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A refined method for the photoaffinity labelling of the nitrobenzylthioinosine-sensitive nucleoside transport protein: Application to cell membranes of calf lung tissue

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A refined method for the photoaffinity labelling of the NBI-sensitive nucleoside transport protein is described. It involves the use of low concentrations of the photolabile probe [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI), whereas the usual inclusion of dithiothreitol in the protocol is omitted. The method was successfully applied to cell membranes of calf lung tissue, which was shown to be a rich source of this physiologically important protein with all the characteristics (both in membrane bound and solubilized form) known from similar proteins on other cell types. Specific covalent incorporation of radioactivity appeared to be pH independent. SDS-polyacrylamide gel electrophoresis revealed a specifically labelled protein with an apparent molecular weight of 55 kDa.

Introduction

The permeation of nucleosides and nucleoside analogues across the plasma membrane of mammalian cells is mediated by nucleoside specific transport proteins. Three types of these nucleoside carriers can be discriminated: (i) proteins that transport nucleosides by facilitated diffusion and that are inhibited by low concentrations (< 10 nM) of the transport inhibitor nitrobenzylthioinosine (NBI), (ii) similar proteins that are NBI-resistant, i.e. inhibition occurs at high concentrations of NBI (> 1 μM), and (iii) Na^+-dependent transport systems in brush-border membranes, the so-called Na^+-cotransporter for nucleosides. Under physiological circumstances blockade of these proteins leads to an increase in the extracellular concentration of nucleosides. This rise may cause profound pharmacological effects, for instance vasodilation in the cardiovascular system in case of adenosine (for reviews, see Refs. 1 and 2).

From the foregoing it is obvious that tritiated [^{3}H]NBI has been and still is of great help in the characterization of the NBI-sensitive proteins [3]. The compound is highly photolabile, which enables photoaffinity labelling of the NBI-sensitive transport protein in various cell types [4–6]. Recently, Jarvis and Young reviewed the protocols used in the labelling procedure [7]. Usually, high concentrations of [^{3}H]NBI (up to 100 nM) are used, together with high concentrations of dithiothreitol (DTT), up to 50 mM.

In this report a refined method is described for the photoaffinity labelling of the NBI-sensitive carrier. Low concentrations of [^{3}H]NBI are used, whereas DTT is omitted. The method was applied to cell membranes of calf lung tissue. Lung tissue is a major site for the removal of circulating adenosine, and it is a rich source of this physiologically important protein [6].

Materials and Methods

Chemicals

[^{3}H]NBI (26 Ci/mmol) was from NEN, Dreieich, F.R.G. The following compounds were gifts, which are gratefully acknowledged: dipyridamole (Karl Thomae, Biberach, F.R.G.), mioflazine (Janssen Pharmaceutica, Beerse, Belgium) and dilazep (Asta-Werke, Frankfurt a/M, F.R.G.). All reagents for SDS-polyacrylamide gel electrophoresis were from LKB (Bromma, Sweden). Protein markers were obtained from Pharmacia (Up-

Abbreviations: [^{3}H]NBI, [^{3}H]nitrobenzylthioinosine; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecylsulfate; kDa, kilodalton.

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psala, Sweden). All other chemicals were supplied by Sigma (St. Louis, MO, U.S.A.).

Membrane preparation and solubilization

Fresh calf lung tissue was obtained from a local slaughterhouse. After dissection of main blood vessels and trachea the tissue was homogenized, and a membrane preparation obtained (P_2-fraction) by differential centrifugation ($8000 \times g$ for 10 min, and $20000 \times g$ for 60 min, respectively). The final pellet was washed once, resuspended and stored at −80 °C. The composition of the buffer was 50 mM Tris-HCl, 5 mM EDTA and 0.1 mM PMSF (pH 7.4).

Solubilization of the membrane bound proteins was achieved in buffer (pH 7.4) containing 20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 20% glycerol, 1% CHAPS and a mixture of proteinase inhibitors (1 mM DTT, 0.5 mM PMSF, 0.1 mM iodoacetamide, 0.1 mM benzamidine). Incubation was at 0 °C for 30 min. After centrifugation ($175000 \times g$ for 60 min) the supernatant was stored at −80 °C. Protein was determined by the bicinchoninic acid (BCA) method with bovine serum albumin as standard [8].

Binding studies

All assays were performed in duplicate in a final volume of 300 μl Tris-HCl (pH 7.4, 25 °C) containing the following components: (i) 100 μl of graded concentrations of inhibitors (displacement studies) or 100 μl buffer (saturation experiments), (ii) 100 μl [^{3}H]NBI (0–10 nM in saturation experiments, 1.5 nM in displacement studies), (iii) 100 μl of a membrane suspension or solubilized protein solution (0.03 mg protein/ml). Incubations were carried out at 25 °C for 30 min, and were terminated by the addition of 1 ml icecold buffer and rapid vacuum filtration over Whatman GF/C filters in the cold room (in case of the solubilized solution two filters presoaked in 0.3% polyethyleneimine [9], were used). Filters were washed twice with 2 × 2 ml cold buffer and dried (1 h, 75 °C). Radioactivity retained on the filters was counted in 3.5 ml OptiPhase MP counting liquid on a Packard TriCarb 4640 spectrometer with 50% efficiency. Specific binding was defined as the difference between radioactivity bound in the absence and presence of 3 μM dipyridamole or 0.1 μM nitrobenzylthioinosine, which yielded identical results. Results are the means of three separate experiments.

Photoaffinity labelling

Calf lung membranes (0.4 mg/ml) were equilibrated (25 °C, 30 min) with [^{3}H]NBI (1.5 nM) in the absence or presence of dipyridamole (3 μM). Photolysis was then carried out in 3 ml perspex spectrophotometer cuvettes with a 450 W xenon-mercury lamp (Oriel, Stamford, CT, U.S.A.) during 30 s at a distance of 30 cm from the lamp housing. After irradiation samples were diluted 10-fold with buffer (50 mM Tris-HCl, pH 7.4) to which 10 μM dipyridamole was added, and either allowed to stand at room temperature for 1 h before being filtered and washed twice to determine covalently bound [^{3}H]NBI or centrifuged (20 min, $50000 \times g$) to recover membrane pellets. In the latter case the pellets were washed once with dipyridamole containing buffer and dissolved in gel electrophoresis buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% mercaptoethanol, pH 6.8) and heated at 60 °C for 1 min.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [10] on a 12% acrylamide gel. Radioactivity in the various protein bands was then determined by the following procedure. Gels were cut into 1.2 mm slices, which were placed by two in scintillation vials and dissolved in 150 μl perchloric acid (60%) and 300 μl hydrogen peroxide (30%). After three hours at 75 °C dissolution was complete. Then 5 ml liquid scintillation fluid (OptiPhase MP) was added, and radioactivity determined (30% efficiency). Lanes with protein markers were stained with Coomassie brilliant blue.

Results

The specific equilibrium binding of [^{3}H]NBI to both membrane bound and solubilized binding sites in calf lung cell membranes was saturable and reversible, displaying high affinity ($K_d = 0.65 \pm 0.05$ nM and 0.68 ± 0.06 nM, respectively) and capacity ($B_{max} = 2.5 \pm 0.2$ and 3.7 ± 0.2 pmol/mg protein, respectively). Dipyridamole, mioflazine and dilazep were effective displacers of specific [^{3}H]NBI binding to membranes with K_i values of 8.8 ± 1.2 nM, 5.1 ± 0.3 nM and 2.1 ± 0.6 nM, respectively. The natural nucleosides adenosine ($K_i = 140 \pm 40$ μM) and inosine ($K_i = 130 \pm 20$ μM) were equipotent in inhibiting [^{3}H]NBI binding. DTT was capable of displacing [^{3}H]NBI as well. Rather high concentrations were needed ($IC_{50} = 6 \pm 1$ mM, at 1.5 nM [^{3}H]NBI), although substantially lower than those that are generally used in photoaffinity labelling studies [7]. Similar results for all displacers were obtained on solubilized preparations.

In Fig. 1 the effects of the inclusion of DTT in photoaffinity labelling experiments are shown. Bars A and B represent control experiments in which reversible equilibrium [^{3}H]NBI binding was quantified under conditions used in the photoaffinity labelling experiments. Without the addition of DTT specific covalent [^{3}H]NBI binding represented 35–40% (bar C) of specific reversible [^{3}H]NBI binding (bars A and B). The addition of a low concentration of DTT (10 μM) only slightly af-

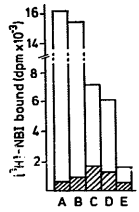

Fig. 1. Binding of [^{3}H]NBI to cell membranes of calf lung tissue. Open bars represent specific, shaded bars represent non-specific binding. Bars A and B: reversible equilibrium binding. Bars C–E: covalent binding. A: control membranes, no irradiation. B: membranes pretreated by irradiation, no further irradiation; C: photoaffinity labelling, no scavenger; D: Photoaffinity labelling in the presence of 10 μM DTT; E: photoaffinity labelling in the presence of 1 mM DTT.

fected these results (bar D), whereas 1 mM DTT in this experimental setup drastically reduced specific covalent binding of [^{3}H]NBI due to a far more pronounced decrease in total than in non-specific covalent binding (bar E).

In order to study a possible pH dependency of the covalent incorporation of [^{3}H]NBI [11], we performed experiments as described in Fig. 1, bar C, over a pH range varying from 7.0 to 9.5 (0.5 pH unit intervals). No significant deviations (< 10%) from the results in Fig. 1, bar C, were noticed, neither with respect to total nor to non-specific binding. SDS-polyacrylamide gel electrophoresis of covalently labelled lung cell membranes (obtained under the conditions described for bar C in

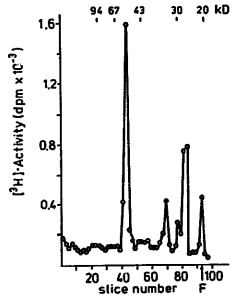

Fig. 2. SDS-polyacrylamide gel electrophoresis of membrane proteins covalently labelled by [^{3}H]NBI. F is front. For procedure see Materials and Methods. Similar results were obtained on five different occasions.

Fig. 1) showed that most radioactivity migrates as a symmetrical peak with an apparent molecular weight of 55 kDa. In some membrane preparations smaller peaks, e.g. a polypeptide of 26 kDa, were also noticed (Fig. 2). Membranes, similarly treated in the presence of 3 μM dipyridamole, did not show any specific accumulation of radioactivity (maximal incorporation per slice did not exceed 100 dpm).

Discussion

Calf lung tissue is a rich source of the nucleoside transport protein as identified by [^{3}H]NBI binding. B_{max} and K_d values are in the same range as the capacities and affinities found by others for peripheral tissues in several species, e.g., guinea pig cardiac [12] and rat lung membranes [6]. Solubilization of the membranes with the mild detergent CHAPS concomitantly yields a slight purification of the NBI binding polypeptide, as can be inferred from the increase in B_{max}. Although chemically unrelated, dipyridamole, mioflazine and dilazep are all potent displacers of [^{3}H]NBI binding. To our knowledge this is the first report that demonstrates the potency of mioflazine in inhibiting specific [^{3}H]NBI binding. The data for dipyridamole and dilazep are in agreement with other findings, although, as an exception, in rat tissue generally higher K_i values are observed [13,14]. Thus, the NBI binding sites present in calf lung tissue display similar characteristics as described for the transporters from other sources.

Protocols for photoaffinity labelling of this physiologically important protein generally advise the use of high concentrations of [^{3}H]NBI (up to 100 nM) and high concentrations of DTT as a radical scavenger (50 mM) to reduce non-specific binding (for review see Ref. 7). In our initial displacement studies of reversibly bound [^{3}H]NBI we observed that 50 mM DTT is capable of almost fully inhibiting [^{3}H]NBI (1.5 nM) binding (see Results). This finding prompted us to avoid the use of DTT in photoaffinity labelling experiments and to look for suitable conditions in the absence of this scavenger. Instead of using quartz cuvettes, we carried out our experiments in conventional perspex cuvettes (light cutoff 285 nm), thereby avoiding the influence of high intensity UV irradiation of lower wavelength on both [^{3}H]NBI and the membranes. Since NBI displays an UV absorption maximum at 290 nm and its spectrum is extended to almost 360 nm (results not shown, see also Ref. 4), still sufficient radiation is supplied to induce time-dependent changes in the spectrum of NBI (results not shown) and covalent incorporation to the membranes (Fig. 1, bar C). Without DTT, concentrations of [^{3}H]NBI can be kept low (1.5 nM), probably the cause of the observed low non-specific binding. The tremendous effects of DTT in these circumstances are also represented in Fig. 1 (bars D and E): as low as 1

mM DTT reduces specific covalent binding considerably. From bars A and B it can be derived that irradiation in our protocol hardly affects the integrity of the NBI-sensitive transport protein with respect to ligand binding. Similar experiments performed in quartz cuvettes strongly affected reversible [^{3}H]NBI binding, causing a 40% reduction in specific binding. The covalent incorporation of radioactivity upon irradiation under identical experimental conditions was approx. twice as high in quartz as in perspex cuvettes. Addition of an excess of unlabelled NBI (100 nM) during the irradiation procedure led to a decrease in photolabelling efficiency, whereas the detrimental effects of DTT were not overcome (results not shown).

In contrast to findings for the peripheral benzodiazepine binding sites [11], no influence of pH on the covalent incorporation of radioligand was observed for this nucleoside transport protein.

The apparent molecular weight of the protein in calf lung tissue is estimated to be 55 kDa (Fig. 2). This value is in agreement with reports on many other cell types, e.g., human erythrocytes, rat lung and liver, and guinea pig liver, lung, cardiac muscle and brain [4–6,12,15]. In these tissues, NBI binding polypeptides have an apparent molecular weight ranging between 66 kDa and 45 kDa. The radioactivity retained at the smaller 26 kDa protein band (Fig. 2) indicates that some proteolysis in our preparation may have occurred. A more rigorous use of protease inhibitors in the early steps of membrane preparation may help to avoid this phenomenon.

In conclusion, in this report a refined method for the photoaffinity labelling of the NBI-sensitive nucleoside transport protein is described. The method was applied to calf lung tissue, a rich source of this protein with characteristics that are shared by NBI binding polypeptides from most other sources. These findings could be of help in further characterization, isolation and purification of this physiologically important protein.

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