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Nitrobenzylthioinosine Binding Cooperativity in Chromaffin Tissue Membranes*

T. Casillas, R. P. Sen, E. G. Delicado and M. T. Miras-Portugal*

Departamento de Bioquímica
Facultad de Veterinaria
Universidad Complutense de Madrid
28040 Madrid (Spain)

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T. CASILLAS, R. P. SEN, E. G. DELICADO and M. T. MIRAS-PORTUGAL. *Nitrobenzylthioinosine Binding Cooperativity in Chromaffin Tissue Membranes.* Rev. esp. Fisiol., 48 (1), 1-6, 1992.

The nucleoside transporter present in chromaffin tissue membranes has been studied by [^{3}H]nitrobenzylthioinosine (NBTI) binding. This ligand presents a high affinity, with a K_d value of 2.1 ± 0.2 nM and a B_{max} of 1.7 ± 0.2 pmol/mg protein. From the Scatchard and the semilogarithmic graphical representations a positive cooperativity was deduced, with a Hill coefficient of 1.7 ± 0.4. In displacement studies of NBTI by the non labelled compound, the Hill coefficient was also higher than 1 (1.44 ± 0.11) in the presence of ATP. This nucleotide seems necessary to maintain the number of high affinity binding sites.

Key words: Nucleoside transporter, Nitrobenzylthioinosine binding, Positive cooperativity, Chromaffin tissue.

Nitrobenzylthioinosine (NBTI) is a specific potent inhibitor for the facilitated diffusion nucleoside transport in a wide variety of cells (15, 16). This compound, radioactively labelled, can be used as a ligand test for the transporter, and the presence of high-affinity binding sites has been demonstrated (4, 18). Not all the nucleoside transporters are inhibited by NBTI to the same extent, and at least two types, with high and low sensitivity, have been described (14, 19). NBTI is also a photoactivable ligand that can covalently bind the nucleoside transporters after UV-light stimulation. Photoaffinity labelling of the transport protein and subsequent sodium dodecylsulfate-polyacrylamide gel electroforesis reveals the presence of two labelled proteins of approximately 50 and 100 KDa, which may correspond to the monomeric and the dimeric form (10, 18). Studies of radiation-inactivation have enabled the identification of a protein with

* To whom all correspondence should be addressed (Telef: 34 - 1 - 394 38 94 / 92; FAX: 34 - 1 - 394 38 83).

molecular weight close to the dimeric form (11). Furthermore, a new series of compounds related to lidoflazine and mioflazine have been found to inhibit the NBTI binding to nucleoside transporters with pseudo-Hill coefficients greater than one (9).

In the present study we report the positive cooperativity of [^{3}H]NBTI binding to membrane preparations from neurochromaffin tissue. These results are in agreement with the dimeric nature of the transporter.

Materials and Methods

Materials. — Nitrobenzylthioinosine and adenosine 5'triphosphate were supplied by Sigma, adenosine deaminase (E.C. 3.5.4.4) and phosphatase alkaline (E.C. 3.1.3.1) by Boehringer, [^{3}H]nitrobenzylthioinosine (25 Ci/mmol) by New England Nuclear and [^{3}H]nitrobenzylthioinosine (30 Ci/mmol) by Moravek Biochemicals.

The scintillation liquid, Ready Safe, for aqueous and non-aqueous samples were purchased from Beckman. All other reagents were supplied by Merck.

Preparations of subcellular membrane fractions. — Subcellular membrane preparations were obtained as described before (18).

Adrenal medulla was homogenized in a Teflonpestle homogenizer with 0.32 M sucrose containing 5 μM phenylmethanesulfonyl fluoride. The original homogenate was centrifuged at 800 × *g* for 10 min. The pellet was rejected and the supernatant was centrifuged at 10.000 × *g* for 30 min. This pellet was resuspended in 0.32 M sucrose and layered over a discontinuous sucrose gradient (0.95-1.34-1.6). Centrifugation was carried out for 60 min at 100.000 × *g*. The plasma membrane fraction was then collected at the 0.32-0.95 sucrose interface. To eliminate sucrose and other metabolites such as catecholamines and ATP, the membrane fractions were washed twice with Hepes 10 mM pH = 7.0. Controls for the presence of catecholamines and ATP were carried out.

[^{3}H]NBTI binding experiments. — The binding experiments were accomplished as described by TORRES *et al.* (18).

The [^{3}H]NBTI binding was carried out by incubating 0.1-0.25 mg protein in 0.5 ml 10 mM Hepes (pH = 7.0) with graded concentrations of the labelled compound (0.05-10 nM). Routinely 0.5 UI adenosine deaminase was included to eliminate the possible adenosine present in biological samples. Unless otherwise stated the incubations were made at 37 °C during 15 min. After the incubation period membranes were collected onto Whatman GF/C glass fiber filters washed twice with 5 ml icecold buffer containing 10 μM non-labelled NBTI, dried and the radioactivity was counted. The filters were pretreated with the same washing buffer to decrease the non specific retention of labelled NBTI on the filters.

Non specific binding was obtained in the presence of 10 μM non-labelled NBTI and the labelled compound in the same concentration as the assays.

Displacement studies were carried out with 1 nM [^{3}H]NBTI and non-labelled product ranging from 0.25 to 20 nM. Volumes and assay conditions were as described for binding experiments.

Results

[^{3}H]Nitrobenzylthioinosine binding to chromaffin tissue membranes. — The time course of [^{3}H]NBTI association with chromaffin tissue membranes is shown in figure 1A. The equilibrium was reached after a 10 minute incubation at 37 °C and after a 30 minute incubation at 4 °C. The values obtained for the constant associa-

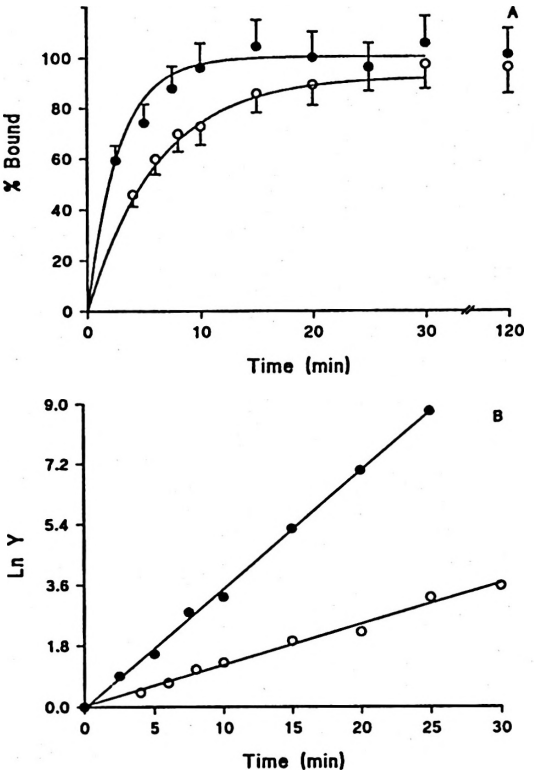

Fig. 1. *Determination of association rate for Nitrobenzylthioinosine to chromaffin tissue membranes.*

A) The association rate was measured to (•) 37 °C and (○) 0 °C, with 1 nM [^{3}H]NBTI. B) With the values obtained from A; Ln Y corresponds with $Y = [B_e] / ([B_e] - [B])$, where B_e is the bound of the equilibrium at this concentration and B is the bound at every experimental time. K_{+1} is deduced from the slope considering K_{+1} = slope / ($[L]B_{max} / [B_e]$).

tion, K_{+1} were 0.088 ± 0,001 nM^{-1} min^{-1} and 0,030 ± 0.001 nM^{-1} min^{-1} at 37 °C and 4 °C respectively, obtained from the slope of fig 1B, according to the equation: K_{+1} = slope / ([L]Bmax / [B_e]). These ligand association studies were necessary to assume that the equilibrium was reached and to discriminate between positive cooperativity or failure of the reaction to reach equilibrium, when the Scatchard representations were done.

In figure 2A the binding data have been represented as a curvilinear Scatchard plot. This shape can result from positive cooperativity between the binding sites, because the reaching of equilibrium has been assessed previously (fig. 1A). The semilogarithmic representation in figure 2B seems to confirm this positive cooperativity. Furthermore, NBTI is a ligand that is not inactivated during the incubation time as occurs with proteic ligands. The K_d value obtained for these membrane preparations was 2.1 ± 0.2 nM (n = 7) ranging from 1.4 to 2.5 nM. The

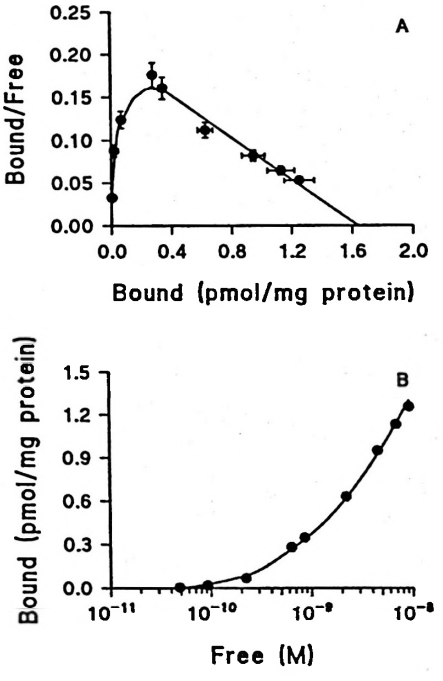

Fig. 2. *Representations of equilibrium binding data of [^{3}H]NBTI to chromaffin tissue membranes.*

A) Scatchard analysis. It represents a reproducible and typical experiment in triplicate. B) Logarithmic representation for the same experiment as in A.

maximal bound capability was 1.7 ± 0.2 pmol/mg protein (n = 7) ranging from 1.4 to 1.9. The Hill coefficient was 1.7 ± 0.4 ranging from 1.3 to 2.0.

Displacement studies with nitrobenzylthioinosine. — Indirect binding assays of NBTI were made by displacement studies of labelled [^{3}H]NBTI. This compound was used at 1 nM concentration, which is half of the K_d value (2.1 nM) in these experimental conditions. In figure 3A the displacement curve obtained for [^{3}H]NBTI is shown. The NBTI concentration necessary to displace half of the bound ligand (IC_{50}) was 5.12 ± 0.16 nM and the K_i obtained from the CHENG and PRUSOFF equation (2) was 3.47 ± 0.20 nM. These values are obtained with isolated membranes, which were not submitted to further treatment. The effect of ATP on displacement studies is represented in figure 3B. As cellular membrane preparations have tightly bound ATP to many of their proteic constituents, a control was made with membranes incubated in the presence of alkaline phosphatase to destroy the remaining ATP. The presence in the assay of 1 mM ATP significantly increased the NBTI binding to cellular membranes. The IC_{50} for membranes in presence or absence of ATP were 4.85 ± 0.12 nM and 26.6 ± 1.99 nM, respectively. The ligand bound in ATP presence was 0.284 ± 0.004 pmol/mg protein and in the presence of alkaline phosphatase was 0.149 ± 0.023 pmol/mg protein. The Hill coefficient in the presence of ATP was 1.46 ± 0.12.

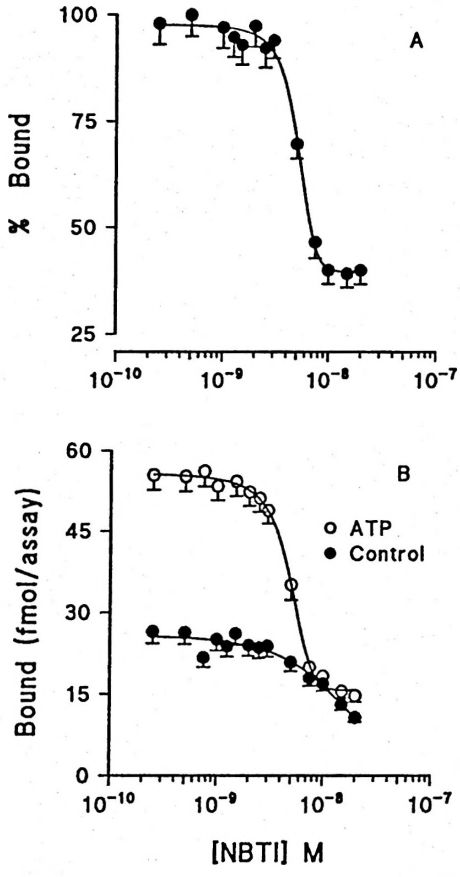

Fig. 3. *Displacement studies of [^{3}H]NBTI binding by non labelled compounds.*
A) Experiment carried out with chromaffin tissue membranes, without further treatment after isolation. B) The displacement studies were carried out after membrane treatment with preincubations for 15 min at room temperature: with Mg-ATP (o) and with alkaline phosphatase 1 UI/ml, in order to destroy the possible residual ATP (•).

Discussion

The work reported here analyzes the NBTI binding to chromaffin tissue membranes. The K_d values obtained are in good agreement with those reported by other authors in membrane preparations from a large variety of tissues and animal species (5, 16, 20). Nevertheless the affinity is significantly lower than that reported for whole cells or synaptosomal preparations, where the K_d values are under the nM range (13, 14, 18). No positive cooperativity has ever been reported for complete cells, but the possible existence of transporters in a dimeric form has been

deduced from radiation-inactivation studies in erythrocytes, and from photolabelling of pig erythrocyte membranes (11, 12, 18). The kinetic data of NBTI binding reported here indicates a positive cooperativity, both by the shape of the Scatchard plot, and by the Hill coefficient higher than one.

Positive cooperativity for nucleoside transporters has been described in displacement studies of NBTI by nucleoside transport inhibitors related with lidoflazine and mioflazine (9). In the experimental conditions reported here the [^{3}H]NBTI binding displacement by non-labelled NBTI presents a Hill coefficient higher than one, which is also in favour of positive cooperativity.

Differences between complete chromaffin cells and isolated membranes concerning the NBTI binding need to be considered. Studies with a similar protein, i.e. the glucose transporter, also indicate different properties in binding and transport, for complete cells and isolated membranes (6, 7). These effects have been interpreted from the loss of intracellular factors, or reversible molecular modifications of these proteins (1). Preliminary studies have shown that the adenosine transporter can be modified by the action of protein kinase A and protein kinase C in both, complete cells and isolated membranes (3, 17). Other intracellular soluble factors can modify the kinetic properties of transport and ligand binding. In the case of glucose transport one of these factors is ATP, which is also tightly bound to most of the membrane transporters (7, 8). Thus, it seems possible that ATP can play a conformational role for nucleoside transporters. A broad careful study of the ATP action on nucleoside transport will be necessary to understand the role of this nucleotide in the cellular control of adenosine transport, and in general, to relate the physiological cellular function of metabolite transport with the cellular energetic levels.

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Resumen

Se estudia por medio de la unión del ligando [^{3}H]nitrobenziltioinosina ([^{3}H]NBTI) el transportador de nucleósidos presente en membranas de tejido cromafín. Este ligando muestra alta afinidad por el transportador, con un valor de K_d de 2,1 ± 0,2 nM y un B_{max} de 1,7 ± 0,2 pmoles/mg de proteína. A partir de las representaciones de Scatchard y semilogarítmica se deduce una cooperatividad positiva, con un coeficiente de Hill de 1,7 ± 0,4. Mediante estudios de desplazamiento de [^{3}H]NBTI por el ligando no marcado radiactivamente, se determina que el coeficiente de Hill también es mayor que 1 (1,44 ± 0,11) en presencia de ATP. Este nucleótido parece necesario para mantener el número de sitios de alta afinidad.

Palabras clave: Transportador de nucleósidos, Tejido cromafín, Nitrobenziltioinosina, Cooperatividad positiva

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