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Solubilization and molecular characterization of the nitrobenzylthioinosine binding sites from pig kidney brush-border membranes

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Abstract

The nitrobenzylthioinosine binding sites from luminal membranes of proximal tubule of pig kidney were solubilized by treatment of the brush-border membrane vesicles with the zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) in 2% solution. The high yield solubilization of a stable form of the transporter took place in the presence of adenosine in the medium of incubation with the detergent and the additional presence of glycerol as stabilizer. The solubilization of the NBTI-sensitive nucleoside transporter from pig kidney brush-border membranes did not change the nitrobenzylthioinosine (NBTI) binding characteristics; the only major change was a 3-fold decrease in the affinity. The carrier molecule was cross-linked to [^{3}H]NBTI and by electrophoretic characterization under reducing conditions it displayed a molecular mass of 65 kDa. Treatment of the samples at low temperature prior to electrophoresis gave rise to the appearance of further bands corresponding to dimeric and tetrameric forms which interacted non-covalently. The removal of the N-linked oligosaccharides by treatment with endoglycosidase F shifted the molecular mass to 57 kDa. The chromatographic behaviour of the solubilized transporter was similar to that of human erythrocytes and differed from that found in pig erythrocytes. Since the molecular mass of the monomer before and after treatment with endoglycosidase F is the same for pig erythrocytes and pig kidney luminal membranes, the different chromatographic behaviour might result from tissue differences due to transcriptional variations or to posttranscriptional modifications of the transporter molecule.

Key words: NBTI-sensitive nucleoside transport; Nucleoside transport; Kidney; Solubilization; Adenosine; Nitrobenzylthioinosine; Brush-border membrane

1. Introduction

The action of adenosine in the kidney is as varied as the many cell types found in this organ. Its regulatory effects in the rate and distribution of renal blood flow, glomerular filtration, hormone and neurotransmitter release and tubular reabsorption have been summarized in a number of reviews [1–4], in which the effect of exogenous adenosine has been analysed and the role of endogenous adenosine as mediator of several physiological and pathophysiological phenomena has been postulated.

The interaction of adenosine with high-affinity specific receptors on the cell surface mediates many of the effects of the nucleoside in mammalian tissues. Both the presence of adenylate cyclase stimulating and inhibiting adenosine receptors and the coupling of these receptors to other effector systems have also been demonstrated in the kidney [5–7].

However, the multiple effects exerted by adenosine in the kidney cannot be explained only on the basis of its interaction with specific receptors and it is clear that other processes, such as the release and uptake of the nucleoside and its intra and extracellular metabolism, must participate in the control of the adenosine actions. Thus, for instance, recovery of the intracellular nucleotide pool from exogeneous adenosine has been demonstrated in guinea pig kidney slices [8]. It becomes clear that a number of events in kidney are

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mediated upon uptake by the cells of extracellular adenosine. It is in this context that we are interested in the study of the nucleoside transport in brush-border membrane vesicles (BBM) and in the proteins functionally involved in it. It has been reported that renal brush-border membrane adenosine transport shows two components, one sodium-independent and the other sodium-dependent [9–12]. In mammalian cell membranes the sodium-independent component is mediated by two equilibrative (facilitated diffusion) transporters with similar kinetic properties, but with differences in their sensitivity to the inhibitor nitrobenzylthioinosine (NBTI). The NBTI-sensitive transporter ('es' as proposed by Vijayalakshmi and Belt [13]) possesses a high-affinity NBTI binding site and is inhibited by nanomolar concentrations of NBTI. Conversely, the NBTI-insensitive equilibrative transporter ('ei' as proposed by Vijayalakshmi and Belt (loc. cit.)) is not affected by NBTI at concentrations up to 1 μM and lacks the high-affinity binding site for the inhibitor (for reviews see [14,15]). On the other hand, the two concentrative transporters that mediate the sodium dependent transport in normal and neoplastic mammalian cells ('cif' and 'cit' in the terminology of Vijayalakshmi and Belt (loc. cit.)) are insensitive to NBTI [9,13,16–18]. Therefore, the binding of the potent inhibitor NBTI is usually used to quantify the presence of the NBTI-sensitive nucleoside transport in agreement with the general assumption that the high affinity NBTI binding sites are associated with the NBTI-sensitive transporter.

Solubilization and characterization of kidney NBTI-binding sites from pig proximal tubules provides a direct means to elucidate the molecular properties of the NBTI-sensitive carriers expressed in these particular membranes of the kidney and to establish the possible influence of the membrane environment on their pharmacological and biochemical characteristics. By means of high affinity probes such as [^{3}H]NBTI, solubilization and characterization of the NBTI-sensitive nucleoside transporter from erythrocytes have been reported [19–23]. Concerning other cell types, only few studies have been reported on the solubilization of the transporter from brain [24] and from Erlich ascites tumour cells [25]. The aim of this study has been the solubilization and characterization of the NBTI-sensitive nucleoside transporter from pig kidney BBM.

2. Materials and methods

Materials

[^{3}H]NBTI (nitrobenzylthioinosine, [benzyl-^{3}H]) (30 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, USA). Inosine, uridine, NBTI (nitrobenzylthioinosine), Triton X-100, 50% polyethylenimine, pepstatin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), deoxycoformycin, cytochalasin B and glucose were from Sigma (St. Louis, USA). Adenosine, adenosine deaminase, leupeptin, bestatin and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate)) were from Boehringer Mannheim (Germany). DEAE-Sepharose, and CM-Sepharose were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Dipyridamole was kindly provided by Dr. Cembrano from Boehringer Ingelheim (Barcelona, Spain). Stock solutions of NBTI (1 μM) were prepared in DMSO.

All other products were of the best grade available and were purchased from Merck (Darmstadt, Germany). Deionized water further purified with a Millipore Milli-Q system was used throughout.

Methods

Preparation of luminal membranes from pig kidney proximal tubule. Luminal membranes (Brush-border membranes, BBM) were isolated by a magnesium precipitation method described by Vannier et al. [26] and modified by Lin et al. [27]. Briefly, after perfusion, cortex was removed from pig kidneys and placed in 10 mM mannitol, 2 mM Tris-HCl buffer (pH 7.1). Homogenization was performed in 5 volumes of the Tris-mannitol buffer using a Polytron disrupter (PTA 20TS rotor, setting 5) (Kinematica, Lucerne, Switzerland) for three periods of 10 s separated by intervals of 20 s. After homogenization, 1 M $MgCl_2$ was added to a final concentration of 10 mM Mg^{2+}. The final mixture was stirred for 15 min and centrifuged for 12 min at 2000 $\times$ g. The supernatant was removed and centrifuged for 1 h at 60 000 $\times$ g. The pellet was suspended in 150 ml of 170 mM KCl, and 2 volumes of 200 mM Tris-HCl buffer (pH 7.8) were added to the suspension. This suspension was centrifuged for 15 min at 3000 $\times$ g, and the supernatant was centrifuged for 1 h at 60 000 $\times$ g. The final pellet (BBM) was suspended in 50 mM Tris-HCl buffer (pH 7.5) to a final protein concentration of 20–30 mg/ml. All steps were carried out at 4°C. Membrane suspensions were stored at −80°C. In the final membrane fraction the marker enzyme alkaline phosphatase (EC 3.1.3.1) was enriched 9–11-fold.

Where indicated, BBM were obtained in the presence of a cocktail of protease inhibitors composed of 1 μM leupeptin, pepstatin, aprotinin, and bestatin, 200 μM PMSF and 100 μM EDTA; then, the cocktail was included in all the buffers used in the BBM preparation.

Protein determination. Protein was measured by the bicinchroninic acid method, BCA, (Pierce), as described Sorensen and Brodbeck [28].

Solubilization. Unless otherwise indicated, solubilization was performed using glycerol and the natural substrate adenosine as stabilisers of the NBTI-sensitive

nucleoside transporter during solubilization, and the removal of the stabilizing products before the assay of solubilized binding sites. Briefly, the membranes (15 mg/ml) were incubated with 20 mM adenosine, 1 h at 25°C, and then with 20% (v/v) glycerol for 5 min at 4°C before solubilization with 32.6 mM (2% w/v) CHAPS. After incubation with the detergent for 30 min at 4°C, the mixture was centrifuged at $80\,000 \times g$ for 90 min at 4°C. The supernatant, which constituted the solubilized preparations, contained 70% of protein in the intact membranes. Solubilized preparations (10 mg protein/ml) were filtered through a Sephadex G-50 column (0.9 × 15, K-9, Pharmacia, Sweden), previously equilibrated with 50 mM Tris-HCl (pH 7.4), containing 2 mM (0.123% w/v) CHAPS. Samples of 1 ml of solubilized preparations were applied and eluted with 50 mM Tris-HCl buffer (pH 7.4), containing 2 mM (0.123% w/v) CHAPS, at a flow rate of 8 ml/h. The protein containing fractions, as measured by the BCA method, were combined and constituted the sample used in the binding assays.

[^{3}H]NBTI binding assays. [^{3}H]NBTI binding to intact BBM was measured after incubating the membranes (final protein concentration 0.7–0.8 mg protein/ml of suspension) with adenosine deaminase (0.2 U/ml) for 30 min at 25°C in 50 mM Tris-HCl buffer (pH 7.4). Radioligand and modulators or displacers were then added at varying concentrations (see Section 3). After standing at 25°C until equilibrium was achieved (usually 1 h for the lowest ligand concentration and 20 min for the highest ligand concentration), free and bound radioligand were separated by rapid filtration of 500-ml aliquots through Whatman GF/C filters, which were subsequently washed in 10 ml of ice-cold Tris-HCl buffer. The filters were presoaked in 0.3% polyethylenimine (2–4 h, pH 10) to improve performance. Radioligand binding to solubilized BBM was performed upon incubation of the eluates (0.7–0.8 mg protein/ml) with adenosine deaminase (0.2 U/ml) for 30 min at 25°C in 50 mM Tris-HCl buffer (pH 7.4). Radioligands, modulators or displacers were then added. After standing at 25°C until equilibrium was achieved (as with membranes), free and bound radioligand were separated by rapid filtration of 500-μl aliquots through Whatman GF/B filters, which were subsequently washed in 10 ml of ice-cold Tris buffer. The filters were also presoaked in 0.3% polyethylenimine (2–4 h, (pH 10)). Nonspecific binding was determined in the presence of an excess (200–500-fold) of cold ligand.

In all cases, after filtration, the filters were placed in standard vials with 10 ml of Formula-989 liquid scintillation cocktail (New England Nuclear). After an interval of at least 12 h, the vials were counted in a Packard 1600 TRICARB liquid scintillation counter with an efficiency of 50%.

Data analysis. Data from saturation isotherms and displacement curves were analysed by non-linear regression using the ENZFITTER program (Elsevier Biosoft) as described elsewhere [29,30]. Total binding was fitted to a function corresponding to the sum of specific binding to the carrier plus nonspecific binding (assuming that this was linear with ligand concentration), to avoid systematic errors. As previously demonstrated [29], the inclusion of the nonspecific binding component in the model gives the same values of equilibrium binding parameters, but avoids the spread of values of experimentally determined nonspecific binding:

$$\text{Total binding} = \frac{R\,[\text{L}]}{K_\text{D} + [\text{L}]} + K_\text{n}\,[\text{L}]$$

were K_n denotes the constant for nonspecific binding and R is the maximum specific binding. Three to five replicates of each point were performed. Goodness of fit was tested according to the reduced χ^2 or S.D. values given by the program.

Photoaffinity labelling. Membranes (0.7 mg protein/ml) were equilibrated in 50 mM Tris-HCl buffer (pH 7.4) with 5 nM [^{3}H]NBTI in the absence or presence of different competing nonradioactive ligands for 60 min at 25°C. Photolysis was then carried out in 3-ml perspex spectrophotometer cuvettes by 10 s exposure to a 200 W mercury lamp (Applied Photophysics) at a distance of 4 cm from the lamp housing. After irradiation, an excess (500-fold) of unlabelled NBTI was added and then allowed to stand at 25°C for 1 h before being filtered and washed in cool buffer to determine the covalently bound [^{3}H]NBTI. Photolabelled membranes were collected by centrifugation at $60\,000 \times g$ for 1 h.

Endoglycosidase F treatment. [^{3}H]NBTI labelled membranes (4 mg protein/ml) were treated with endoglycosidase F as described by Stiles [31]. In short, photolabelled membranes were solubilized (30 min at 37°C with constant stirring) in 1% Triton X-100, 100 mM sodium phosphate buffer (pH 6.1) containing 30 mM EDTA and 0.05% SDS. The suspension was then centrifuged at $80\,000 \times g$ for 90 min at 4°C. The supernatants (2.6 mg protein/ml) were then aspirated and divided into aliquots (80 μl); endoglycosidase F (1 U/mg protein) or the same amount of solubilization buffer, was added and incubation was carried out at 37°C for 18 h. Enzymatic digestion was terminated by the addition of 12 μl of concentrated (8 ×) SDS-PAGE sample buffer solution and treated for 1 min at 100°C.

Ion exchange chromatography. BBM (0.7 mg protein/ml) was radiolabelled as indicated in the absence or presence of 2 μM NBTI in order to determine nonspecific binding. Membranes were then treated with 1% Triton X-100 in 5 mM sodium acetate buffer (pH 5.0) for 30 min at 37°C and then centrifuged at 80 000

$\times g$ for 90 min at 4°C. The detergent extract was applied to a carboxymethyl Sepharose exchanger (CM-Sepharose) column (15 × 80 mm), which was equilibrated with 0.1% Triton X-100 in the sodium acetate buffer. The column was eluted with the same buffer at a flow rate of 20 ml/h and 1-ml aliquots were collected. After 20 ml, a linear gradient (0–0.2 M NaCl) of 100 ml was applied and the column was washed with 0.5 M NaCl. The void-volume fractions (5 ml) of CM-Sepharose were collected and dialysed overnight against an excess of 5 mM Tris-HCl buffer (pH 8.3) containing 0.1% Triton X-100. The resulting extract was applied to a diethylaminoethyl exchanger column (DEAE-Sepharose) equilibrated with the same buffer. Protein adsorbed to the column was eluted with a linear salt gradient 0–0.2 M NaCl in 5 mM Tris-HCl, 0.1% Triton buffer (pH 8.3). Fractions containing [^{3}H]NBTI photolabelled protein (void-volume of DEAE-Sepharose) were pooled and lyophilized, and brought up to 60 μl with SDS-PAGE sample buffer solution and treated for 1 min at 100°C for electrophoretic analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Photolabelled membranes were washed once and dissolved in (2 ×) SDS-PAGE sample buffer (125 mM Tris-HCl, 2% SDS, 20% glycerol, 2% mercaptoethanol, 0.002% Bromophenol blue (pH 6.8)) and treated for 1 min at 100°C or 1 h at 37°C. The electrophoresis was performed according to the method of Laemmli [32] using homogeneous slab gels containing 12.5% acrylamide separating gel, which were run at constant voltage. After running, the gel was fixed (10% acetic acid, 10% methanol and 80% water), treated with Amplify (Amersham) for fluorographic detection and dried at 80°C in a gel drier (Bio-Rad). Dry gels were subjected to autoradiography at −80°C. When low amounts of radioactivity were applied the gel was cut in 1.2-mm slices, which were dissolved with Solvable (Du Pont) for 3 h at 75°C, then 10 ml liquid scintillation was added, and radioactivity was counted.

3. Results

Solubilization of NBTI binding sites from BBM

The presence of the NBTI-sensitive nucleoside transporter in the solubilized membrane fractions and the percentage of non-solubilized binding sites remaining in the pellet was evaluated by measurement of the [^{3}H]NBTI specific binding. Binding assays were performed as described in Section 2 with a fixed [^{3}H]NBTI concentration (0.5 nM); nonspecific binding was determined in the presence of 1 μM NBTI. The incubation of kidney BBM (15 mg/ml) with the non-ionic detergent Triton X-100 caused the total denaturation of the NBTI-sensitive nucleoside transporter, although the yield of solubilized proteins was relatively high (Table 1). Treatment with 2% CHAPS solubilized 52% of total protein but the recovery of [^{3}H]NBTI binding sites was very low (Table 1). With regard to the low percentage of binding sites remaining in the membrane after solubilization, 2% CHAPS solubilized the NBTI-sensitive nucleoside transporter in a form that was unable to bind NBTI. An approach to raise the yield was the addition of glycerol, reported as general stabilizer of proteins in solution [24], and of adenosine, which usually improves the efficacy of solubilization in the case of adenosine binding molecules [33]. In these conditions 41% of the [^{3}H]NBTI binding sites were solubilized (Table 1) though upon elimination of adenosine and excess glycerol the yield rose to 60%. Elimination of the glycerol excess and dilution of the CHAPS below its critical micellar concentration (CMC = 8 mM) was achieved by gel permeation using Sephadex G-50 equilibrated with 50 mM Tris-HCl (pH 7.4) containing 0.123% CHAPS. The recovery was the 75% of the applied protein. Adenosine was eliminated by preincubation of the solubilized fraction with adenosine deaminase before the radioligand binding. Changes in the detergent/protein ratio showed ratio 1 as the most suitable to increase the stability of the solubilized preparations (data not shown). When assayed after 11 days of storage at either 4°C or −20°C all the extracts retained the same [^{3}H]NBTI binding capacity shown shortly after solubilization. Neither an increase in ionic strength by addition of 100 mM NaCl, nor the addition of 1 mM EDTA improved the solubilization by 2% CHAPS plus 20% glycerol (data not shown).

Table 1
Solubilization of [^{3}H]NBTI binding sites

Treatment	% Solubilized protein	% Non-solubilized binding sites	% Solubilized binding sites
0.5% Triton X-100	48 ± 10	0	0
2% CHAPS	52 ± 4	8 ± 1	11 ± 2
2% CHAPS + 20% glycerol + 20 μM adenosine [a]	73 ± 10	12 ± 3	41 ± 16

Pig kidney BBM (15 mg/ml) were incubated (30 min at 25°C) in 50 mM Tris-HCl buffer (pH 7.4) containing the indicated additives. The suspension was centrifuged at 80000 × g for 90 min (4°C). Supernatants and pellets were used for binding assay with a fixed [^{3}H]NBTI concentration (0.5 nM); nonspecific binding was determined in presence of 1 μM NBTI. 100% binding refers to the value found for untreated BBM (0.6 pmol/mg protein) under the same experimental conditions. Values are mean ± S.D. of three experiments.

[a] The membranes were incubated with adenosine 20 μM, 1 h at 25°C, and then with 20% (v/v) glycerol for 5 min at 4°C before their solubilization with 2% (w/v) CHAPS.

Characterization of the [^{3}H]NBTI binding to solubilized pig kidney BBM

Saturation analysis of the [^{3}H]NBTI binding to solubilized BBM led to the determination of one affinity state (Fig. 1). The parameter values at equilibrium deduced from the saturation isotherms (see Section 2; values are mean ± S.D. of four independent experiments) showed that the maximum specific binding (B_{max} = 0.82 ± 0.05 pmol/mg protein) was similar to that of intact BBM (B_{max} = 0.97 ± 0.05 pmol/mg protein), whereas the value of K_d in the detergent extracts was greater (K_d = 3.7 ± 0.9 nM) than that in BBM (K_d = 1.1 ± 0.3 nM), indicating that the process of solubilization causes a decrease in the affinity of the NBTI-sensitive nucleoside transporter. The fact that the binding sites remaining in the detergent-treated membranes presented a K_d of 1.7 ± 0.8 nM, which is similar to that found in intact membranes, suggests that the decrease in affinity is a consequence of the state of the transporter in the solubilized form, rather than an effect of the solubilization treatment. The B_{max} value for detergent-treated membranes was 0.51 ± 0.1 pmol/mg protein.

Displacement experiments were performed using [^{3}H]NBTI as ligand and dipyridamol, adenosine, inosine, uridine, NBTI, cytochalasin B, and glucose as displacers. The order of potency of the NBTI-sensitive nucleoside transport substrates and inhibitors (NBTI > dipyridamole > adenosine > inosine > uridine) as displacers, was the same in the intact BBM and in the solubilized preparations (Table 2), indicating that the NBTI binding sites in the solubilized pig kidney membranes display the same specificity as in the intact BBM vesicles. On the other hand, the increase in the K_i values determined for the solubilized form (Table 2) corroborates the decrease in affinity of the nucleoside tranporter for the NBTI binding in the solubilized preparations. When the displacer was adenosine, an inhibitor of adenosine deaminase (200 nM deoxycoformycin) was used to prevent hydrolysis of the nucleoside during the binding assay (see Section 2).

The high K_i values determined using glucose and cytochalasin B as displacers indicated that the high-affinity binding of [^{3}H]NBTI is to the NBTI-sensitive nucleoside transporter rather than to the glucose trans-

Table 2
Inhibition constants for displacers of [^{3}H]NBTI specific binding to intact and solubilized pig kidney BBM

Displacer	K_i (μM)	
	Intact BBM	Solubilized BBM
NBTI	0.0009 ± 0.0001	0.0065 ± 0.0001
Dipyridamole	0.009 ± 0.002	0.023 ± 0.003
Adenosine	83 ± 9	100 ± 1
Inosine	300 ± 60	900 ± 200
Uridine	780 ± 80	1600 ± 300
Cytochalasin B	11 ± 2	n.d.
Glucose	$> 6 \cdot 10^5$	n.d.

Experimental conditions are those described in Section 2. The fixed [^{3}H]NBTI concentration was that producing a 20% saturation of total binding sites: 0.5 nM in the displacement of [^{3}H]NBTI binding to intact pig kidney BBM, and 2.5 nM in the displacement of [^{3}H]NBTI binding to solubilized BBM. The concentrations of the displacers were variable in the following ranges: NBTI (0.1 nM–10 μM), dipyridamol (1 nM–100 μM), adenosine (316 nM–10 mM), inosine (1 μM–31 mM), and uridine (3 μM–100 mM), cytochalasin B (100 nM–1 mM), and glucose (100 μM–1 M). Results are taken from the displacement curves obtained from five replicates and were fitted using a non-linear regression program as indicated in Section 2. Values are mean ± S.D. given by the fitting program.

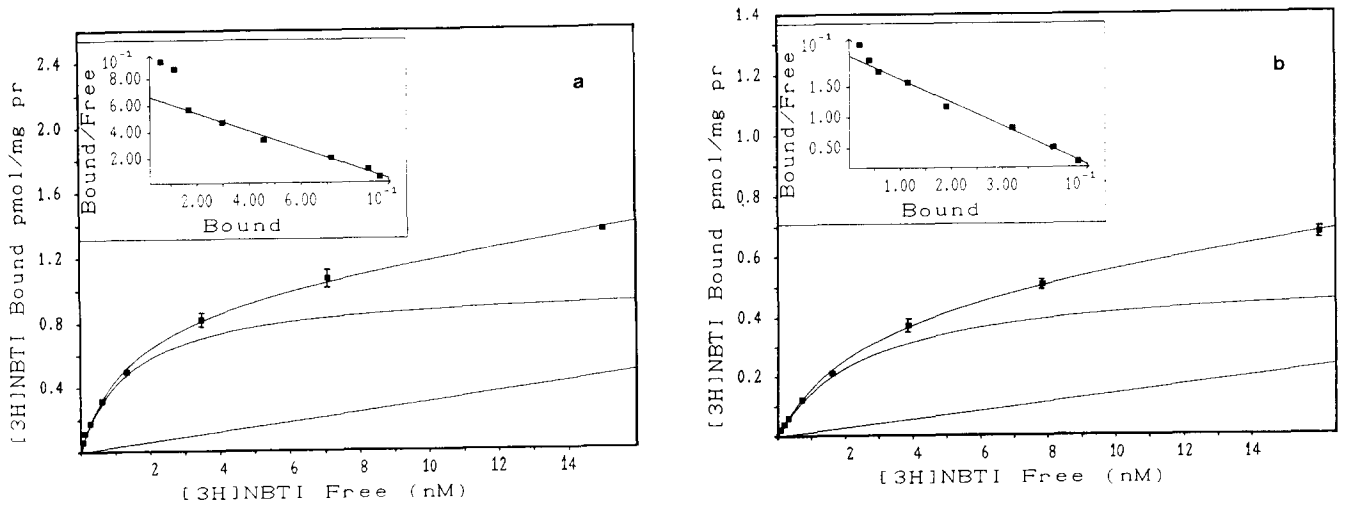

Fig. 1. Isotherms of [^{3}H]NBTI binding to BBM. Saturation analysis of [^{3}H]NBTI binding to (a) intact pig kidney BBM vesicles and (b) soluble extracts, was performed as described in Section 2. The concentration of [^{3}H]NBTI varied in the range 0.1–15 nM. Nonspecific binding was determined in the presence of a 200-fold excess of NBTI. All points represent the mean ± S.E. of five replicates and the curves shown are representative of four separate experiments. Data were adjusted using a non-linear regression program as described in Section 2. Inset: Scatchard plot of the computer-derived specific binding data.

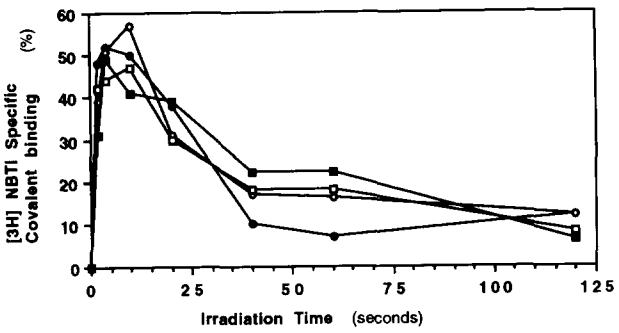

Fig. 2. Efficiency of [^{3}H]NBTI photoaffinity labelling. BBM (0.7 mg protein/ml) were equilibrated with 5 nM [^{3}H]NBTI in 50 mM Tris-HCl buffer (pH 7.4) for 30 min. The incubation mixtures were then exposed to UV light at different distances from the lamp (cm): (●) 2; (○) 4; (□) 10; (■) 20. At the indicated exposure times, 2 μM NBTI was added to each sample in order to determine the covalent binding (see Section 2). Values are given as percent of the total binding.

porter. In fact, when analysing the [^{3}H]NBTI binding to the glucose transporter, Jhun et al. [34] reported K_c values in the micromolar range.

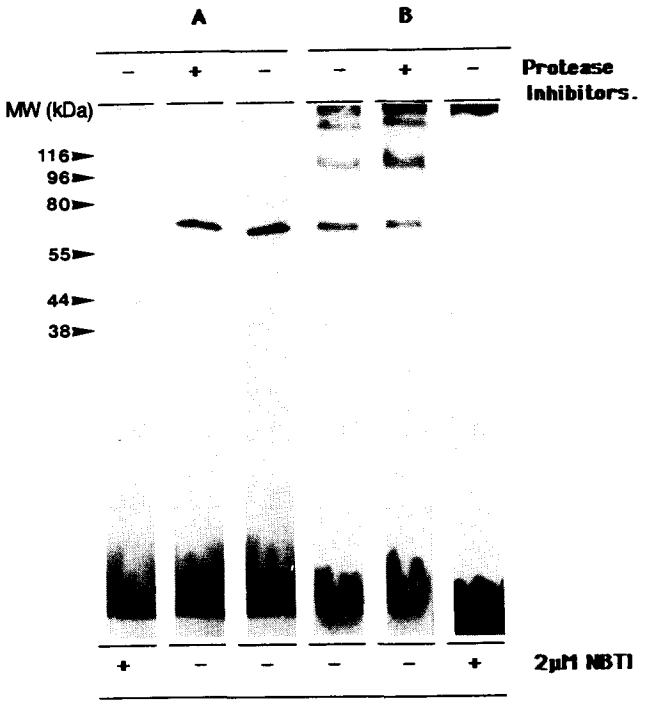

Fig. 3. SDS-PAGE of the NBTI-sensitive nucleoside transporter labelled with [^{3}H]NBTI. BBM (0.7 mg protein/ml) were equilibrated in 50 mM Tris-HCl buffer (pH 7.4) with 5 nM [^{3}H]NBTI in absence and the presence of 2 μM NBTI and/or a cocktail of protease inhibitors (1 μM of leupeptin, pepstatin, aprotinin, and bestatin; 200 μM PMSF; 100 μM EDTA). Samples were placed at 4 cm from the lamp and exposed to UV light for 10 s. Unreacted ^{3}H-ligand was removed by washing with buffer. Labelled BBM were dissolved for SDS-PAGE as indicated in Section 2 and treated for 1 min at 100°C (A) or 1 h at 37°C (B) prior to electrophoresis. The gel was run, dried and autoradiographed as described in Section 2.

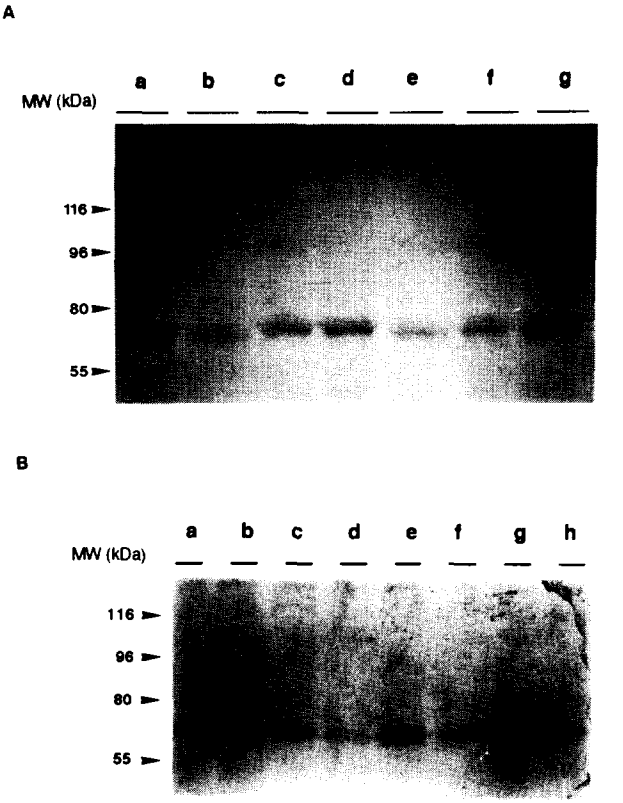

Fig. 4. Specificity of the [^{3}H]NBTI photoaffinity labelling. Membranes (0.7 mg protein/ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) with 5 nM [^{3}H]NBTI in absence (control) or in presence of glucose (A) or nucleoside transporter (B) -specific ligands. Samples were placed at 4 cm from the lamp and exposed to UV light for 10 s. Membranes were washed with buffer to eliminate the excess reagents and treated for SDS-PAGE for 1 min at 100°C. The gel was run, dried and autoradiographed as described in Section 2. Quantitative densitometry of the autoradiographs was performed using a Computing Densitometer (Molecular Dynamics). Values are mean ± S.D. of three separate experiments. (A) Control (a): 222 ± 16; 666 mM glucose (b): 116 ± 9; 200 mM glucose (c): 169 ± 2; 1 mM glucose (d): 261 ± 10; 25 μM cytochalasin B (e): 155 ± 5; 5 μM cytochalasin B (f): 162 ± 8; 0.5 μM cytochalasin B (g): 189 ± 30. (B): 5 nM NBTI (a): 88 ± 11; Control (b): 185 ± 12; 10 nM dipyridamole (c): 113 ± 9; 10 μM dipyridamole (d): 81 ± 14; 100 μM adenosine (e): 140 ± 13; 10 mM adenosine (f): 62 ± 9; 800 μM uridine (g): 146 ± 2; 10 mM uridine (h): 86 ± 9.

Photoaffinity labelling. The photoaffinity labelling of NBTI-sensitive nucleoside transporter by [^{3}H]NBTI was performed under the conditions described in Section 2. Using different distances from the lamp and different times (Fig. 2) the greatest efficiency in the [^{3}H]NBTI cross-linking (57% of the total nucleoside transporter present at the BBM) was found when the irradiation was for 10 s at 4 cm of the lamp. At higher irradiation times the specific covalent binding of [^{3}H]NBTI was reduced due to a pronounced increase in nonspecific binding.

SDS-PAGE of affinity labelled BBM was developed after treatment of the samples in two different ways.

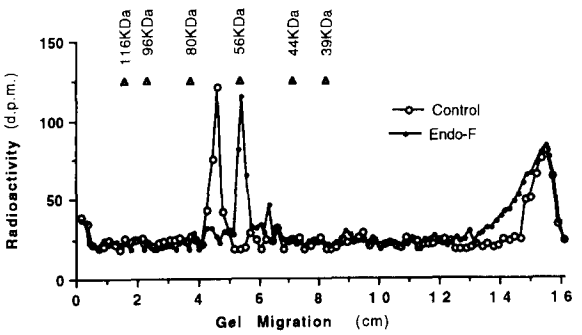

Fig. 5. Effect of endoglycosidase F on the NBTI-sensitive nucleoside transporter electrophoretic mobility. [^{3}H]NBTI affinity labelled BBM (see legend of Fig. 4 for the control samples) were solubilized (4 mg protein/ml) with 30 mM EDTA, 0.05% SDS, 100 mM NaH_2PO_4 buffer (pH 6.1) containing 1% Triton X-100. Then, 1 U/mg protein of endoglycosidase F (•) or the same amount of solubilization buffer in a control sample (○), were added to the extracts. After 18 h at 37°C samples were treated with SDS and the SDS-PAGE was performed. The gel was run and dried as indicated in Section 2. The gel was cut into 1.2-mm slices, which were dissolved with Solvable (Du Pont) for 3 h at 75°C and the radioactivity was counted in a liquid scintillation counter.

The lane corresponding to the labelled membranes treated with the SDS sample buffer for 1 min at 100°C showed a single sharp radioactive band (Fig. 3, panel A) of 65 kDa which disappeared in the presence of 2 μM NBTI. However, when the labelled membranes were treated with sample buffer for 1 h at 37°C (Fig. 3, panel B) three molecular polypeptides were specifically photolabelled with [^{3}H]NBTI; their approximate molecular masses were 65 kDa, 110 kDa, and 230 kDa, which suggests that they correspond to the monomeric, dimeric and tetrameric froms of the NBTI-sensitive nucleoside transporter. No differences were found when the membranes were obtained in the presence or absence of a cocktail of protease inhibitors (see Section 2).

The specificity of the photoaffinity binding of NBTI was assessed by competition with ligands specific for the glucose or the NBTI-sensitive nucleoside transporter. The conditions of the photolabelling were those described above but, with the addition of the corresponding reagent: glucose or cytochalasin B as specific for the glucose transporter, and NBTI, dipyridamole, adenosine and uridine as specific for the nucleoside transporter. Pelleted membranes were treated with SDS sample buffer for 1 min at 100°C and subjected to SDS-PAGE. As shown in Fig. 4, neither glucose nor cytochalasin B competed for the covalent binding (panel A) whereas all ligands specific for the nucleoside transporter led to the disappearance of the control band (panel B). This demonstrates that the [^{3}H]NBTI photoaffinity cross-linking was highly specific for the NBTI-sensitive nucleoside transporter.

Treatment with endoglycosidase F of the monomeric form of the transporter led to a shift in molecular mass, from 65 kDa to 57 kDa, indicating that the molecule of the NBTI-sensitive nucleoside transporter has N-linked oligosaccharides (Fig. 5).

Characterization of the transporter. The NBTI-sensitive nucleoside transporter from BBM was partially purified using ionic exchange chromatography. Due to the fact that CHAPS is a zwitterionic detergent that would interfere in the chromatographies, the non-ionic detergent Triton X-100 (1%) was used to solubilize the BBM in which the transporter molecules were previously cross-linked to [^{3}H]NBTI. Both anionic and cationic exchange were sequentially attempted. Thus, detergent extracts from [^{3}H]NBTI affinity labelled membranes were first chromatographed using CM-Sepharose equilibrated with 0.1% Triton X-100 in sodium acetate buffer at pH 5. The active fractions, as measured by the radioactivity of the eluates, were found in the void volume, whereas no sign of the transporter appeared during the salt gradient (Fig.

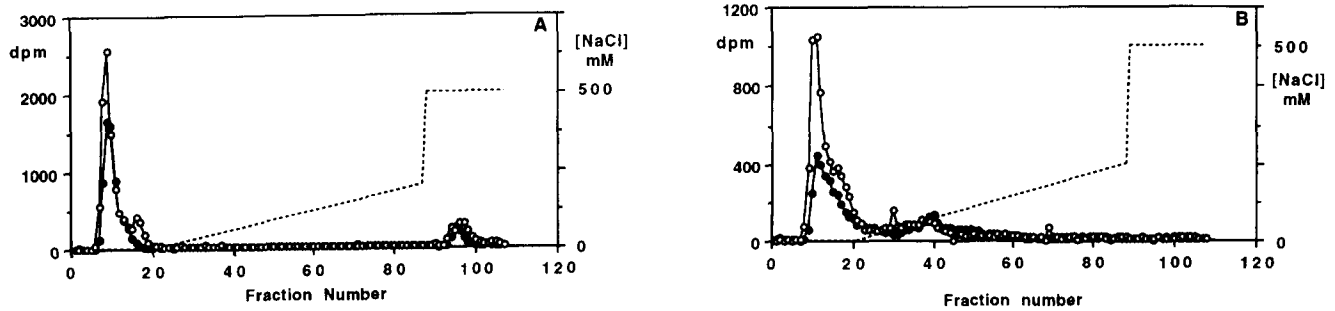

Fig. 6. Ionic exchange chromatography of the NBTI-sensitive nucleoside transporter. [^{3}H]NBTI radiolabelled BBM in the presence (•) or in the absence (○) of 2 μM NBTI (see legend of Fig. 4 for the control samples) were solubilized with 1% of Triton X-100 in 5 mM sodium acetate buffer (pH 5.0). The soluble fraction was chromatographed through a carboxymethyl column (CM-Sepharose) equilibrated with the same buffer but containing 0.1% Triton X-100 (A). The void-volume fractions of the CM-Sepharose elution were collected and dialysed overnight against an excess of 5 mM Tris-HCl buffer (pH 8.3) containing 0.1% Triton X-100. The dialysed fraction was chromatographed through a diethylaminoethyl (DEAE) Sepharose gel (B) equilibrated with the same buffer. In both cases, elution was performed with a linear gradient of 0–0.2 M NaCl (100 ml total volume of gradient, at a flow rate of 1 ml/min). Radioactivity of the eluted samples was determined as described in Section 2.

6A). The degree of purification in this step was 2.5. Active fractions were pooled, dialysed against 0.1% Triton X-100 in a Tris-HCl buffer (pH 8.3) and chromatographed in a DEAE-Sepharose column equilibrated with the same buffer (Fig. 6B). Under these conditions, active fractions also appeared in the void volume and the degree of purification in this step was 5.3, allowing a total degree of purification of 13.

4. Discussion

The procedure established for the solubilization of proteins from pig kidney BBM is very effective, as indicated by the high yield of the solubilization of the NBTI-sensitive nucleoside transporter. The binding of the specific ligand, [^{3}H]NBTI, was used to quantify the presence of this nucleoside transporter in the intact membranes and in the solubilized preparations. The use of glycerol and adenosine considerably increased the stability of the solubilized transporter, as can be deduced from the values of the binding sites that remain in the membranes after detergent treatment. Moreover, the reconstitution of the transporter by the elimination of the excess of glycerol and detergent by gel filtration provides a notable recovery of the NBTI binding sites, suggesting that the transporter is more accessible to [^{3}H]NBTI binding in the absence of glycerol rather than in its presence and after decreasing the CHAPS concentration below its critical micellar concentration (CMC = 8 mM). Although high yield procedures for solubilization of NBTI-sensitive nucleoside transporter from erythrocytes have been reported [19,20,22,23,35], no procedure for the solubilization of the transporter from kidney has yet been described. The solubilization of the NBTI-sensitive nucleoside transporter from pig kidney BBM did not change its NBTI-binding characteristics, as shown by the order of potency of nucleosides as displacers; the only major change was a 3-fold decrease in affinity. On the other hand, the high K_i found for glucose and cytochalasin B as displacers of the [^{3}H]NBTI binding indicates that the glucose transporter dit not interfere in the characterization of the NBTI-sensitive transporter. Furthermore, the [^{3}H]NBTI photoaffinity labelling performed on intact BBM leads to the cross-linking of the ligand to this nucleoside transporter, since the specific band obtained by SDS-PAGE is only displaced by using adenosine, NBTI, dipyridamole or uridine (Fig. 4B). The NBTI binding is highly specific for this nucleoside transporter, since glucose and cytochalasin B did not compete with [^{3}H]NBTI in these assays (Fig. 4A).

The characterization by SDS-PAGE of the nucleoside transporter cross-linked to [^{3}H]NBTI led to different results, depending on how the samples were prepared. When treatment was for 1 min at 100°C with SDS sample buffer, only one band of 65 kDa was detected for the NBTI-sensitive nucleoside transporter in pig kidney BBM, which is similar to the mass found for the transporter in pig erythrocytes [22]. This molecular mass is higher than those found in human erythrocytes (40–55 kDa) [23,34,36], Ehrlich ascites tumour cells (42 kDa) [25] and chromaffin cells (51 kDa) [37,38]. When the treatment of the samples was for 1 h at 37°C (Fig. 3B), further bands appeared which, according to their approximate molecular mass, probably correspond to dimers and tetramers of the 65 kDa form. The existence of dimers was suspected in human erythrocytes by radiation-inactivation studies performed by Jarvis et al. [39] and found in chromaffin cells by SDS-PAGE [37,38]. The physiological significance of the existence of dimers and tetramers requires further investigation but, based on the conditions required for their appearance (37°C, 1 h), they may involve hydrophobic interactions.

It should be noted that the chromatographic behaviour of the NBTI-sensitive nucleoside transporter from pig kidney BBM is very interesting if compared with that of human and pig erythrocytes. Under similar conditions as those used in this paper, and with DEAE cellulose, the transporter from pig erythrocytes eluted in the 80–110 mM NaCl range [21]. In contrast, the behaviour of the transporter from pig kidney BBM (Fig. 6) and from human erythrocytes [19] upon passage through a DEAE-based column was identical, both eluting in the void volume. The difference in chromatographic behaviour between the transporter in two different locations (kidney and erythrocytes) of the same species (pig) may result from the existence of two different genes, two splicing products or from posttranscriptional modifications such as variation in the composition of the carbohydrates in the glycoprotein. According to the latter hypothesis, the removal of N-linked oligosaccharides by treatment with endoglycosidase F leads in both cases, kidney (Fig. 5) and erythrocytes [21], to a single band of 57 kDa. The composition of the carbohydrate side chains of the NBTI-sensitive nucleoside transporter seems to reflect specific expression patterns, since Hammond and Johnstone [25] have demonstrated that transporter molecules expressed in different cell types and which differ in molecular mass may lead to a single band of identical size upon removal of carbohydrates.

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