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Biochimica et Biophysica Acta 1190 (1994) 185–187

BBA Biochimica et Biophysica Acta

Rapid Report

The in situ size of the dopamine transporter is a tetramer as estimated by radiation inactivation

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(Received 16 November 1993)

Abstract

Radiation inactivation analysis of the mazindol-sensitive binding of the dopamine transporter inhibitor, [^{3}H]GBR-12935 to canine striatal membranes yielded a radiation inactivation target size of 278 ± 16 kDa. This result, in conjunction with other findings in the literature demonstrating that the molecular mass of the dopamine transporter is ~ 70 kDa, suggests that the native form of the dopamine carrier in the neuronal membranes is a tetrameric assembly of identical 70 kDa subunits.

Key words: Dopamine transporter; Radiation inactivation; GBR-12935; (Canine brain membrane)

Transport of dopamine that has been released into the synaptic cleft back into the nerve terminals occurs via a Na^+/Cl^--dependent transporter on the neuronal membrane and is the predominant means of inactivating the actions of this neurotransmitter [1]. Potent specific inhibitors of this transporter have been described including the aryl 1,4-dialk(en)ylpiperazines such as GBR-12935 [2,3]. GBR-12935 has been used as a selective marker of dopamine transporters and binds with high affinity (apparent K_d 1.8–4.9 nM) to striatal membranes from a number of different species [3,4]. Photoaffinity ligands for the transporter have also been developed and covalently bound radioactivity has been reported to be specifically incorporated into a glycoprotein of M_r 58000–80000 depending on the type of molecular weight markers used and the nature of the probe [5–7]. In addition, the cloned CNS dopamine transporter has a predicted molecular mass of ~ 69 kDa [8–10] and is a member of an emerging family of Na^+/Cl^--dependent neurotransporters [11,12]. Examination of the sequences of the cloned transporters reveals the possibility of a degenerate 'leucine zipper' motif that has been proposed as a possible oligomerization signal [12]. Despite these significant advances in identifying and cloning the biogenic amine transporters, little is known regarding the structural organisation of these proteins in the membrane.

Radiation inactivation is a noninvasive technique that has been used to determine the in situ size of membrane-associated proteins without the need for isolation and purification procedures [13]. In the present study we have used the technique of radiation inactivation to determine the apparent target size of the dopamine transporter as detected by the specific binding of [^{3}H]GBR-12935.

Fig. 1 shows the results of a typical binding experiment in which the amount of [^{3}H]GBR-12935 bound to canine striatal membranes is plotted against the steady state concentration of free ligand. Binding was resolved into two components: (a) a saturable association with binding constants of 5.4 ± 0.80 pmol/mg protein for the B_{max} with an apparent K_d of 2.7 ± 0.50 nM and (b) a nonsaturable component responsible for the binding of 0.86 pmol/mg protein at 1.0 nM. The saturable component of binding was abolished in the presence of 3 μM mazindol. Other studies demonstrated that the dopamine-transport inhibitors, GBR-12909, mazindol, cocaine, (+)-diclofensine, (−)-diclofensine and dopamine, blocked the mazindol-sensitive [^{3}H]GBR-12935 binding with K_i values of 11, 35,

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SSDI 0005-2736(93)E0367-J

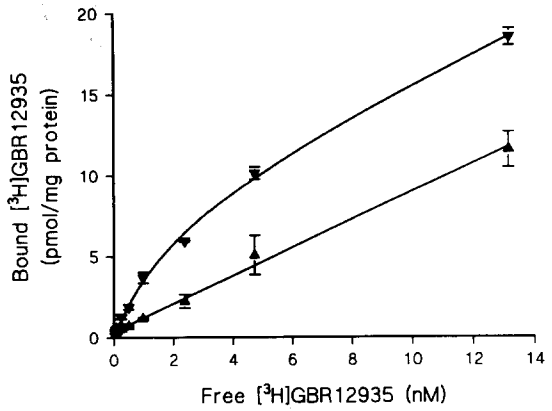

Fig. 1. Concentration dependence of [^{3}H]GBR-12935 binding to canine striatal membranes. The striatal region (caudate nucleus and putamen) of freshly thawed frozen canine brains were dissected and homogenised in 20 volumes (wt/vol) of ice-cold sucrose (0.32 M), Tris (5 mM), Hepes (5 mM) (pH 7.4) with a glass-teflon homogeniser. After centrifugation for 10 min at $1700\times g$, the suspension was retained and the pellet rehomogenised and centrifuged as before. The supernatants were pooled and recentrifuged at $48000\times g$ for 1 h. The final pellet was resuspended in 2 ml buffer/g original wet weight of tissue. Membranes (0.05 mg protein) were incubated with graded concentrations of [^{3}H]GBR-12935 (final concentration 0.2–10 nM; 44.2 Ci/mmol) (NEN, Stevenage, Herts, UK) in 1 ml of buffer (9.6 mM $NaHCO_3$/0.4 mM NaH_2PO_4/0.1 mM EDTA (pH 7.4) containing 60 mM NaCl and 0.01% (w/v) bovine serum albumin). Incubations were performed at 25°C for 1 h in the presence (▲) and absence (▼) of 3 μM mazindol and the reaction terminated by vacuum filtration over Whatman GF/B filters (prewetted with 0.1% BSA). The filters were rinsed twice with 5 ml of ice-cold buffer and the radioactivity remaining on the filters measured by liquid scintillation spectrometry. Specific binding constants for the data shown are K_d 2.7 ± 0.50 nM and B_{max} 5.4 ± 0.80 pmol/mg protein.

120, 100, 2800 and 12000 nM, respectively. This pharmacological profile is similar to that obtained previously with rat brain membranes [3] and demonstrates that the binding of [^{3}H]GBR-12935 in canine striatum is to the dopamine transporter.

In subsequent binding assays, differences between the membrane content of [^{3}H]GBR-12935 in the presence and absence of 3 μM mazindol were used to quantify the specific binding of the inhibitor. Initial control studies demonstrated that freeze-drying the canine membranes and reconstitution did not result in significant loss of [^{3}H]GBR-12935 binding activity ([^{3}H]GBR-12935 at 1.0 nM bound to lyophilised samples was 96% of the value for control membranes).

Irradiation of canine striatal membranes resulted in a decrease in [^{3}H]GBR-12935 binding activity that declined as a simple exponential function of radiation dosage and, thus, a single target size (Fig. 2). Estimates of the molecular weight where obtained by substituting D_{37t}, the radiation dose (in Mrad) required to inactivate the original activity to 37% of its initial value, at an irradiation temperature t (in °C) into the empirical equation [14]:

$$\log M_r = 5.89 - \log D_{37t} - 0.0028t$$

D_{37t} values were obtained from linear regression analysis of log[activity (%)] versus radiation dose (Mrad) using least-squares fit. Based on four independent experiments the D_{37} value was estimated to be 2.3 ± 0.13 Mrad resulting in a molecular size of 278 ± 16 kDa for the [^{3}H]GBR-12935 binding component. Inclusion of the free-radical scavenger, dithiothreitol (5 mM), to eliminate the possibility of inactivation of binding activity due to active radiolytic and free radical products formed from residual molecular oxygen during irradiation, had no effect on the irradiation profile (data not shown).

The loss of [^{3}H]GBR-12935 binding activity with irradiation could have resulted from a radiation-induced decrease in the affinity of [^{3}H]GBR-12935 for the dopamine transporter. Samples were thus irradiated at 2 Mrad, a radiation dose that decreases the binding of [^{3}H]GBR-12935 by at least 50%, and the ability of unlabelled GBR-12935 to compete with [^{3}H]GBR-12935 binding to control and irradiated canine striatal membranes compared. Inhibition constants (K_i) of 5.9 ± 0.65 nM and 5.1 ± 0.9 nM were found for untreated and irradiated samples, respectively, indicating that the affinity of [^{3}H]GBR-12935 for the dopamine transporter was not affected by the radiation treatment.

As a further control, acetylcholinesterase was used as an endogenous internal standard (Fig. 2, triangles). A D_{37} of 8.2 ± 0.31 ($n=4$) Mrad was determined yielding a functional mass of 78 ± 3 kDa, in good

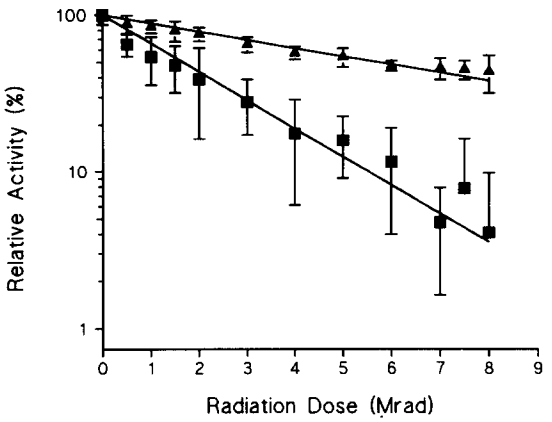

Fig. 2. Effect of increasing radiation dose on specific GBR-12935 binding activity and acetylcholinesterase activity in canine striatal membranes. Samples of lyophilised membranes were irradiated at 30°C in a Gammacell model 220 at a dose rate of about 1 Mrad/h. After irradiation the samples were kept at −20°C until assayed for mazindol-sensitive [^{3}H]GBR-12935 binding at 0.5 nM (■) as described in the legend to Fig. 1 and acetylcholinesterase activity (▲) by the method of Chow and Islam (1970) [17]. The data were expressed relative to the unirradiated samples and plotted as log relative activity versus radiation dose. Based on four independent experiments, the average target size of [^{3}H]GBR-12935 binding and acetylcholinesterase activity was 278 ± 16 and 78 ± 3 kDa, respectively.

agreement with previously reported values of approx. 78 kDa for acetylcholinesterase in human brain membranes [15]. By comparing the slopes of the inactivation curves for the [^{3}H]GBR-12935 binding component and acetylcholinesterase and applying the known molecular weight of acetylcholinesterase, the molecular size of the [^{3}H]GBR-12935 binding component was calculated to be 285 ± 27 kDa, a value similar to that determined by the D_{37} method.

In conclusion, the present results demonstrate that the dopamine transporter in canine striatal membranes has an apparent molecular weight in situ of $\sim 280\,000$, as estimated by the binding of the transport inhibitor [^{3}H]GBR-12935. Radiation techniques on a variety of glycoproteins have shown that only the protein portion of the molecule is measured [13]. Recent cDNA cloning studies of the rat, bovine and human CNS dopamine transporters have revealed an unmodified protein molecular mass of ~ 69 kDa [8–10]. Thus, the target size of 278 ± 16 kDa could be due to the cloned 69-kDa peptide plus other, as yet unidentified, proteins. However, expression of the cloned protein results in both dopamine transport activity and the binding of specific inhibitors [8–10]. Thus, this data and the present results suggest that the Na^+-dependent dopamine transporter exists in the membrane as an oligomer composed of four identical subunits of approximate M_r 69 000. The presence of 'leucine zipper' motifs within the amino acid sequence of the transporter further supports the possibility of polypeptide oligomerization. It is interesting to note that the functional size of another Na^+ cotransporter, the Na^+-dependent renal glucose transporter, has also been proposed to be a tetramer [16]. This raises the possibility that other members of these families of cotransporters may also be present in the membrane as oligomers.

This work was supported in part by grants from SmithKline (1982) Foundation, the Medical Research Council (UK) and NSERC (Canada). H.E.M. is in receipt of an SERC postgraduate studentship.

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