, 1994; Eriksson *et al*. 1995) and depression of the electrically evoked activity in the submuscular ventral longitudinal nerve cords of *Notoplana acticola* (Keenan *et al*. 1979). In schistosomes, the trematode responsible for human schistosomiasis, there was no direct evidence for a GABA transmission pathway until our recent description of GABA immunoreactivity (GABA-IR) in nerve cells and fibres of the cerebral ganglia and longitudinal nerve cords and the nerve plexuses ramifying throughout the parenchyma of male adult *Schistosoma mansoni*. In addition, strong GABA-IR was also found associated with the oral and ventral suckers as well as in testes, indicating a role for GABA in fixation to the host vascular wall and spermatogenesis (Mendonça-Silva *et al*. 2004). Furthermore, measurement of a glutamate decarboxylase (GAD) activity and Western blot analysis with antibodies raised against mammalian GAD showed that *S. mansoni* was able to synthesize GABA from glutamate (Mendonça-Silva *et al*. 2004). Thus, GABA is to be considered a putative neurotransmitter in *S. mansoni*, although not all classical criteria that define a neurotransmitter have been met hitherto, such as a detectable influence of GABA on any neurophysiological end-point and the identification of a specific receptor. Such receptors could be $GABA_A$-like ionotropic receptors since this is the type of GABA receptor that is the most widely studied in invertebrates (Walker *et al*. 1996) and have been well characterized in somatic smooth muscles of *A. suum* (Holden-Dye *et al*. 1989). Furthermore, our recent data showing that picrotoxin, a classical blocker of the $GABA_A$ channel, is able to modify the motor activity of adult *S. mansoni* support this idea (Mendonça-Silva *et al*. 2004).

The objective of the present work was to search for, and characterize, $GABA_A$ receptors in adult

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S. mansoni using [^{3}H]-flunitrazepam, a classical tool for labelling the allosteric benzodiazepine site present on the (mammalian) $GABA_A$ receptor/channel complex (central benzodiazepine receptor). This strategy was also stimulated by the existence of one study describing the characteristics of a benzodiazepine binding site in *S. mansoni* (Bennett, 1980) and the necessity to revise this relatively old work in the light of the recent knowledge on benzodiazepine binding sites. In fact, enormous advances have been made in the molecular biology and pharmacological analysis of central benzodiazepine receptor subtypes (Barnard *et al*. 1998; Möhler *et al*. 2001). Also of importance is the recent discovery of peripheral benzodiazepine receptors that constitute a totally distinct molecular entity (Gavish *et al*. 1999), mainly present in the internal and external mitochondrial membranes where it is part of the mitochondrial permeability transition pore (McEnery *et al*. 1992; Papadopoulos *et al*. 1994). Finally, our work characterizes a new putative target for the development of anti-parasitic agents related to the neuromuscular system of this flatworm.

MATERIALS AND METHODS

Preparation of subcellular fractions of S. mansoni

The investigation was in accord with the Institutional Ethical Committee for animal care (Federal University of Rio de Janeiro, Brazil). Adult male *S. mansoni* (BH strain) were obtained from mice, sacrificed by cervical dislocation, that have been infected 45 days earlier with approximately 150 male cercariae each, as previously described (Cunha *et al*. 1992). About 1500 male worms were then homogenized in a Dounce homogenizer at 4 °C in 0·25 M sucrose solutions (5 mMTris (hydroxymethyl) aminomethane, Tris-HCl, pH 7·4) using 3 sequences of 10 passes of the pestle. The homogenate was centrifuged to obtain 4 pellets (P_1, P_2, P_3, P_4) sedimenting respectively at 300 g_{av} (5 min); 1000 g_{av} (10 min); 8000 g_{av} (10 min) and 100 000 g_{av} (1 h). These fractions have been previously characterized by electronic microscopy as heterogeneous (P_1), nuclear (P_2), mitochondrial (P_3) and microsomal (P_4) (Cunha *et al*. 1988). The pellets were resuspended in buffered sucrose solution and stored at −70 °C until use. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Preparation of rat brain synaptosomes

Brains, without cerebellum and brain stem, were obtained from adult male Wistar rats sacrificed by decapitation. Briefly, tissues were homogenized in a Potter apparatus with a motor-driven Teflon pestle at 4 °C in 15 volumes of ice-cold 0·32 M buffered sucrose (pH 7·4) per gram of organ. After centrifuging at 1000 g_{max} for 10 min, the supernatant was centrifuged at 48 000 g_{av} for 20 min to obtain the crude synaptosomes that were resuspended in buffered Krebs solution (1·4 ml/g tissue) and stored at −80 °C until use. Protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Preparation of rat kidney mitochondrial fraction

Kidneys from adult male Wistar rats were homogenized in a Potter apparatus with a motor-driven Teflon pestle at 4 °C in 10 volumes of ice-cold 50 mM Tris-HCl (pH 7·4) per gram of organ. After centrifuging at 500 g_{av} for 10 min, the supernatant was centrifuged at 15 000 g_{av} for 15 min. After resuspension of the pellet in 3 volumes of 50 mM Tris-HCl (pH 7·4), the material was centrifuged at 50 000 g_{av} for 20 min to obtain the mitochondrial fraction that was resuspended in the buffer (3 ml/g tissue) and stored at −80 °C until use.

[^{3}H]flunitrazepam binding assay

P_1-P_4 fractions of *S. mansoni* (100–200 μg protein) or rat synaptosomes (200 μg protein) were incubated at 4 °C for 90 min in a buffered Krebs solution containing 0·2 nM or 1 nM [^{3}H]flunitrazepam (71–85 Ci/mmol, New England Nuclear Life Science Products, USA). After incubation, samples were rapidly diluted with 3 ml of ice-cold Krebs buffer and immediately filtered on glass fibre filters (GMF 3, Filtrak, Germany) under vacuum. Filters were then washed once more with 3 ml of buffer, dried, immersed in a scintillation mixture [1,4-bis-[2-(5-phenyloxazolyl)]-benzene, POPOP, (0·1 g/l) and 2,5-diphenyloxazole, PPO, (4·0 g/l) in toluene], and the radioactivity retained in the filters was counted with a Packard Tri-Carb 1600 TR liquid scintillation analyser. Saturation experiments were performed by the addition of increasing concentrations of unlabelled flunitrazepam (0·1 nM to 5·0 nM) to 0·2 nM [^{3}H]flunitrazepam. This protocol, classically referred to as a competition experiment, has already been used with the same radioligand (Lopes *et al*. 2004) and is of particular interest for binding studies with *S. mansoni* since it allows a great economy of radioligand and protein (Mendonça-Silva *et al*. 2002). Non-specific binding was estimated in the presence of 5 μM unlabelled flunitrazepam.

[^{3}H]PK11195 binding assay

P_1-P_4 fractions of *S. mansoni* (100–200 μg protein) or rat kidney mitochondrial fraction (100 μg protein) were incubated at 4 °C for 60 min in 50 mM Tris-HCl buffer (pH 7·4) containing 0·5 nM [^{3}H]PK11195 (83·5 Ci/mmol, New England Nuclear Life Science

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Products, USA), a selective antagonist of the peripheral binding receptor that directly interacts with its 'isoquinoline-binding protein' subunit. After incubation, samples were rapidly diluted with 4 ml of ice-cold buffer and immediately filtered on glass fibre filters (GMF 3, Filtrak, Germany) under vacuum. Filters were then washed twice more with 4 ml of buffer and treated as described above. Non-specific binding was estimated in the presence of 10 μM unlabelled PK11195.

Statistics

Statistical comparisons of recoveries of flunitrazepam or PK11195 binding in the different subcellular fractions were determined by using ANOVA followed by Bonferroni test for multiple comparisons. Binding data from saturation experiments were presented as a classical Scatchard plot. The model parameters (B_{max} and K_d, for saturation assays; IC_{50}, for competition assays) were estimated using a computerized non-linear regression analysis of the untransformed data (Prism 4.0, GraphPad Software Inc.), assuming a single population of binding sites. The time-course of flunitrazepam binding was analysed using the model of one phase exponential association.

Drugs

Bicuculline, DMCM, GABA, flunitrazepam, pentobarbital, picrotoxin, PK11195 and Ro5-4864 were obtained from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). Clonazepam, diazepam, midazolam and zolpidem were kindly supplied by Roche Indústrias Químicas (Brazil). Flumazenil was from Cristália Produtos farmacêuticos. DMCM, flunitrazepam, PK11195, Ro5-4864, clonazepam, diazepam and midazolam were dissolved in 100% ethanol whereas flumazenil and picrotoxin were dissolved in 100% dimethyl sulfoxide (DMSO) to obtain stock solutions (usually 30 mM) and further diluted in water or Krebs solution. Pentobarbital was dissolved in water (5 mM). At the final concentrations used, ethanol and DMSO had no effect in our assays.

RESULTS

[^{3}H]*flunitrazepam binding*

Specific binding sites for 1 nM [^{3}H]flunitrazepam were detected in all subcellular fractions (P_1-P_4), but a higher specific binding was obtained in the mitochondrial (P_3) fraction (Table 1). Therefore this fraction was used to further characterize the binding of [^{3}H]flunitrazepam to *S. mansoni*. As [^{3}H]flunitrazepam binding to its allosteric site present on the $GABA_A$ receptor complex of mammalian brain is sensitive to temperature (Maguire *et al.* 1992), we compared the binding of 1 nM [^{3}H]flunitrazepam to the P_3 fraction of *S. mansoni* at 4 °C, 25 °C and 37 °C (Table 2). The binding was significantly higher at the lowest temperature, in good accordance with the increase of affinity observed in rat brain when lowering the temperature (Maguire *et al.* 1992). These data also support the use of this temperature (4 °C) throughout our study in *S. mansoni*, as is generally the case for studies in mammals. Figure 1 shows the time-course of 0·2 nM [^{3}H]flunitrazepam binding to P_3 in our standard conditions (4 °C). [^{3}H]flunitrazepam binding is very rapid (half-life = 5 min) reaching a plateau after about 30 min and remaining stable during the 90-min incubation period. After having determined these optimal experimental conditions, we studied the concentration-dependence of flunitrazepam binding to *S. mansoni* by incubating fraction P_3 at 4 °C during 90 min, in the presence of 0·2–5 nM of the ligand (see Materials and Methods section for details). As shown in Fig. 2 for a typical experiment, the Scatchard plot was linear, indicating

Table 1. Subcellular distribution of 1 nM [^{3}H]flunitrazepam binding in subcellular fractions of *Schistosoma mansoni*

Fractions	[^{3}H]flunitrazepam bound (fmol/mg protein)	Recovery (%)†
P_1	51·5 ± 13·6	16·9 ± 3·7
P_2	60·7 ± 18·5	14·2 ± 2·3
P_3	202 ± 26·7*	30·8 ± 2·5
P_4	72·7 ± 18·6	38·1 ± 2·8$^+$

† % Recovery = 100 × binding (*specific binding* × *protein content*) in each individual fraction divided by the sum of the binding of the 4 fractions. Values represent means ± S.E.M. from 3 different preparations, each experiment being performed in triplicate.
* $P < 0{\cdot}05$, ANOVA followed by Bonferroni test (P_3 *vs* P_1, P_2 and P_4 fractions).
$^+$ $P < 0{\cdot}05$, ANOVA followed by Bonferroni test (P_4 *vs* P_1 and P_2).

Table 2. Effect of temperature on [^{3}H]flunitrazepam binding to the P_3 fraction of *Schistosoma mansoni*

(Approx. 100 μg protein were incubated for 90 min at different temperatures in a modified Krebs solution containing 1 nM [^{3}H]flunitrazepam. Values represent means ± S.E.M. from 3 different preparations, each experiment being performed in triplicate.)

Temperature	[^{3}H]flunitrazepam bound (fmol/mg protein)
4 °C	216·3 ± 27·5*
25 °C	81·0 ± 5·5
37 °C	30·7 ± 2·7

* $P < 0{\cdot}05$, ANOVA followed by Bonferroni test (4 °C *vs* 25 °C and 37 °C).

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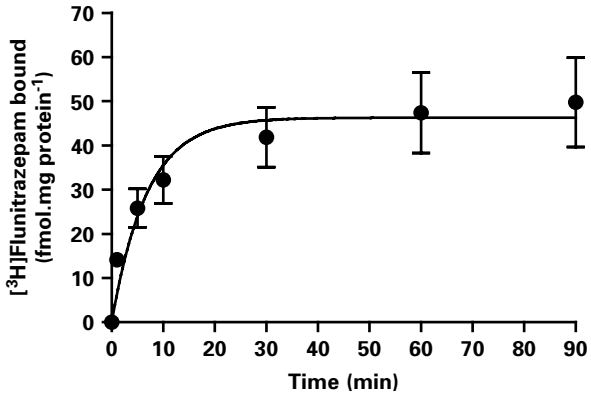

Fig. 1. Time-course of [^{3}H]flunitrazepam binding to the *Schistosoma mansoni* P_3 fraction. Approximately 100 μg protein were incubated at 4 °C in a modified Krebs solution containing 1 nM [^{3}H]flunitrazepam. The theoretical curve was obtained by non-linear regression analysis using the model of one phase exponential association. Each point represents the mean ± S.E.M. obtained from experiments performed in triplicate with 3 different preparations.

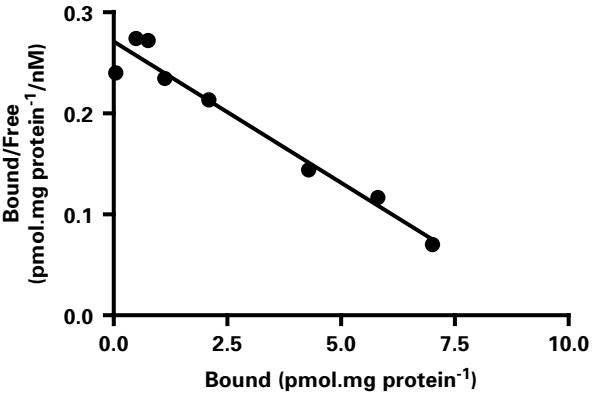

Fig. 2. Typical Scatchard plot for [^{3}H]flunitrazepam binding to the P_3 fraction of *Schistosoma mansoni*. The curve was drawn using the parameters fitted by non-linear regression analysis with the model of a single class of independent binding sites. Each point represents the mean of triplicate determinations in a typical experiment. Non-specific binding accounted for 10–30% of total [^{3}H]flunitrazepam binding.

the labelling of a single homogeneous population of receptors. These experiments were repeated twice in order to obtain good estimations of the parameters K_d and B_{max}, by averaging the values calculated independently in 3 different preparations (Table 3). Flunitrazepam binds in the nanomolar range of concentration, i.e with a relatively high affinity, to a very large number of sites. When compared to rat brain synaptosomes (Table 3), a preparation enriched in $GABA_A$ receptors, the density of flunitrazepam binding sites is about 4 times higher in the mitochondrial fraction of *S. mansoni* whereas the affinity is about 15 times lower. In order to further characterize the flunitrazepam binding sites present

Table 3. Equilibrium constants for the specific binding of [^{3}H]flunitrazepam

(Values of parameters represent means ± S.E.M. of 3 individual experiments performed in triplicate.)

	K_d (nM)	B_{max} (pmol . mg protein^{-1})
S. mansoni (P_3 fraction)	33·6 ± 1·5	8·25 ± 1·1
Rat synaptosomes	2·3 ± 1·1	2·1 ± 1·0

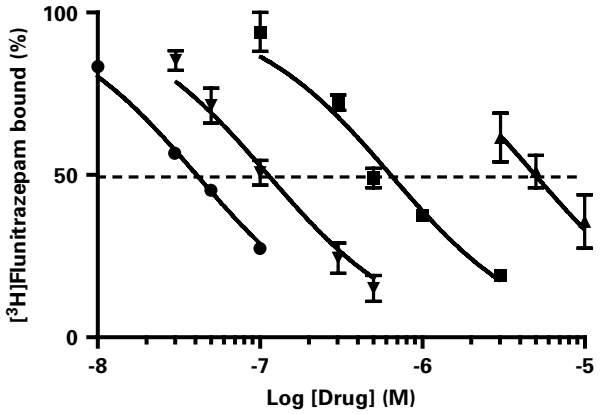

Fig. 3. Competition curves for 0·2 nM [^{3}H]flunitrazepam binding to P_3 fraction of *Schistosoma mansoni*. Flunitrazepam (●), zolpidem (▼), diazepam (■) and clonazepam (▲). The curves were drawn using the parameters fitted by non-linear regression analysis using the model of a single class of independent binding sites. Each point represents the mean ± S.E.M. obtained from 2–4 experiments performed in quadruplicate.

in *S. mansoni*, we firstly tested the effect of allosteric modulators of the mammalian benzodiazepine binding site present on the $GABA_A$ receptor complex (central benzodiazepine receptor). Neither GABA (1 mM) nor pentobarbital (100 μM) altered the binding of [^{3}H]flunitrazepam to P_3, in contrast to observations in mammalian brain preparations washed from endogenous GABA (Lopes *et al*. 2004). Bicuculline (30 μM) and picrotoxin (100 μM), which modulate the binding of benzodiazepine to mammalian brain preparations in the presence of GABA (Lopes *et al*. 2004), were also without effect in our preparation from schistosomes. As the pharmacological modulation of a binding site is a powerful tool for its classification, we performed whole competition curves for [^{3}H]flunitrazepam binding to the P_3 fraction of *S. mansoni* using different putative ligands of the benzodiazepine receptor and compared their IC_{50} values. Figure 3 shows competition curves for 4 classical ligands of the mammalian central benzodiazepine receptors. The IC_{50} values for zolpidem, flunitrazepam and diazepam were not very different from the values observed in rat brain, being

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Table 4. Inhibition of [^{3}H] flunitrazepam binding in rat brain synaptosomes and in P_3 fraction of *Schistosoma mansoni* by different ligands of benzodiazepine receptors

(IC_{50} values were calculated by non-linear regression analysis of competition curves like those shown in Fig. 3. Drugs were incubated along with 0·2 nM [^{3}H] flunitrazepam at 4 °C for 90 min in a buffered Krebs solution in the presence of 100–200 μg protein of rat synaptosomes or P_3 fraction of *S. mansoni*. #No inhibition was obtained even at 10 μM flumazenil in *S. mansoni*, indicating that the IC_{50} should be much higher than this concentration, or that flumazenil has no affinity at all for these binding sites. In mammals, clonazepam and flumazenil are selective ligands of the central benzodiazepine receptors whereas PK11195 and Ro5-4864 are selective for the peripheral benzodiazepine receptors (see Table 5).)

	IC_{50} (nM)		
Drug	Rat (brain)	*S. mansoni* (P_3 fraction)	Ratio (Schisto/rat)
Zolpidem	49	110	2·2
Flunitrazepam	3	41	14
Diazepam	11	640	58
Clonazepam	1	4940	4940
Flumazenil	2	#	$\gg$5000#
PK11195	19 400	4710	0·24
Ro5-4864	33 100	110	0·0033

Table 5. Affinities (IC_{50}) of various benzodiazepine ligands for central and peripheral benzodiazepine receptors of the rat

(IC_{50} values were calculated by non-linear regression analysis of competition curves for [^{3}H] flunitrazepam binding in rat brain synaptosomes (central benzodiazepine receptor) or for [^{3}H]PK11195 binding in rat kidneys (peripheral benzodiazepine receptor). The ratio (PK/FLU) is a measure of the selectivity between these 2 benzodiazepine receptors: values higher than 1 indicates a selectivity for central benzodiazepine receptors and values lower than 1 indicate a selectivity for peripheral benzodiazepine receptors.)

	Rat – IC_{50} (nM)		
Drug	Brain [^{3}H]FLU	Kidney [^{3}H]PK11195	Ratio PK/FLU
Zolpidem	49	490	10
Flunitrazepam	3	30	10
Diazepam	11	220	20
Clonazepam	1	11 500	11 500
Flumazenil	2	$>$30 000[a]	$>$15 000[a]
PK11195	19 400	17	0·00088
Ro5-4864	33 100	25	0·00076

[a] Less than 40% inhibition was obtained even at 30 μM flumazenil, indicating that the IC_{50} should be higher than this concentration.

about 2–58 times higher (Table 4). On the other hand, the IC_{50} for clonazepam (4940 nM) was surprisingly very high, about 5000 times higher than in rat brain. The fact that clonazepam is particular in that it is very selective for the central benzodiazepine receptors in mammals (it has a very low affinity for the peripheral benzodiazepine receptors (Hirsch *et al.* 1988)), together with the lack of effect of GABA and other classical modulators of the central benzodiazepine receptor led us to investigate if the [^{3}H]flunitrazepam binding sites detected in *S. mansoni* could correspond to peripheral-like binding sites instead of central-like benzodiazepine binding sites. Firstly, we tested flumazenil, another benzodiazepine which is very selective for the mammalian central benzodiazepine receptor; in this case, no effect was observed even using a concentration as high as 10 000 nM (Table 4). A second approach was to assay 2 ligands very selective for the mammalian peripheral benzodiazepine receptor, namely PK11195 and Ro5-4864 (Maguire *et al.* 1992). PK11195 exhibited an IC_{50} value (4710 nM) similar to the one measured in rat brain for competition to the central benzodiazepine receptor (19 400 nM, Table 4). On the other hand, the IC_{50} value of Ro5-4864 was much lower in *S. mansoni* (110 nM) than in rat brain (33 100 nM) being even similar to the IC_{50} measured for binding to the mammalian peripheral benzodiazepine receptor present in rat kidney (25 nM, Table 5). This result is compatible with the idea that [^{3}H]flunitrazepam could be labelling a peripheral-like benzodiazepine receptor in *S. mansoni*. On the other hand, the IC_{50} value of PK11195 should be too high for such peripheral sites, based on comparison with values for rat kidney (Hirsch *et al.* 1988 and Table 5), indicating that PK11195 is here competing with [^{3}H]flunitrazepam for a schistosomal central benzodiazepine receptor. In an attempt to directly challenge this hypothesis, we decided to search for PK11195 binding sites with higher affinity (hence, presumably of the peripheral type), using [^{3}H]PK11195 as a radioligand.

[^{3}H]PK11195 binding

Specific binding sites for 0·5 nM [^{3}H]PK11195 were detected in all subcellular fractions (P_1-P_4) with a similar value of specific binding (Table 6). Here too, the mitochondrial (P_3) fraction was chosen for further characterization of these binding sites. The best way to study the pharmacological modulation of a binding site is to compare the IC_{50} values calculated from whole competition curves performed with different putative ligands. Unfortunately, such a protocol was not possible here due to the technical limitation imposed by the very low specific binding (only about 20–50% of total binding at 0·5 nM [^{3}H]PK11195, in the absence of competitor). This low level of specific binding also explains why we did not obtain sufficiently reliable saturation curves in order to calculate K_d and B_{max} in P_3 (5 assays) but

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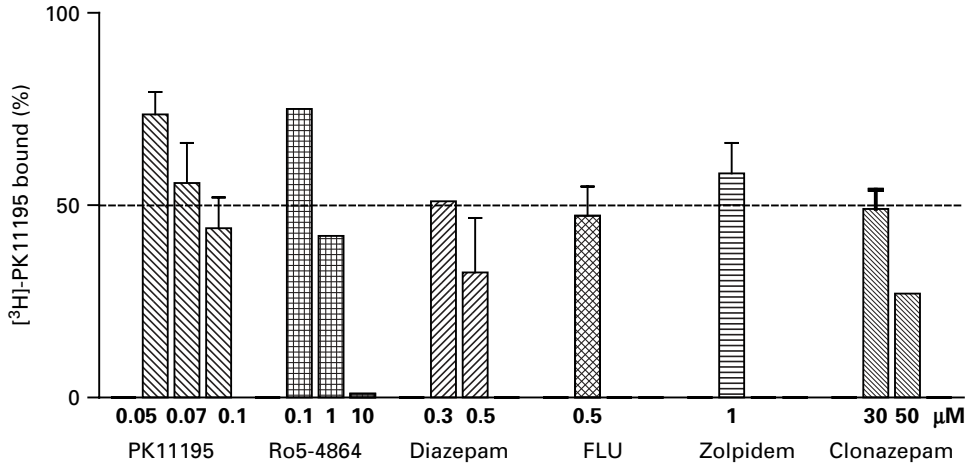

Fig. 4. Pharmacological modulation of 0·5 nM [^{3}H]PPK11195 binding to P_3 fraction of *Schistosoma mansoni*. Each point represents the mean ± S.E.M. obtained from 1–4 experiments performed in quadruplicate. Non-specific binding accounted for 48–82% of total [^{3}H]flunitrazepam binding. FLU = flunitrazepam.

indicates that the number of [^{3}H]PK11195 binding sites in P_3 fraction is lower than of [^{3}H]flunitrazepam binding sites, the first indication that the two ligands label different sites. Note that the scarcity of material (3·17 ± 0·26 mg protein of P_3 per 1000 worms, $n = 15$) was an additive constraint throughout the present work. As a surrogate, we tested only 1–3 concentrations of each putative ligand, near their IC_{50} values that we previously estimated in a screening assay. Figure 4 shows that we were able to obtain good estimates of IC_{50} for all drugs tested with the exception of flumazenil, which produced only 10% inhibition at 10 μM. As a whole, the IC_{50} values for [^{3}H]PK11195 binding in our schistosomal fraction were very similar to the values for the mammalian peripheral benzodiazepine receptor, as measured in the rat kidney preparation (compare Table 5 with Table 7). Only flunitrazepam and Ro5-4864 exhibited higher IC_{50} values in schistosomes than in rat (17 and 28 times higher, respectively). The fact that we were able to obtain good estimates of IC_{50} values for both [^{3}H]flunitrazepam and [^{3}H]PK11195 in schistosome and rat preparations, in the same experimental conditions, allowed us to compare the selectivity of these drugs for the 2 benzodiazepine binding sites, in both species. Comparison of the ratio of PK11195/flunitrazepam IC_{50} values (Tables 5 and 7) indicated that no drug was very selective in *S. mansoni*, since only PK11195 exhibited a noticeable selectivity (55 times) for the 'peripheral' binding site (ratio = 0·018). Note that this drug had a 1140 times selectivity for the peripheral benzodiazepine receptor in the rat (ratio = 0·00088) just as R05-4864 (ratio = 0·00076). On the other hand, clonazepam that was 11 500 times more selective for the central benzodiazepine receptor in the rat (ratio = 11500) was nearly equipotent for competing at both [^{3}H]flunitrazepam and [^{3}H]PK11195 binding sites, in schistosomes (ratio = 6). With respect to IC_{50} values for the mammalian peripheral benzodiazepine receptors (Table 5), we performed whole competition curves for 0·5 nM [^{3}H]PK11195 binding in our rat kidney mitochondrial fraction. Note that this preparation contains a relatively high density of peripheral benzodiazepine receptors, as calculated from saturation curves performed either with [^{3}H]PK11195 ($B_{max} = 6·6$ pmol . mg protein^{-1}, $n = 1$) or with [^{3}H]flunitrazepam ($B_{max} = 8·26 \pm 1·04$ pmol. mg protein^{-1}, $n = 3$), allowing good technical conditions (specific binding about 80% at 0·5 nM [^{3}H]PK11195).

DISCUSSION

Our recent evidence for the presence of a GABAergic neurotransmission in the trematode *S. mansoni* (Mendonça-Silva *et al.* 2004) led us to investigate the presence of ionotropic $GABA_A$ receptors using [^{3}H]-flunitrazepam, a classical tool for labelling the allosteric benzodiazepine site (central benzodiazepine receptor) present on the mammalian $GABA_A$ receptor/channel complex. The specific binding of [^{3}H]flunitrazepam was higher in the P_3 fraction of adult male *S. mansoni*, a fraction previously characterized as a mitochondrial fraction (Cunha *et al.* 1988), not coinciding with the binding sites for kainic acid, which labels another putative amino acid neurotransmitter receptor (glutamate), that have been previously detected only in the P_1 fraction (Mendonça-Silva *et al.* 2002). This was the first reason to imagine that the benzodiazepine binding sites present in this worm could be different from the classical central benzodiazepine receptors of mammals and even to suspect flunitrazepam labelling of peripheral benzodiazepine binding sites, initially discovered in mitochondria (Gavish *et al.* 1999; McEnery *et al.* 1992). This hypothesis had to be seriously considered since flunitrazepam is known to

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Table 6. Subcellular distribution of 0·5 nM [^{3}H]PK11195 binding in subcellular fractions of *Schistosoma mansoni*

(Values represent means ± S.E.M. from 3 different preparations, each experiment being performed in triplicate.)

Fractions	[^{3}H]PK11195 bound (fmol/mg protein)	Recovery (%)†
P_1	12·5 ± 2·7	44·8 ± 8·3*
P_2	9·3 ± 2·1	9·5 ± 4·7
P_3	15·9 ± 2·6	16·4 ± 1·9
P_4	10·9 ± 1·5	29·3 ± 2·2

† % Recovery = 100 × binding (*specific binding* × *protein content*) in each individual fraction divided by the sum of the binding of the 4 fractions.
* $P < 0·05$, ANOVA followed by Bonferroni test (P_1 *vs* P_2 and P_3 fractions).

Table 7. Affinities (IC_{50}) of various benzodiazepine ligands for 'central' and 'peripheral' benzodiazepine receptors of *Schistosoma mansoni*

(IC_{50} values were calculated by non-linear regression analysis of competition curves for [^{3}H]flunitrazepam ('central'-like benzodiazepine receptor) or roughly estimated from partial inhibition assays (see Fig. 4) for [^{3}H]PK11195 binding ('peripheral'-like benzodiazepine receptor) in the P_3 fraction of *S. mansoni*. The ratio (PK/FLU) is a measure of the selectivity between these two benzodiazepine receptors: values higher than 1 indicates a selectivity for central benzodiazepine receptors and values lower than 1 indicate a selectivity for peripheral benzodiazepine receptors.)

	S. mansoni – IC_{50} (nM)		
Drug	P_3 fraction [^{3}H]FLU	P_3 fraction [^{3}H]PK11195	Ratio PK/FLU
Zolpidem	110	2000	18
Flunitrazepam	41	500	12
Diazepam	640	300	0·47
Clonazepam	4940	30 000	6·1
Flumazenil	≫10 000[a]	≫1000[b]	—
PK11195	4710	85	0·018
Ro5-4864	110	700	6·4

[a] No inhibition was obtained at 10 μM flumazenil, indicating that the IC_{50} should be much higher than this concentration.
[b] Only 10% inhibition was observed at 1 μM flumazenil, indicating that the IC_{50} should be much higher than this concentration.

be poorly selective for the central benzodiazepine receptors, labelling the peripheral benzodiazepine receptors present in rat kidney mitochondria with a IC_{50} of about 30 nM i.e. a value very similar to that measured in our P_3 fraction of *S. mansoni* (41 nM). Note that IC_{50} values reported in the literature for flunitrazepam binding to peripheral benzodiazepine receptors vary largely, from 20 to 500 nM (Hirsch *et al.* 1988; Lueddens and Skolnick, 1987), reinforcing the importance of comparing drugs and species in experiments performed under the very same conditions, as we did here. Another strong argument apparently supporting flunitrazepam labelling of peripheral benzodiazepine receptors was the pharmacological profile of [^{3}H]flunitrazepam binding that was very different from that expected for the 'classical' (mammalian) central binding site present on the $GABA_A$ receptor/channel complex and much more similar to that observed for the peripheral benzodiazepine receptor (compare first column of Table 7 to first and second columns of Table 5). On the other hand, zolpidem and, more significantly, PK11195 IC_{50} values did not fit with this hypothesis. The IC_{50} value for PK11195 (4710 nM) was more similar to the value for the rat central benzodiazepine receptor (19400 nM) than for the rat peripheral benzodiazepine receptor (17 nM). Another argument favouring the initial hypothesis that [^{3}H]flunitrazepam was effectively labelling a central-like benzodiazepine receptor, and not a peripheral-like receptor, was the temperature-dependence of the binding, with higher affinity at lower temperature. Finally, the direct evidence that [^{3}H]PK11195 labelled a different population of benzodiazepine binding sites, with a somewhat different subcellular distribution, (apparently) lower density, high affinity for PK11195 and a pharmacology very similar to the rat peripheral benzodiazepine receptor supports the existence of 2 distinct populations of benzodiazepine binding sites in adult *S. mansoni*. We will now discuss the characteristics of these 2 populations, in the light of our knowledge on mammalian and invertebrate benzodiazepine receptors.

The first population of schistosomal benzodiazepine binding sites should correspond to a central-like benzodiazepine receptor, albeit significantly different in various aspects to the mammalian site present on the $GABA_A$ receptor/channel complex, as discussed below. (1) Preferential localization in the mitochondrial fraction. (2) Lack of effect of GABA and other classical modulators of the central benzodiazepine receptor like pentobarbital, bicuculline and picrotoxin. (3) Singular pharmacological modulation, that does not fit well with a mammalian central (nor a peripheral) benzodiazepine receptor, due to a very high affinity for Ro5-4864 and a very low affinity for clonazepam. These differences of affinities could be explained by putative structural peculiarities of the worm receptors as a function of the large phylogenetic distance between mammals and platyhelminths. Note that platyhelminths are the first metazoan group to possess a centralized nervous system, being considered as the link between lower and higher invertebrates (Day and Maule, 1999). Indeed, numerous authors reported differences between mammalian and invertebrate $GABA_A$

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receptors (Walker *et al*. 1996). As an example, the pharmacological profile of the benzodiazepine sites present in GABA-activated chloride channels in insects is more similar to mammalian peripheral than central benzodiazepine receptors (Sattelle *et al*. 1991). In the somatic musculature of the nematode *Ascaris suum* the GABA receptors are insensitive to the antagonists bicuculline and picrotoxin and to allosteric modulators including the benzodiazepines and barbiturates (Holden-Dye *et al*. 1989). At the molecular level, such differences could be due to a different arrangement of the subunits constitutive of the pentameric receptor complex, since the benzodiazepine binding site is localized at the interface of the α and γ subunits and their affinities depend on the composition of these 2 subunits (Smith and Olsen, 1995). The combination of a α_1, α_2, α_3, or α_5 subunit with a γ_2 or γ_3 subunit, for example, is necessary for a high affinity (McKernan *et al*. 1995; Barnard *et al*. 1998) whereas receptors formed with α_4 or α_6 are virtually insensitive to most benzodiazepines. The peculiar pharmacology of the central-like benzodiazepine receptor of *S. mansoni* could also be partially explained by the presence of an ε subunit as suggested by analysis of the *S. mansoni* transcriptome (Verjovski-Almeida *et al*. 2003), since this subunit can substitute the γ_2 in the pentameric complex being responsible for an atypical binding of the benzodiazepines, as reported in the mammalian locus coeruleus (Moragues *et al*. 2002) for example. Note that we looked in the genomic (http://www.genedb.org/genedb/smansoni/) and EST (Schistosoma mansoni EST Genome Project website at: http://cancer.lbi.ic.unicamp.br/schisto6/) data sets and found 2 pieces of indirect evidence for a central-type benzodiazepine receptor: an EST compatible with the above-cited ε subunit of the pentameric $GABA_A$ receptor complex (SmAE 709016.1) as well as an EST (SmAE 604360.1) and a coding sequence (Smp_073790) compatible with a $GABA_A$ receptor associated protein.

The sites labelled with [^{3}H]flunitrazepam in our P_3 fraction of *S. mansoni* are probably similar to those detected by Bennett in his pioneering, and unique, work on benzodiazepine binding sites in schistosomes, using [^{3}H]clonazepam and [^{14}C]3-methylclonazepam as probes (Bennett, 1980). Although this author worked in a non-equilibrium condition (3 min incubation) and at a higher temperature (37 °C), that decreases the affinity of flunitrazepam to its binding sites, the IC_{50} reported for clonazepam and diazepam (4 μM and 6 μM, respectively) are roughly similar to our values.

The second population of schistosomal benzodiazepine binding sites should correspond to a peripheral-like benzodiazepine receptor, with pharmacology very similar to the mammalian correspondent. (1) The IC_{50} values for [^{3}H]PK11195 binding in our schistosomal fraction are very similar to the values for the mammalian peripheral benzodiazepine receptor, as measured in the rat kidney preparation. (2) The sequence of potency, a classical pharmacological tool for receptor classification, is nearly identical to that characterizing the mammalian peripheral benzodiazepine receptor (PK11195 = Ro5-4864 = Flunitrazepam > Diazepam $\approx$ Zolpidem $\gg$ Clonazepam > Flumazenil) but not the mammalian central benzodiazepine receptor (Clonazepam = Flumazenil = Flunitrazepam > Diazepam > Zolpidem $\gg$ PK11195 = R05-4864). Note that a gene encoding a protein with homology to the human peripheral benzodiazepine receptor has been cloned supporting our hypothesis (Santos *et al*. 1999). With respect to the localization and functional role of such receptors, we can just speculate that they could be localized in the mitochondria or even in the tegument, based on their main recovery (55%) in the P_1 fraction, a heterogeneous fraction containing pieces of tegument. Note that there is evidence for the presence of mammalian peripheral benzodiazepine receptors in the plasma membrane of different cells (Olson *et al*. 1988; Woods and Williams, 1996).

As a conclusion, this work not only contributes to the basic knowledge of *S. mansoni* biology but also should enable investigations on the likelihood that these worm benzodiazepine receptors are involved in the proposed GABA neurotransmission and are the molecular targets for the schistosomicidal effect of clonazepam and 3-methylclonazepam (Stohler, 1978). We are now investigating this relevant question for the putative development of new antiparasitic agents related to the neuromuscular system of this flatworm (Geary *et al*. 1992).

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