Biochimica et Biophysica Acta, 1105 (1992) 291–299

BBAMEM 75564

Down-regulation and recycling of the nitrobenzylthioinosine-sensitive nucleoside transporter in cultured chromaffin cells

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(Received 23 May 1991)
(Revised manuscript received 25 September 1991)

Key words: Chromaffin cell; Cycloheximide; Down regulation; Nitrobenzylthioinosine; Nucleoside transport; Protein recycling; Protein turnover

The dynamics of the nitrobenzylthioinosine (NBTI)-sensitive nucleoside transporter were studied in cultured chromaffin cells. Photolabelling of transporters with [^{3}H]NBTI induced a down-regulation of this protein from the plasma membrane with a half-life value of 2.31 ± 0.61 h, measured by specific isolation of plasma membrane on polycationic beads. In this internalization step 50–60% of transporters were destroyed. The remaining labelled protein reappeared in plasma membranes and underwent a new disappearance cycle with a longer half-life period (34.65 ± 3.9 h). A similar pattern of internalization and reappearance of nucleoside transporters was observed in cells cross-linked with non-labelled NBTI, with a half value of reappearance of 33 h. Chromaffin cells cultured in the presence of the protein synthesis inhibitor, cycloheximide, had a component of disappearance for NBTI binding sites with a half-life value of 24.6 ± 1.4 h.

Introduction

The plasma membrane is a dynamic macromolecular assembly with possibilities to regulate the variety and the number of its membrane proteins. One of best known components is the non-concentrative glucose transporter, the presence of which can be modified in several ways at the plasma membrane: translocation from an intracellular pool, modulation by the effects of protein kinases and expression of several mRNA [1–5].

A similar membrane protein is the non-concentrative nucleoside transport which is expressed by all the studied mammalian cells [6]. Nevertheless, there has been no investigation so far of its membrane dynamics and turnover, in spite of the increasing importance of the nucleoside adenosine, as an extracellular signal [7]. In neural tissues extracellular adenosine seems to originate from the exocytotically released ATP, degraded by the action of ectonucleotidases to adenosine [8–10]. To recover the synaptic vesicle functionality, the adenosine transport, salvage, and storage as ATP in the recycled vesicles are necessary [11,12].

To study the dynamics of nucleoside transporters, chromaffin cells were selected as a neuronal model. These cells present the advantage of being largely employed as a neurosecretory model, where the membrane recycling of secretory granules is well known. The kinetic parameters for nucleoside transport are very similar in these cells, when compared with other neural preparations [6,11,13,14]. Furthermore, the majority of nucleoside transporters in chromaffin cells are very sensitive to inhibition by nitrobenzylthioinosine (NBTI). This homogeneous population of transporters with respect to this photoactivable ligand is of interest in undertaking the recycling studies [15].

The study of the nucleoside transporter metabolism in cultured chromaffin cells is the purpose of this experimental work. Direct approaches were made using covalently binding of radiolabelled nitrobenzylthioinosine. The disappearance of covalently bound radioactivity was studied, both from the whole cells and from plasma membranes, specificaly isolated on polycationic beads. Nucleoside transporter metabolism was

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measured indirectly with cells cultured in the presence of inhibitors of protein synthesis, the number of transporters measured as a function of time.

In this study we demonstrate for the first time that exposure of chromaffin cells to covalently bound nitrobenzylthioinosine, leads to down regulation of nucleoside transporters from plasma membrane, with a half-life close to 2 h. The half-life for transporters disappearance in presence of cycloheximide was 25 h.

Materials and Methods

Materials

Cycloheximide, cytosine arabinofuranoside, digitonin, nitrobenzylthioinosine and other purine bases and nucleosides were obtained from Sigma Chemical Co., MO; adenosine deaminase (EC 3.5.4.4) and collagenase (EC 3.4.24.3) from Boehringer Mannheim, culture media from Gibco, Percoll and cytodex from Pharmacia (Upsala, Sweden); the scintillation liquid Ready Safe from Beckman; [2,8-^{3}H]adenosine (22 Ci/mmol), *S*-adenosyl[*methyl*-^{14}C]methionine (50 Ci/mmol), [7-^{14}C]tyramine hydrochloride (60 Ci/mmol) from Amersham. [^{3}H]Nitrobenzylthioinosine (32 Ci/mmol) was obtained from New England Nuclear. All other reagents were obtained from Merck.

Isolation and culture of chromaffin cells

Chromaffin cells were prepared by the method of Miras-Portugal et al. [16] in a Ca^{2+} free Locke's solution (mM, NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, glucose 5.6, Hepes 5, (pH 7.4)) supplemented with collagenase (1 mg/ml) and 0.25% (w/v) albumine. After digestion, cells were purified through a Percoll gradient, carefully collected and washed with Locke's solution. Cells were then suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 50 μM cytosine arabinofuranoside and 10 μM 5'-fluoro-2-deoxyuridine. Cells were plated in 3.5 cm/diameter Petri dishes at a density of $(3–5) \cdot 10^6$ cells/dish. The incubation was at 37°C in 5% CO_2/95% air. Cells were employed during the first 6 days after plating. Controls for cellular viability were routinely done by Trypan blue exclusion and vesicular accumulation of neutral red dye, which is specifically taken up by monoaminergic cells [17].

Cultured chromaffin cells were then preincubated under the following conditions: (1) basal, cells were incubated (with no additions) in DMEM, and processed at different times; (2) cycloheximide; this compound (10 μg/ml) was added to culture medium and the cells were incubated for the experimental time required.

The digitonin permeabilization of chromaffin cells was carried out with $5 \cdot 10^6$ cells plated in 3.5 cm/diameter Petri dishes, according to the experimental procedure described by Dunn and Holz [18], with minor modifications. Briefly, the culture media was aspirated, the cells were washed three times with Ca^{2+}-free Locke's solution and then they were preincubated for 5 min at 37°C with Ca^{2+}-free permeabilizing medium containing: digitonin 20 μM; sodium glutamate 140 mM; piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Pipes) 20 mM; glucose 5 mM; $MgSO_4$ 5 mM; KCl 5 mM; and EGTA 5 mM; adjusted to pH 6.8. After that, the cells were washed twice with Ca^{2+}-free Locke's solution. The binding study was done as described in the next section, using 1 nM of [^{3}H]NBTI in Ca^{2+}-free Locke's solution.

Nitrobenzylthioinosine binding studies

The NBTI-binding to nucleoside transport sites in chromaffin cells were carried out as described by Torres et al. [14].

$(3–5) \cdot 10^6$ cultured cells in Petri dishes were incubated with 2 ml of Locke's solution, containing 1 U of adenosine deaminase and a graded concentration of [^{3}H]NBTI ranging from 0.01 to 10 nM for 30 min at 37°C, in order to reach the equilibrium; the medium was then aspirated and the cells washed twice with 5 ml of cold Locke's solution containing 10 μM non-labelled NBTI. 0.5 ml of trichloroacetic acid (10% w/v) was added and the cells scraped out of the dish and the radioactivity counted. In order to determine the non-specific binding, controls were done in the presence of 10 μM non-labelled nitrobenzylthioinosine and labelled compound in the same concentrations as in the assay.

Binding experiments with hypoosmotic lysed cells were carried out in the same conditions as above, but in the absence of 154 mM NaCl in the incubation medium. Once the equilibrium was reached, the cellular lysate was filtered on GF/C Whatman filters and washed twice with 5 ml of cold hypoosmotic buffer.

In experiments where the K_d value determination was not necessary, 1 nM of [^{3}H]NBTI was routinely used. This ligand concentration was twice the K_d value obtained for this compound by Scatchard analysis, and assures a high specific binding.

Photochemical cross-linking of cultured chromaffin cells with nitrobenzylthioinosine

Cultured chromaffin cells were incubated in Locke's solution, supplemented with 5 mM ascorbic acid, at 37°C with 25 nM of [^{3}H]NBTI or 25 nM of non-labelled NBTI. After 30 min of ligand–transporter interaction, the cells were then photochemically cross-linked by using a 450 watt mercury arc lamp at 6 cm distance from the cells for 2 min, according to the procedure of Gati et al. [19]. The remaining non-covalently bound ligand was eliminated by two cycles of washing with 5 ml of Locke's solution with 5-min intervals between

cycles to reach equilibrium. 5 ml of culture medium were added at the end of washing and the cells were ready to be processed at the required times. Controls for non-covalently bound ligand displacement were done in the presence of 25 nM [^{3}H]NBTI without irradiation. After the equilibrium was reached the remaining radioactivity was about 0.3% after the second cycle of washing. Trypan blue exclusion and neutral red dye accumulation were employed as probes of viability at the end of the photolabelling procedure.

The molecular type that covalently binds to NBTI after photolabelling was studied by using one aliquot of plasma membranes covalently cross-linked to [^{3}H]NBTI. Membranes were boiled for 5 min in Laemmli [20] sample buffer for solubilization, and then analysed by SDS-polyacrylamide gel electrophoresis as previously described by Torres et al. [14]. The acrylamide concentration in the resolving gel was 10%.

Migration distances of molecular-mass marker proteins (Pharmacia) were used to estimate the molecular weight of [^{3}H]NBTI-labelled proteins.

Radioactivity was measured in a liquid scintillation system after extraction of 2 mm slices of the gel lanes in a solution of 30% H_2O_2.

Isolation of chromaffin cell plasma membranes on polycationic beads

The original procedure described by Jacobson et al. [21] and modified by Van der Meulen et al. [22] was adapted to the small cellular preparations of cultured chromaffin cells, in order to recover the radioactivity covalently cross-linked to plasma membranes.

After photochemical cross-linking at the required times, the cultured chromaffin cells ($5 \cdot 10^6$ cells/dish) were processed as follows: the culture medium was aspirated from the dishes and 1 ml of sucrose-acetate buffer (0.3 M sucrose, 10 mM sodium acetate (pH 5.2)) containing 5 mg of cytodex, was gently added. The polycationic beads of cytodex had been hydrated and equilibrated previously with the sucrose-acetate buffer. The mixture was carefully stirred for 10 min and cells were detached from the culture dish and bound to the cytodex. The cationic charges of cytodex not bound to chromaffin cells were blocked with 5 mg of heparin sulfate added in 1 ml of sucrose-acetate buffer. The mixture was then transferred from Petri dishes to 10 ml tubes. The cytodex beads with the bound cells were sedimented and the supernatant aspirated. The cells were then lysed by hypotonic shock with 10 ml of 10 mM Tris–HCl (pH 7.4) and washed and sedimented three times with the same buffer. All procedures were carried out between 0 and 4°C.

Finally, the plasma membranes recovered on polycationic beads were transferred to counting vials and the radioactivity measured.

Marker-enzyme assays

Among the enzymes assayed, acetylcholinesterase (EC 3.1.1.7) activity was measured by the method of Ellman, according to the procedure of Low and Finean [23]. 5′-Nucleotidase (EC 3.1.3.5) activity was determined by the method of Aronson and Touster [24], dopamine-β-hydroxylase (EC 1.14.17.1) activity was assayed as described by Miras-Portugal et al. [25]. The more sensitive radiometric method of Goldstein et al. [26] modified by Muñoz et al. [27] was employed to quantify the presence of chromaffin granule membranes on the plasma membrane fraction which were recovered on polycationic beads. Monoamine oxidase (EC 1.4.3.4), used as a mitochondrial marker enzyme, was assayed by the method of Goridis and Neff [28]. Protein determination was carried out by the method of Bradford.

All values in the text are given as means ± S.D. Kinetic analysis of transporter turnover need not take into account cell growth, because chromaffin cells are non proliferating.

Results

Cycloheximide effects on NBTI-sensitive nucleoside transporters

Cultured chromaffin cells presented high-affinity binding sites for nitrobenzylthioinosine, the specific ligand for nucleoside transporters. In Fig. 1 a Scatchard representation is shown. The K_d value obtained was 0.54 ± 0.12 nM and the number of high-affinity binding

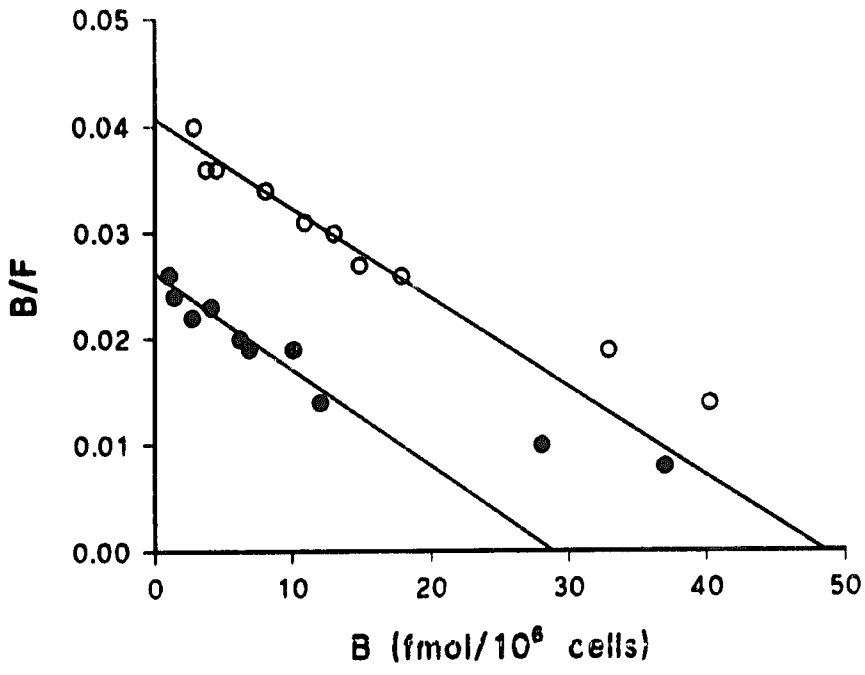


Fig. 1. Scatchard analysis of [^{3}H]NBTI binding to chromaffin cells. Effect of cycloheximide. Cultured chromaffin cells ($3 \cdot 10^6$ cells/dish) were incubated with variable concentrations of [^{3}H]NBTI in the presence or absence of 10 μM non-labelled NBTI as described in Methods. Specific binding of NBTI represents the difference between cell-associated [^{3}H]NBTI in the presence and absence of non labelled NBTI. The binding studies were made with control cells, in the absence of cycloheximide (○———○) and with cultured cells in the presence of cycloheximide (10 μg/ml) for 30 h (●———●).

sites was 48 ± 8 fmol/10^6 cells which corresponded to $32\,000 \pm 6\,000$ high-affinity binding sites per cell. As NBTI is a lipid soluble compound it was necessary to carry out experiments in order to know if the ligand was bound to the plasma membranes or to intracellular binding sites. The first approach was to measure the number of binding sites in hypoosmotic lysed cells. In this situation the accesibility of [^{3}H]NBTI to the totality of binding sites was permitted. The B_{max} value obtained was $115\,200 \pm 13\,000$ binding sites per lysed cell. The second approach was carried out with digitonin permabilized cells. This experimental situation allows the accessibility of the ligand to the cytosol, but the integrity of subcellular structures was maintained. The binding values for 1 nM [^{3}H]NBTI concentration were 35 ± 4 ($n = 8$) and 36 ± 5 ($n = 8$) fmol/10^6 cells for controls and permeabilized cells, respectively. These results pleaded for the almost exclusive measurement of plasma membrane binding sites in intact chromaffin cells.

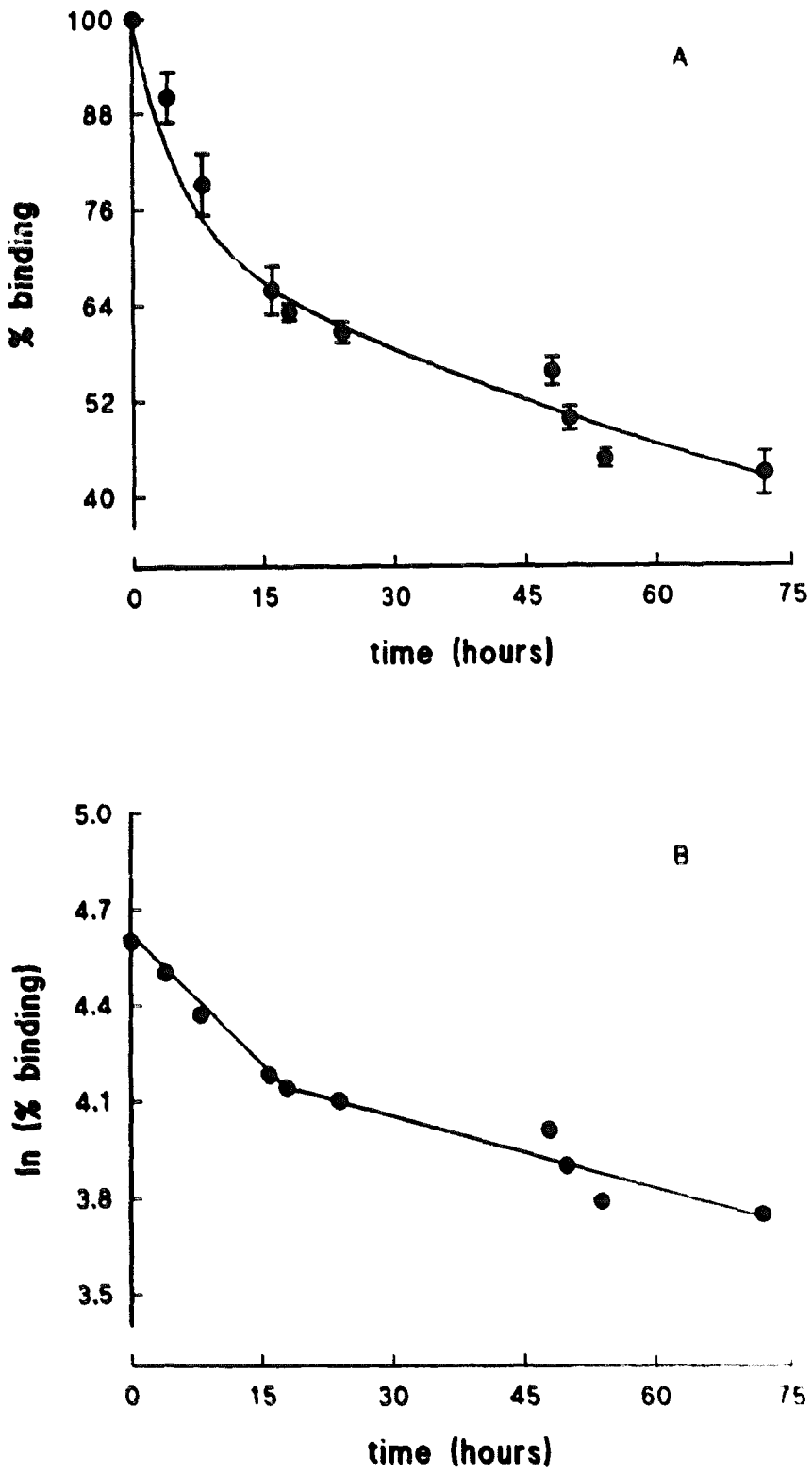


Fig. 2. Cyclohexamide effect on [^{3}H]NBTI binding to chromaffin cells as a function of time. Cultured chromaffin cells ($3 \cdot 10^6$ cells/dish) were preincubated in the presence of cyclohexamide (10 μg/ml). At required times the culture medium was aspirated and [^{3}H]NBTI binding was measured, as described in Methods. (A) [^{3}H]NBTI binding (as percentage with respect to control) at different preincubation times with cyclohexamide. Binding values were done with 1 nM of [^{3}H]NBTI. At this concentration the 100% of specific binding was 35 ± 4 fmol/10^6 cells (1600 ± 150 cpm/10^6 cells). Values are the means $\pm$ S.D. of three experiments in triplicate. No changes were observed in binding values for this concentration of [^{3}H]NBTI to cultured cells in absence of cyclohexamide. (B) Semilogarithmic plot from NBTI binding data from Fig. 2A as a function of preincubation time.

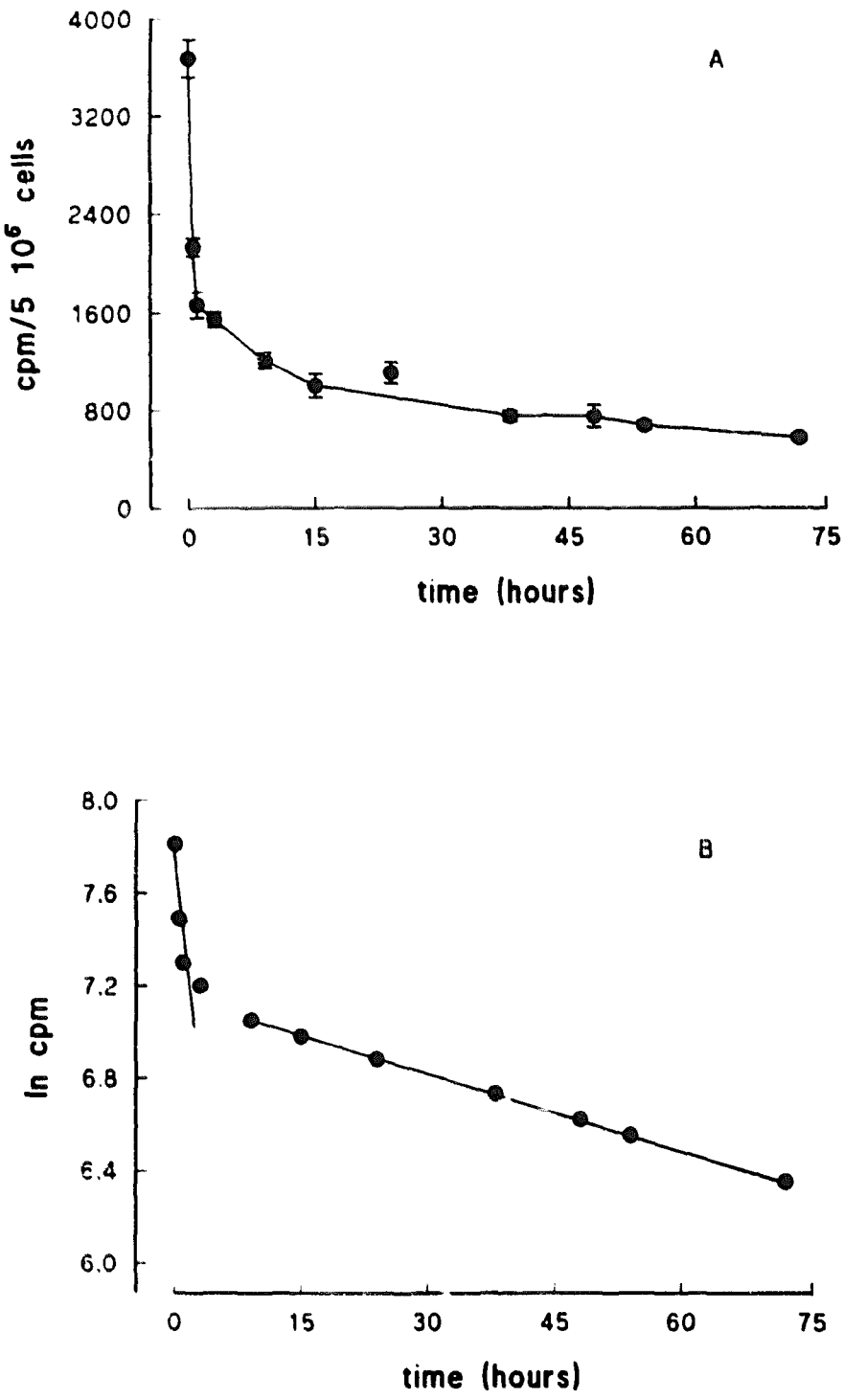


Fig. 3. Dynamics of covalently-bound [^{3}H]NBTI to whole chromaffin cells. (A) Cultured chromaffin cells ($5 \cdot 10^6$ cells/dish) were photostimulated in the presence of [^{3}H]NBTI 25 nM as described in Methods. At required times the culture medium was removed and cells were washed twice with 5 ml Locke's solution and then scraped out of the plastic, and the radioactivity counted. Values are the means $\pm$ S.D. of three experiments in triplicate. (B) Semilogarithmic plot of covalently-bound NBTI to chromaffin cells, as a time function after photostimulation. Values are from Fig. 3A.

To study the half-life of the nucleoside transporters the protein synthesis was inhibited and the [^{3}H]NBTI binding was measured as a function of time. The protein synthesis inhibitor, cycloheximide, added to the culture medium (10 μg/ml) caused a significant decrease of NBTI binding sites (Fig. 1). After 30 h the B_{max} was 27 ± 3 fmol/10^6 cells ($18\,000 \pm 2\,000$ sites/cell). No change was observed at the K_d value in cells cultured in the presence of cycloheximide.

In Fig. 2A the effect of protein synthesis inhibition on the transporter number is represented as a function of time. The remaining nucleoside transporters were measured with 1 nM [^{3}H]NBTI, which corresponded approximately to twice the K_d value for this ligand. The exponential representation from Fig. 2A can be linearized as shown in Fig. 2B. Two velocity constants for the disappearance of nucleoside transporters can be deduced with half-lives of 24.6 ± 1.4 ($n = 3$) and 121.5 ± 15.6 h ($n = 3$). In the absence of cycloheximide, the number of NBTI binding sites was not modified in cultured chromaffin cells throughout the same experimental time. The decrese in transport capacity in the presence of cycloheximide correlates well with the decrease in the number of binding sites. The half-life obtained from these experiments was 24.3 ± 2.1 h [29].

Changes in radioactivity after photolabelling of chromaffin cells with [^{3}H]NBTI

Direct studies of the disappearance of the nucleoside transporters can be accomplished by photolabelling of chromaffin cells with tritiated nitrobenzylthioinosine, and then by measuring the radioactivity covalently bound as a function of time. This experimental approach allows a direct measurement of the transporters' half-life in the whole cell, or specifically at the plasma membranes.

Cultured chromaffin cells were photostimulated in the presence of 25 nM of [^{3}H]NBTI, as described in Methods. In our experimental conditions the covalently-bound ligand reached 44–50% of the maximal specific binding values obtained without UV light stimulation (Fig. 1).

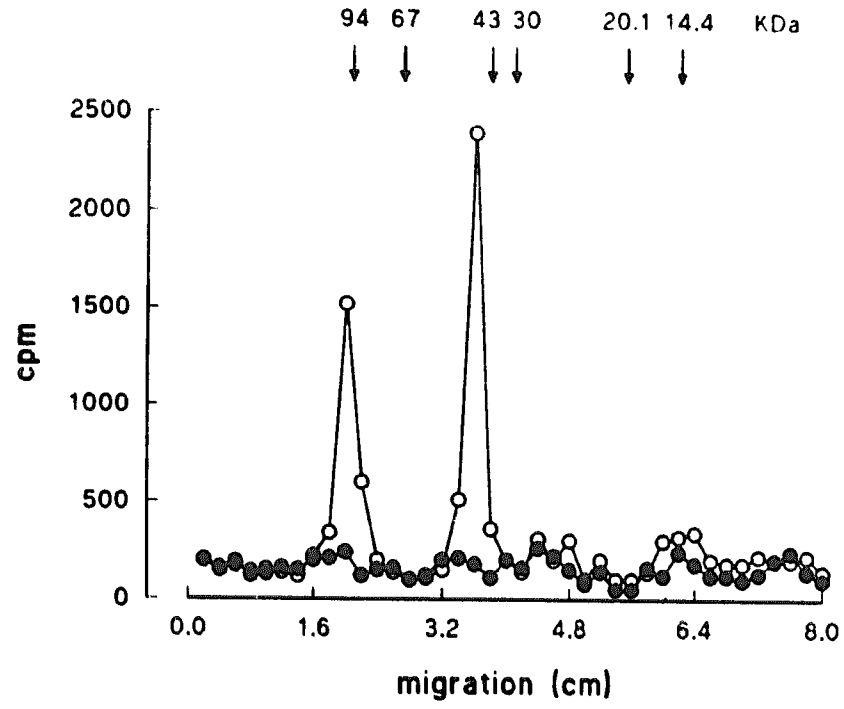


Fig. 4. SDS-polyacrylamide gel electrophoresis radioactivity profiles of chromaffin cell plasma membranes covalently labelled with [^{3}H]NBTI. Plasma membranes were photostimulated in the presence (●) or absence (○) of 10 μM nonlabelled NBTI and 25 nM [^{3}H]NBTI in both. This figure is representative of a typical and very reproducible experiment. The electrophoresis was carried out as described in Methods with 10% polyacrylamide concentration in the resolving gel.

In Fig. 3A the covalently bound NBTI to the whole cell preparation is shown as a function of time. Two components can be observed in the radioactivity decrease. The first sharp decrease could reflect the destruction of transporters after a fast recycling of membrane components. Not all the transporters with [^{3}H]NBTI covalently bound were destroyed, and the remaining, representing about 40–50% of the total radioactivity covalently bound, underwent a slow decrease. This second component could indicate the renewal of nucleoside transporters in the whole cells.

TABLE I

Specific activities of plasma membrane markers isolated on cytodex beads from cultured chromaffin cells

$25 \cdot 10^6$ cultured chromaffin cells were processed to study the parameters showed in the first column. (1) Specific enzyme activities are given in nmol of substrate converted per min per mg of protein. (2) Covalently bound [^{3}H]nitrobenzylthioinosine, after photolabelling treatment, as described in Experimental procedures are given in cpm per mg of protein. The reported values are given as means ± S.D. of (n) determinations done in triplicate.

(1) Enzymes and (2) Transporters	Cultured cells homogenate	Plasma membranes	Ratio	Recovery percentage	(n)
5'-Nucleotidase	2.3 ± 0.3	22.5 ± 2.3	9.8	30.15	(3)
Acetylcholinesterase	17.1 ± 0.6	173.7 ± 15.8	10.16	31.24	(3)
Monoamine oxidase	1.7 ± 0.1	0.1 ± 0.02	0.06	0.18	(3)
Dopamine-β-hydroxylase	31.5 ± 4.0	1.3 ± 0.3	0.04	0.13	(3)
Covalently bound [^{3}H]NBTI	7059 ± 830	74063 ± 2300	10.49	32.28	(5)
Proteins (mg per $25 \cdot 10^6$ cultured cells)	2.6 ± 0.3	0.08 ± 0.01	0.03	3.10	(5)

The half-life values obtained from Fig. 3B were 2.2 ± 0.74 h for the fast component and 61.14 ± 4.26 h for the slow component.

The molecular species of protein that covalently bind [^{3}H]NBTI were studied. In Fig. 4 the electrophoretic pattern after 1 h of photostimulation is shown. No significant changes in the radioactivity profile distribution were observed at longer experimental time period. Two main molecular species were observed. The molecular weights obtained were 51 000 and 104 000, probably corresponding to the monomeric and the dimeric forms [6,14,19].

Changes in radioactivity at plasma membrane level after photolabelling with [^{3}H]nitrobenzylthioinosine as a function of time

The photolabelling technique with [^{3}H]NBTI also allowed the study of nucleoside transporters at the plasma membrane level by combination with specific isolation of plasma membranes on polycationics beads.

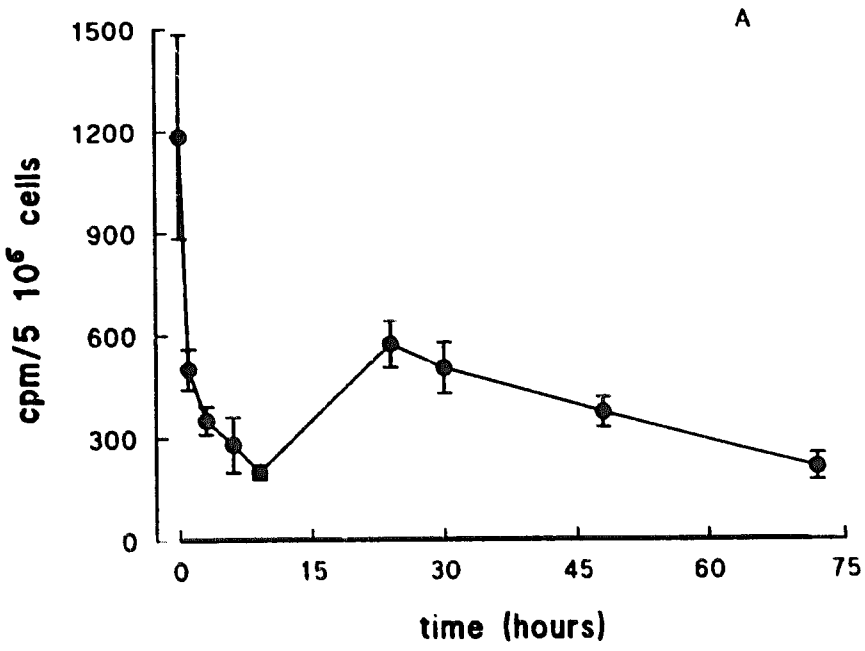
The plasma membranes isolated by this procedure presented good specific activities of marker enzymes, such as acetylcholinesterase and 5′-nucleotidase. The low specific activities of dopamine-β-hydroxylase and monoamine oxidase indicated a poor contamination by chromaffin granule membranes and mitochondria, respectively (Table I).

Nucleoside transporters, measured by binding with [^{3}H]NBTI showed a similar purification ratio and percentage recovery on polycationics beads as the enzyme markers of plasma membranes, which, in our experimental conditions, was 30% (Table I).

These results support the polycationic beads technique as a useful tool to specifically study the plasma membrane dynamics at short and long time periods.

Cultured chromaffin cells photostimulated in the presence of 25 nM [^{3}H]NBTI showed different covalently bound radioactivity at their plasma membranes, according to the time elapsed between the photolabelling procedure and the membrane isolation on polycationic beads.

In Fig. 5A, the changes in radioactivity at the plasma membranes are represented as a function of time. It is necessary to take into account that the recovery of plasma membranes on polycationic beads from cultured cells is close to 30%. This important observation allows comparison between Figs. 3A and 5A. A quick internalisation of [^{3}H]NBTI covalently bound to plasma membranes was observed. This entrance reached 80–85% of the total bound radioactivity. Afterwards, the covalently bound [^{3}H]NBTI returned to the plasma membranes but in a lower percentage, usually 40–45% of the initial values. The recovered radioactivity at the plasma membranes then underwent a progressive decrease. The semilogarithmic plot for the fast and slow component of radioactivity lost at the plasma membranes gave two velocity constants with half-lives of 2.31 ± 0.61 h and 34.65 ± 3.9 h (Fig. 5B). The quick internalisation corresponded to a significant decrease in the total radioactivity measured in the whole cells (Fig. 3B). Thus processing and eventual destruction tookplace. Nevertheless, not all the nucleoside transporters with the ligand covalently bound were destroyed, and significant amounts returned to plasma membranes, where they entered a new dynamic step with a longer half-life.



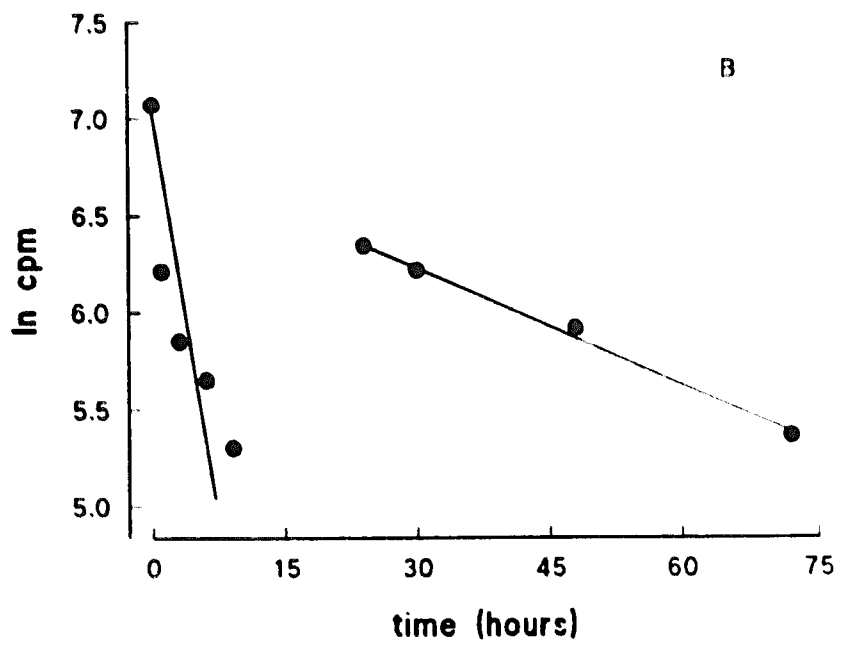


Fig. 5. Dynamics of covalently bound [^{3}H]NBTI to plasma membranes of chromaffin cells. (A) Cultured chromaffin cells ($5 \cdot 10^6$ cells/dish) were photostimulated in the presence of 25 nM [^{3}H]NBTI (32 Ci/mmol) as described in Methods. The plasma membranes were obtained on polycationic beads at the indicated times, and the radioactivity measured. This value is expressed as cpm/$5 \cdot 10^6$ cells as a time function. Values are the means ± S.D. of three experiments in triplicate. (B) Semilogarithmic plot of covalently-bound NBTI to plasma membranes of chromaffin cells from Fig. 5A as a time function after photostimulation. The recovery of plasma membranes on polycationic beads is about 30% according to the values reported in Table I.

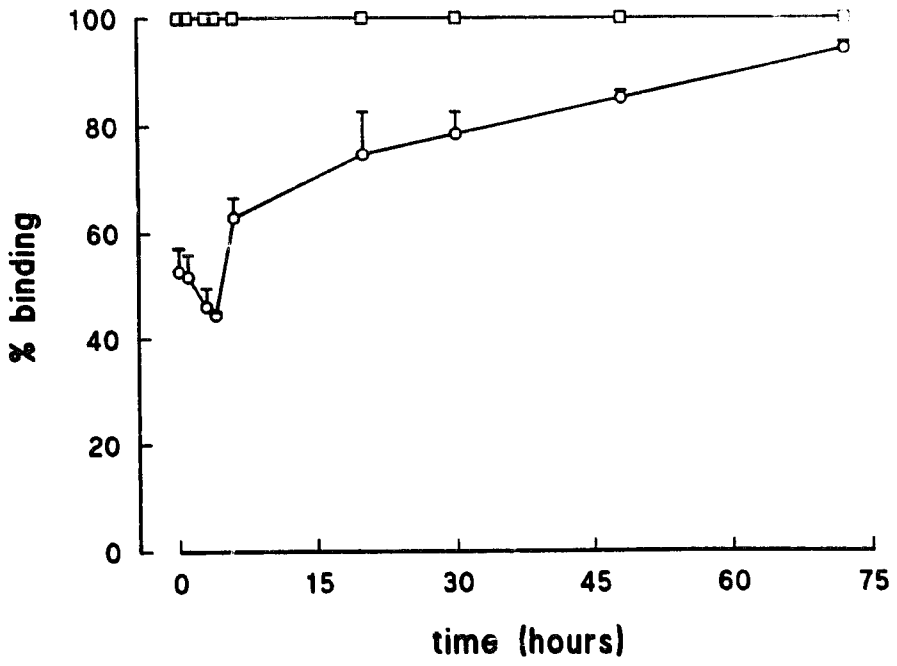


Fig. 6. Recovery of nucleoside transporter binding sites after photostimulation with non-labelled NBTI. Cultured chromaffin cells ($3 \cdot 10^6$ cells/dish) were photostimulated in the presence (○——○) or absence (□——□) of 25 nM non-labelled NBTI, as described in Methods. Cells were cultured until the required times. [^{3}H]NBTI binding was done at 1 nM ligand concentration and represented as percentage of control binding values of cells before cross-linking with non-labelled NBTI. The binding value for control was $35 + 4$ fmol/10^6 cells (1600 ± 150 cpm/10^6 cells).

Recovery of [^{3}H]NBTI binding in chromaffin cells after cross-linking with the nonlabelled ligand

The [^{3}H]NBTI covalently bound to the nucleoside transporter was a useful tool to study the dynamics of the modified protein at the plasma membrane. Furthermore, this compound can be employed to study the dynamics of the non-modified transporters. This other experimental approach is possible by cross-linking the nucleoside transporters with non-labelled NBTI. The reappearance of new functional transporters able to bind [^{3}H]NBTI is then measured as a function of time. In this case, only the non-modified protein is measured.

Cultured chromaffin cells were photochemically cross-linked with 25 nM of non-labelled NBTI, as described in Methods. As shown in Fig. 6, the efficiency of this cross-linking was 48% of the total nucleoside transporters present at the plasma membrane of the cell at the starting time. It is interesting to point out that the decrease in binding sites continued during the first hours of the experiment. This result could only be explained if some non-covalently bound transporters were internalized during the first step, as occurs with the [^{3}H]NBTI-labelled protein (Fig. 5A). The lowest level of nucleoside transporters at the plasma membrane was reached between three and four hours from the beginning, the minimal value reached was 43% of the control.

The reappearance of transporters presented two components. The first sharp recovery reached 63% of the control. The incorporation of these new transporters to the plasma membrane could be explained by a preexisting intracellular pool, or the recycling of non covalently cross-linked transporters previously internalized.

The slow recovery component showed a half-value for the appearance of new nucleoside transporters of 33 h.

To exclude the actions of UV light radiation on chromaffin cells a cellular control was made. In our experimental conditions there was no effect and no changes were observed at the transporter level in the plasma membranes after UV light stimulation in the absence of the specific ligand (Fig. 6).

Discussion

The major goal in initiating these studies was to gain further insight into the metabolism and recycling of nucleoside transporters. Cultured chromaffin cells were chosen as the cellular model because they are homogeneous neural cells and their nucleoside transporter is highly sensitive to nitrobenzylthioinosine inhibition [15]. The number of binding sites for [^{3}H]NBTI (B_{max}) was 32 000 per cell. Due to the hydrophobicity of the ligand, the question is to know where they are localized. The experiments reported here showed that [^{3}H]NBTI bound only to the plasma membranes, when the subcellular integrity was maintained. This statement was based on: (a) The non-modification in binding values in digitonin permeabilized cells. (b) The increase (3–4-times) in the binding values in hypoosmotic lysed cells. It is necessary to take into consideration that subcellular organellas have an acidic pH (close to 5) and the extracellular side of transporters at the plasma membranes, is at the inside of the intracellular vesicles. Once the localization of binding sites for [^{3}H]NBTI was established, this compound was employed in two ways: first, as a ligand to measure the number of binding sites; second as an irreversible blocking agent that covalently binds to nucleoside transporters after photochemical activation [14,19]. The change in radioactivity, after photostimulation of chromaffin cells in the presence of [^{3}H]NBTI, was a direct approach to nucleoside transporter metabolism.

The disappearance and reappearance of radioactively labelled nucleoside transporters can be specifically measured by isolation of plasma membranes on polycationic beads [22,30]. With this technique, the amplitude of the first internalization step was quantified. This value was 80–85% of the total cross-linked nucleoside transporters present at the plasma membrane. The endocytosed transporters (half-life = 2.31 ± 0.61 h) were recycled to an extent of 40–50% compared with the values at the starting time. The rate and extent of nucleoside transporters recycling following internalization was lower when compared to the recycling of photoaffinity-labelled insulin receptors in rat

adipocytes at the same temperature [31], but it was very similar to that found for some adrenergic receptors down-regulated in the presence of specific agonists [32–36]. As in the case of other photoaffinity-labelled receptors, the chemically modified protein was not completely destroyed in this first step of internalization and reappearance to the cell surface [31].

The down-regulation of transporters was also observed using an indirect method, by which, the nucleoside transporters were covalently cross-linked with non-labelled ligand and the remaining transporters were measured. In this situation the ligand induced the internalization of transporters. These results, altogether, seem to confirm the down-regulation step for nucleoside transporters in the presence of a high-affinity ligand. No bibliographical data have yet shown that this event occurs with other cellular transporters. Even in the case of the glucose transporter, the appearance of this protein at the plasma membrane is triggered by insulin through membrane receptors or the transport capacity modulated by protein kinases [5].

The second step of metabolism and recycling of nucleoside transporters presented variable half-life values, according to the methodological approach; and seems to be a complex event due to the peculiar characteristics of chromaffin cell which contains a huge number of cytoplasmic granules. Direct approaches with [^{3}H]NBTI gave a half-life of 34 h for the disappearance of the nucleoside transporters, specifically at plasma membrane. This value was very similar to the 33 h necessary to recover half of binding sites for NBTI after cross-linking with the non-labelled compound.

Previous to stimating of the half-life of nucleoside transporters in the presence of cycloheximide, the effect of this compound on NBTI binding parametters was studied (Fig. 1). The K_d values were the same as controls, but a significant, decrease of high-affinity binding sites in the presence of cycloheximide was found. The *B/F* deviation observed at the highest NBTI concentrations could be explained by the interaction of this ligand with glucose transporters. In this regard it has been shown that NBTI affinity for glucose transporters is 10^3–10^4-times lower than that for the adenosine transporters [37,38]. In chromaffin cells the presence of glucose transporters has been reported to be 15-times more abundant than nucleoside transporters [39].

The kinetics of nucleoside transporters metabolism in the presence of the protein synthesis inhibitor, cycloheximide, clearly showed two components. The first with a half-life of 24 h was in the same order as that obtained for plasma membranes. Similar values are described in the literature for half-life of receptors in cultured cells and in basal conditions [32–34,37]. The same half-life value was obtained for adenosine transport in the presence of cycloheximide [29]. Thus, functionality and binding sites had similar behaviour. Not all the nucleoside transporters were destroyed at this rate and about 60% presented a longer half-life (> 100 h). This slow component in cycloheximide cultured cells could be related either to a loss of the degradative cellular machinery or to the peculiar subcellular structure of chromaffin cells [14,40]. In the absence of cycloheximide, with [^{3}H]NBTI labelled cells, the slow component gave a half-life of 60 h. This value could reflect the nucleoside transporters metabolism of the whole cell, including the granular pool, and not only that of plasma membranes [41–43].

The presence of a specific ligand for nucleoside transporters (NBTI) was essential to start the down-regulation step. This process is reported here for the first time. This event needs some additional comments and raises the question of how they have been modified to be specifically internalized. It is well known that plasma membrane receptors undergo down-regulation by binding to an agonist that can induce an allosteric modification at the cytoplasmic side of this protein and then be chemically modified by phosphorylation [44–48]. Additional information reported that the activation of protein kinase A inhibit the nucleoside transport [49]. The same results, but to a lower extent, were obtained with activators of PKC or extracellular signals going through the Ca^{2+}/phosphatidyl inositol pathway [50,51].

Further work is necessary to understand the effects of relevant physiological situations, mediated by the action of secretagogues or other extracellular signals, on the dynamics of the nucleoside transporter, in neural and non neural tissues.

Acknowledgements

This work was supported by grant PS 89-0095 from the Spanish Comisión Interministerial de Ciencia y Tecnología, Ministery of Education and Science of Spain and a grant from the Rectorado de la Universidad Complutense. Thanks are also extended to Erik Lundin for his help in the preparation of this manuscript.

References

1 Berger, J., Biswas, C., Vicario, P.P., Strout, H.V., Saperstein, R. and Pilch, P.F. (1989) Nature 340, 70–74.
2 Gould, G.W. and Bell, G.I. (1990) Trends Biochem. Sci 15, 18–23.
3 Kitagawa, T., Tanaka, M. and Akamatsu, Y. (1989) Biochim. Biophys. Acta 980, 100–108.
4 Simpson, I.A. and Cushman, S.W. (1986). Annu. Rev. Biochem. 55, 1059–1089.
5 Witters, L.A., Vater, C.A. and Lienhard, G.E. (1985) Nature 315, 777–778.
6 Plagemann, P.G.W., Wohlhueter, R.M. and Woffendin, C. (1988) Biochim. Biophys. Acta 947, 405–143.

7 Williams, M. (1987) Annu. Rev. Pharmacol. Toxicol. 27, 315–345.
8 Grondal, E.J.M. and Zimmermann, H. (1988) in Cellular and Molecular Basis of Synaptic Transmission (Zimmermann, H., ed.), NATO ASI Ser. H 21, 395–410.
9 Richardson, P.J., Brown, S.J., Bailyes, E.M. and Luzio, J.P. (1987) Nature 327, 232–234.
10 Torres, M., Pintor, J. and Miras-Portugal, M.T. (1990) Arch. Biochem. Biophys. 279, 37–44.
11 Miras-Portugal, M.T., Torres, M., Rotllán, P. and Aunis, D. (1986) J. Biol. Chem. 261, 1712–1719.
12 Winkler, H. (1988) in Handbook of Experimental Pharmacology (Tredelenburg, U. and Weiner, N., eds.), Vol. 90/I, pp. 43–118 Springer-Verlag, Berlin.
13 Lee, C.W. and Jarvis, S.M. (1988) Biochem. J. 249, 557–564.
14 Torres, M., Delicado, E.G. and Miras-Portugal, M.T. (1988) Biochim. Biophys. Acta 969, 111–120.
15 Torres, M., Fideu, M.D. and Miras-Portugal, M.T. (1990) Neurosci. Lett. 112, 343–347.
16 Miras-Portugal, M.T., Rotllan, P. and Aunis, D. (1985) Neurochem. Int. 7, 89–93.
17 Role, L.W. and Perlman, R.L. (1980) J. Neurosci. Methods 2, 253–265.
18 Dunn, L.A. and Holz R.W. (1983) J. Biol. Chem. 258, 4989–4993.
19 Gati, W.P., Belt, J.A., Jakobs, E.S., Young, J.D., Jarvis, S.M. and Paterson, A.R.P. (1986) Biochem. J. 236, 665–670.
20 Laemmli, U.K. (1970) Nature 227, 680–685.
21 Jacobson, B.S. and Branton, D. (1977) Science 195, 302–304.
22 Van der Meulen, J.A., Emerson, D.M. and Grinstein, S. (1981) Biochim. Biophys. Acta 643, 601–615.
23 Low, M.G. and Finean, J.B. (1977) FEBS Lett. 82, 143–147.
24 Aronson, N.N. and Touster, O. (1974) in Methods in Enzymology, Vol. 31, Biomembranes, Part A (Fleisher, S. and Packer, L., eds.), pp. 90–91, Academic Press, New York.
25 Miras-Portugal, M.T., Aunis, D. and Mandel, P. (1973) FEBS Lett. 34, 140–142.
26 Goldstein, M., Freedman, L.S. and Bonnay, M. (1971) Experientia 27, 632–633.
27 Muñoz, A., Serrano, C., García-Estañ, J., Quesada, T. and Miras-Portugal, M.T. (1984) Diabetes, USA 33, 1127–1132.
28 Goridis, C. and Neff, N.H. (1971) J. Neurochem. 18, 1673–1682.
29 Fideu, M.D. and Miras-Portugal M.T. (1991) 4th Protuguese-Spanish Biochemistry Congress, Povoa de Varzim (Portugal), Abstr. 32.
30 DeGrella, R.F. and Simoni, R.D. (1982) J. Biol. Chem. 257, 14256–14262.
31 Hueksteadt, T., Olefsky, J.M., Brandenberg, D. and Heidenreich, K.A. (1986) J. Biol. Chem. 261, 8655–8659.
32 Homburger, V., Pantaloni, C., Lucas, M., Gozlan, H. and Bockaert, J. (1984) J. Cell. Physiol. 121, 589–597.
33 Hughes, R.J. and Insel, P.A. (1986) Mol. Pharmacol. 29, 521–530.
34 Mahan, L.C. and Insel, P.A. (1986) Mol. Pharmacol. 29, 7–15.
35 Van Echten, G., Eckel, J. and Reinauer, H. (1986) Biochim. Biophys. Acta 886, 468–473.
36 Zaremba, T.G. and Fishman, P.H. (1984) Mol. Pharmacol. 26, 206–213.
37 Jarvis, S.M., Young, J.D., Wu, J.S.R., Belt, J.A. and Paterson A.R.P (1986) J. Biol. Chem. 261, 11077–11085.
38 Jhun, B.H., Rampal, A.L., Berenski, C.J. and Jung, C.Y. (1990) Biochim. Biophys. Acta 1028, 251–260.
39 Delicado, E.G. and Miras-Portugal, M.T. (1987) Biochem. J. 243, 541–547.
40 Mahan, L.C., McKernan, R.M. and Insel, P.A. (1987) Annu. Rev. Pharmacol. Toxicol. 27, 215–235.
41 De Camilli, P. and Jahn, R. (1990) Annu. Rev. Physiol. 52, 625–645.
42 Patzak, A. and Winkler, H. (1986) J. Cell. Biol. 102, 510–515.
43 Phillips, J.H. and Pryde, J.G. (1987) Ann. NY. Acad. Sci. 493, 27–40.
44 Findlay, D.M., Michelangeli, V.P. and Robinson, P.J. (1989) Endocrinology 125, 2656–2663.
45 Hansdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G. and Lefkowitz, R.T. (1989) J. Biol. Chem. 264, 12657–12665.
46 Mayor, F., Benovic, L., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 6468–6471.
47 Pearce, B., Morrow, C. and Murphy, S. (1988) J. Neurochem. 50, 936–944.
48 Sibley, D.R. and Lefkowitz, R.J. (1985) Nature 317, 124–129.
49 Sen, R.P., Delicado, E.G. and Miras-Portugal, M.T. (1990) Neurochem. Int. 17, 523–528.
50 Miras-Portugal, M.T., Sen, R.P. and Delicado, E.G. (1991) Nucleosides Nucleotides 10, 965–973.
51 Delicado, E.G., Sen, R.P. and Miras-Portugal, M.T. (1991) Biochem. J. 279, 651–655.