Proc. Estonian Acad. Sci. Chem., 2004, **53**, 3, 116–124

Kinetics of [^{3}H]WAY100635 binding to 5-HT$_{1A}$ receptors in rat hippocampal membranes

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Received 4 May 2004, in revised form 12 May 2004

Abstract. The binding of [^{3}H]-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)-cyclohexanecarboxamide ([^{3}H]WAY100635) to 5-HT$_{1A}$ receptors in rat hippocampal membranes was studied. Saturation experiments showed that [^{3}H]WAY100635 binds to a single class of binding sites with very high affinity ($K_D = 87 \pm 4$ pM, $B_{max} = 15.1 \pm 0.2$ fmol/mg protein). The binding of [^{3}H]WAY100635 was reversible, but slow. The dissociation of [^{3}H]WAY100635 from its complex with 5-HT$_{1A}$ receptors was characterized with the $k_{off} = (7.8 \pm 1.1) \times 10^{-3}$ min^{-1}, which means that at low concentrations of the radioligand equilibrium cannot be achieved before 7 h incubation at 25 °C. The obtained data indicate that [^{3}H]WAY100635 is a valuable tool for the determination of the number of 5-HT$_{1A}$ receptor binding sites, but the determination of its affinity is complicated as it hardly reaches equilibrium at concentrations close to its K_D.

Key words: [^{3}H]WAY100635, 5-HT$_{1A}$ receptor, equilibrium, kinetics, affinity, rat hippocampus.

INTRODUCTION

The 5-HT$_{1A}$ (serotonin$_{1A}$) receptor is a member of the family of serotonin (5-HT) receptors, which belong to the superfamily of seven-transmembrane spanning G protein-coupled receptors [1]. The 5-HT$_{1A}$ receptor system has been implicated in a variety of physiological functions and behaviours, including the mood (anxiety and depression) [2], learning, memory, sexual behaviour, and feeding [3]. Therefore the 5-HT$_{1A}$ receptors have become important targets for various psychotherapeutic drugs and an attractive object in screenings for highly selective ligands. For a long time the tritiated derivative of 8-hydroxy-2-(*n*-dipropylamino)tetraline ([^{3}H]-8-OH-DPAT) [4] was the only commercially available specific radioligand for the characterization of 5-HT$_{1A}$ receptors. Over

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the last years some new radioactive antagonists have been proposed: [^{3}H]-(*R*)-3-*N*, *N*-dicyclobutylamino-8-fluoro-3, 4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (2*R*, 3*R*) tartrate monohydrate ([^{3}H]NAD-299 [5]), and [^{3}H]-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)-cyclo-hexane-carboxamide ([^{3}H]WAY100635 [6]). By now, [^{3}H]WAY100635 has become commercially available and has been found to be a useful tool for pharmacological experiments in membrane preparations [7] as well as in brain sections [8]. However, only limited information is available about the kinetic aspects of the binding of [^{3}H]WAY100635 to 5-HT$_{1A}$ receptors [6, 9]. In the present study we used a kinetic approach for characterizing the interactions of [^{3}H]WAY100635 with the 5-HT$_{1A}$ receptors in rat hippocampal membranes. The results indicate that [^{3}H]WAY100635 is a valuable tool for the determination of the number of 5-HT$_{1A}$ receptor binding sites, but the very slow binding seriously complicates the correct determination of the binding affinity of the radioligand.

METHODS

Hippocampi from Wistar rats were homogenized in 30 vol (ww/v) of 50 mM Tris-HCl buffer (pH 7.4) by Bandelin Sonoplus sonificator (2 passes, both 10 s), incubated for 30 min at room temperature and centrifuged at 43 000 *g* for 20 min at 4 °C. The membrane pellet was washed by re-suspending in 50 mM Tris-HCl buffer and centrifugation was repeated twice. The final pellet was re-suspended in 30 vol (ww/v) of the buffer and stored at –80 °C until use.

In saturation binding experiments the suspension of hippocampal membranes (100 μg/mL) in the incubation buffer containing 50 mM Tris-HCl (pH 7.4) and 0.1 mg/mL bovine serum albumine (BSA) was incubated with different concentrations (0.009–0.75 nM) of [^{3}H]WAY100635 (81 Ci/mmol; Amersham Pharmacia Biotech UK Limited) at 25 °C for an appropriate time. The reaction was terminated and bound radioactivity was separated from free by rapid filtration through a Whatman GF/B glass microfibre filter by washing 3 times with 3 mL of 20 mM K-phosphate-buffer (pH 7.4) containing 100 mM NaCl. The radioactivity content of the filters was counted in 5 mL of scintillation cocktail OptiPhase HiSafe®3 (Wallac Perkin Elmer Life Sciences) by Beckman LS 1800 scintillation counter [10]. The specific binding was determined as the difference between total and non-specific binding, measured in the absence and presence of 10 μM (S)-N-tert-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride (WAY100135) or 8-OH-DPAT, respectively. Displacement studies were performed the same way, including 0.5–0.9 nM [^{3}H]WAY100635 and appropriate concentrations of non-labelled ligands.

Association kinetic experiments were started with the addition of [^{3}H]WAY100635 (final concentration 0.08–4.5 nM) to a membrane suspension

in the incubation buffer. At timed intervals aliquots (200–1000 μl depending on the radioligand concentration) were taken and filtered on GF/B as described above. Parallel incubations with the same concentration of [^{3}H]WAY100635 and 10 μM 5-HT or WAY100135 were used to estimate non-specific binding.

Dissociation kinetics was measured after preincubation of membranes with 0.5 nM [^{3}H]WAY100635 for 60 min at 25 °C. Dissociation was then initiated by addition of non-labelled 5-HT (28 μM final concentration) and at timed intervals aliquots (200 μL) were filtered on GF/B and the bound radioactivity was determined as described above.

All data were analysed by means of the non-linear least squares regression method as described in [11] using a commercial program GraphPad PRISM™ (GraphPad, San Diego, CA, USA). Data are presented as mean ± SEM of at least two independent determinations carried out in duplicates. The statistical significance of differences was determined by the Student–Newman–Keuls test, where $P < 0.05$ was taken as the criterion of significance.

RESULTS AND DISCUSSION

The kinetic curves of [^{3}H]WAY100635 binding to rat hippocampal membranes were measured under the excess of radioligand over the receptor concentration. The association of [^{3}H]WAY100635 was well described by the exponential function for the first-order reactions and the observed rate constants (k_{obs}) were obtained at different radioligand concentrations as described earlier [11]. At 0.48 nM of the radioligand the steady-state was achieved within 40 min ($\tau_{1/2} = 7.1 \pm 0.3$ min, Fig. 1), and no additional binding was found during the prolonged incubation time of up to 120 min. Increasing the concentration of the radioligand caused a proportional increase in the association rate, which reached the half life of $\tau_{1/2} = 2.2 \pm 0.2$ min at 3.0 nM of [^{3}H]WAY100635 (Fig. 1, open circles). The analysis of the dependence of the observed association rate constants on the radioligand concentration according to a simple bimolecular reversible ligand binding model revealed the second order rate constant $k_1 = 0.16 \pm 0.02$ nM^{-1} min^{-1}.

The dissociation of [^{3}H]WAY100635 from its complex with 5-HT$_{1A}$ receptors in hippocampal membranes was complete and the equation of the one phase exponential decay revealed a rate constant $k_{off} = (7.8 \pm 1.1) \times 10^{-3}$ min^{-1}, which corresponds to the half time of the dissociation $\tau_{1/2} = 89 \pm 11$ min (Fig. 2). The monophasic exponential decay model was preferred by the F-test in comparison with the two phase exponential decay ($P > 0.05$) in analysis of the dissociation data. The obtained k_{off} value in these experiments agrees with the $k_{-1} = (11 \pm 6) \times 10^{-3}$ min^{-1} calculated from the intercept of the k_{obs} versus [A] plot. The obtained data indicate that the complete dissociation of the radioligand was achieved after at least 7.5 h incubation.

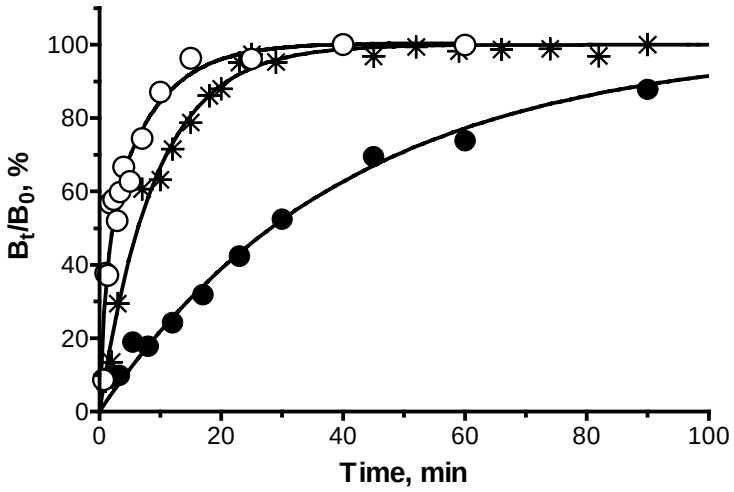

Fig. 1. Time course of specific binding of [^{3}H]WAY100635 to rat hippocampal membranes. The receptor preparations were incubated with 0.07 nM (●), 0.48 nM (∗), or 3.0 nM (○) [^{3}H]WAY100635, and at timed intervals the specifically bound radioactivity in aliquots was determined as described in Methods. The data are presented as the percentage of maximal specific binding at the respective radioligand concentration.

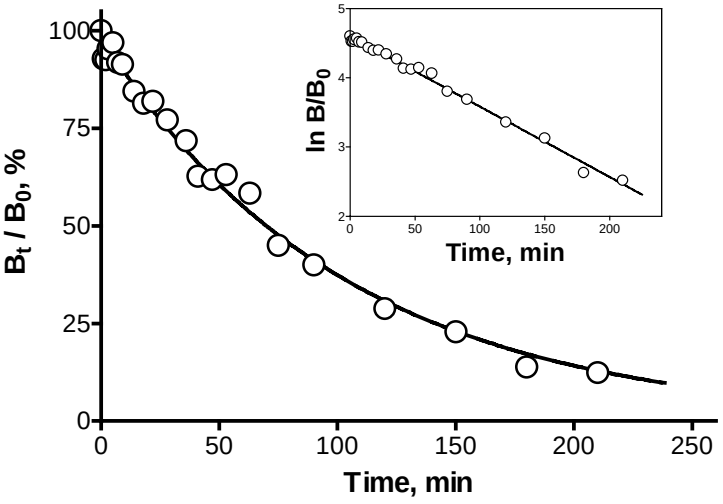

Fig. 2. Dissociation of [^{3}H]WAY100635 from the complex with 5-HT_{1A} receptors in rat hippocampal membranes. The complex was formed by incubation of membranes with 0.5 nM [^{3}H]WAY100635 for 60 min at 25 °C. Dissociation was initiated by addition of large excess (28 μM) of 5-HT. Data are presented as the percentage of the maximal specific binding. **Inset:** Data in semilogarithmic coordinates.

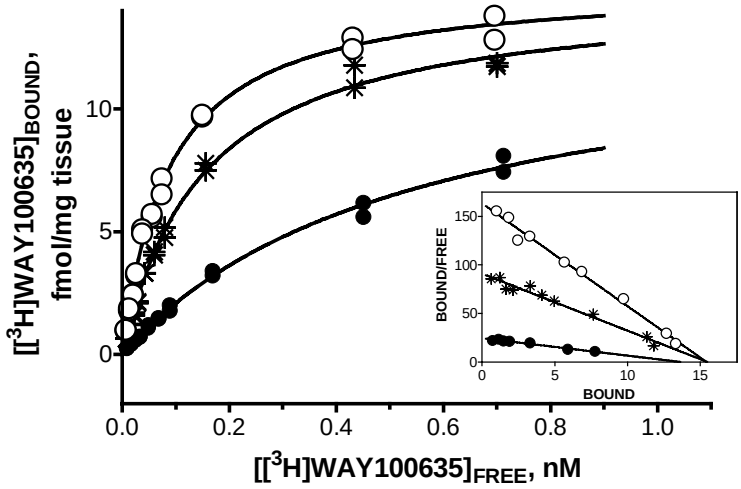

Fig. 3. Specific binding of [^{3}H]WAY100635 to rat hippocampal membranes. The membranes in the incubation buffer (50 mM Tris-HCl (pH 7.4) and 0.1 mg/mL BSA) were incubated with different concentrations of [^{3}H]WAY100635 in the absence (total binding) and presence (nonspecific binding) of 10 μM WAY100135 for 15 min (●), 60 min (∗), or 240 min (○) at 25 °C. The specific binding was defined as the difference between the total and the nonspecific binding. **Inset:** Scatchard plot of the corresponding data.

In the saturation binding experiments the binding of [^{3}H]WAY100635 to rat hippocampal membranes was specific and saturable, and it was characterized with the affinity $K_D = 87 \pm 4$ pM and with the number of the specific binding sites $B_{max} = 15.1 \pm 0.2$ fmol/mg protein (Fig. 3, open circles). The Scatchard plot of these data revealed a straight line and the single-site binding model was preferred also by the F-test in comparison with the two binding site receptor model ($P < 0.05$; Fig. 3, inset). The obtained affinity constant was in general agreement with the constant $K_D = k_{-1}/k_1 = 69$ pM, calculated from the rate constants of the simple reversible reaction. The binding studies with [^{3}H]WAY100635 were carried out in the incubation buffer containing 50 mM Tris-HCl (pH 7.4) and 0.1 mg/mL BSA, which was found to be optimal for the high-affinity binding of the radioligand. The presence of at least 3 mM of Mg^{2+} is usually required for effective coupling of heptahelix receptors with G proteins [12, 13], but for the [^{3}H]WAY100635 binding $MgCl_2$ had a concentration dependent inhibitory effect. Thus, in the presence of 5 mM Mg^{2+} the binding was characterized with the affinity $K_D = 85 \pm 8$ pM and the number of the specific binding sites $B_{max} = 11.1 \pm 0.3$ fmol/mg protein (74% of the control).

Kinetic data indicate that at least 7 h incubation is required to achieve equilibrium at all [^{3}H]WAY100635 concentrations. Shorter incubation times reveal steady-state binding only at higher radioligand concentrations and allowed correct estimation of the number of specific binding sites, but led to under-estimation of the binding affinity. Thus, incubation of the reaction medium for

15 min gave K_D = 540 ± 50 pM, while after 60 min incubation the affinity K_D = 150 ± 10 pM and after 240 min, K_D = 87 ± 4 pM (Fig. 3). Slow kinetics of the [^{3}H]WAY100635 binding might explain the large discrepancy between the previously determined dissociation constants (56 pM [9] and 400 pM [14]). However, it is quite complicated to overcome the problem of slow kinetics in experiments with [^{3}H]WAY100635, as we here meet the limits of the method of radioligand binding. First, very high affinity of that radioligand complicates maintenance of the pseudo-first-order reaction conditions ([R]<<[L]) at low concentrations. The reaction will start following the regularities of the second order reaction kinetics, where in addition to the decrease of the receptor concentration also the decrease of the free radioligand concentration during the reaction must be taken into account. Secondly, the receptors have to remain intact during the incubation time. The hippocampal membranes used in our experiments were quite stable loosing the [^{3}H]WAY100635 binding ability with a half life of 27 h (data not shown), which means that within 7.5 h incubation no more than 14% of the binding sites were lost. Such high stability of antagonist binding properties has been also found for other heptahelix G-protein-coupled receptors [11, 15]. In contrast, the high-affinity agonist binding is much more labile [16, 17] and it is shown that in rat hippocampal membranes the half-life of disappearance of binding sites of [^{3}H]-8-OH-DPAT at 25 °C is 29 min [16], indicating that an essential part of the high-affinity agonist binding sites may be lost in our experiments as the displacement experiments were carried out with the incubation period of 120 min.

We found that all studied serotonergic ligands were able to displace [^{3}H]WAY100635 binding in concentration-dependent manner and with affinities that were generally in agreement with data reported earlier for 5-HT_{1A} receptors [14, 18–20] (Table 1). Here we saw that Mg^{2+} is required for high-affinity agonist binding and activation of G proteins since 30 μM guanosine-5'–O–(γ-thio) triphosphate (GTPγS) turned the receptors into low-affinity state (Table 1). A slight influence of GTPγS in the presence of Mg^{2+} was also found in the displacement curves of antagonists WAY100135 and 1-(2-methoxyphenyl)-4-(4-[2-phtalimido]butyl)-piperazine (NAN-190), which indicates that binding sites of these ligands are affected by the coupling of G protein and they possess some partial agonist properties as it was proposed also earlier [21, 22]. The good agreement of the obtained data with previously reported data indicates that the use of [^{3}H]WAY100635 seems to be very reasonable for the estimation of affinities of various compounds in the displacement experiments. However, it must be kept in mind that if the binding rate of the competitive ligand is comparable with the [^{3}H]WAY100635 binding, overshot of the radioligand will take place, which may lead to an underestimation of the competitor's affinity [23].

Table 1. The influence of Mg^{2+} and GTPγS on the affinities of serotonergic ligands in the displacement of [^{3}H]WAY100635 in rat hippocampal membranes

Ligand	Control		+30 μM GTPγS	
	K_i^{HIGH}, K_i^{LOW}, nM	α_{HIGH}	K_i^{HIGH}, K_i^{LOW}, nM	α_{HIGH}
5-HT				
EDTA	10 ± 2 780 ± 180	0.40 ± 0.03	758 ± 219	–
5 mM Mg^{2+}	2.1 ± 0.4 110 ± 42	0.63 ± 0.04	525 ± 136	–
8-OH-DPAT				
EDTA	2.1 ± 0.7 35 ± 23	0.67 ± 0.11	15 ± 1	–
5 mM Mg^{2+}	0.21 ± 0.03 22 ± 12	0.78 ± 0.03	0.2 ± 0.1 22 ± 3	0.22 ± 0.03
WAY100135				
EDTA	3.9 ± 0.4	–	4.3 ± 0.3	–
5 mM Mg^{2+}	2.5 ± 0.2	–	4.6 ± 0.3	–
NAN-190				
EDTA	6.8 ± 1.0	–	7.9 ± 1.4	–
5 mM Mg^{2+}	2.5 ± 0.2	–	6.7 ± 1.0	–

High- (K_i^{HIGH}) and low- (K_i^{LOW}) affinity binding constants were calculated from displacement curves against 0.8 nM [^{3}H]WAY100635 using the equations of Cheng–Prusoff [24]. α_{HIGH} indicates the proportion of high affinity binding sites.
– No heterogeneity was detected. The single-site binding model was preferred ($P < 0.05$).

In summary, kinetic analysis gave evidence that the [^{3}H]WAY100635 interaction with 5-HT_{1A} receptors is a specific, slow, and reversible process. Its very high affinity and slow dissociation rate make it a very valuable tool for the determination of the number of 5-HT_{1A} receptor binding sites, and allows its application as a reporter ligand in competition experiments. However, correct determination of the affinity of [^{3}H]WAY100635 is complicated, as due to its slow binding rate and high affinity it hardly reaches equilibrium at concentrations close to its K_D. Therefore, the application of [^{3}H]WAY100635 for assays of 5-HT_{1A} receptor binding properties has several limitations, which should be taken into consideration when planning experiments with this radioligand and when interpreting the obtained data.

ACKNOWLEDGEMENT

This work was supported by the Estonian Science Foundation (grant No. 4603).

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[^{3}H]WAY100635 sidumise kineetika serotoniin 1A retseptoritele roti hipokampuse membraanidel

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Serotoniini 1A retseptorite spetsiifilise radioligandi [^{3}H]WAY100635 kineetika uurimine roti aju hipokampuse membraanidel näitas, et selle ligandi seostumine 5-HT_{1A} retseptoriga on spetsiifiline, aeglane ja pöörduv protsess. Väga kõrge afiinsuse ja aeglase dissotsiatsiooni tõttu on [^{3}H]WAY100635 suurepärane vahend 5-HT_{1A} retseptorite sidumiskohtade kontsentratsiooni määramiseks ning kasutatav konkureeriva sidumise katsetes teiste ligandide afiinsuste uurimisel. Samas on [^{3}H]WAY100635 afiinsuse määramine problemaatiline, sest aeglase sidumiskiiruse tõttu pole tõeline tasakaal radioligandi K_D-lähedastel kontsentratsioonidel praktiliselt saavutatav.