Flow cytometric studies of nucleoside transport regulation in single chromaffin cells

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Abstract **The present paper reveals that a fluorescent derivative of nitrobenzylthioinosine, 5-(SAENTA-x8)-fluorescein, is a highly specific inhibitor of the neural NBTI-sensitive nucleoside transporter. 5-(SAENTA-x8)-fluorescein inhibited adenosine transport and [³H]NBTI binding with a K_i of 4 nM in cultured chromaffin cells. Flow cytometry demonstrated that 5-(SAENTA-x8)-fluorescein specifically interacted with the NBTI-sensitive nucleoside transporters with high affinity ($K_D = 6$ nM). Activation of protein kinases A and C with forskolin or nicotinic receptor agonists, respectively, resulted in 50% inhibition of the fluorescence bound to the cells. Flow cytometry will allow studying nucleoside transport in single cells from heterogeneous neural cell populations.**
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Key words: Nucleoside transport; Nitrobenzylthioinosine; Flow cytometry; Chromaffin cell

1. Introduction

The equilibrative nucleoside transporters widely distributed in mammalian tissues are responsible for the internalization of nucleosides, preferentially adenosine, finishing the extracellular actions [1,2]. The quantifying and localization of nucleoside transporters as other plasma membrane proteins (transporters or receptors) have been restricted by the use of binding techniques with radiolabelled specific ligands, such as [³H]dipyridamol and [³H]nitrobenzylthioinosine (NBTI), which require a large number of cells [3–5]. This is not the case for the nucleoside transporters that have been cloned, and specific molecular probes are available [6]. An interesting development in the field of equilibrative nucleoside transporters has been the synthesis of fluorescent ligands. These compounds result from the coupling of a fluorescein molecule to the amino group of 5′-S-(2-aminoethyl)-N^6-(4-nitrobenzyl)-thioadenosine (SAENTA) with different spacer arms [7,8]. The fluorescent properties of these compounds make them suitable for use in flow cytometric techniques that, to date, have not yet been applied to the study of the membrane transporters. Flow cytometric studies performed in leukemia cells and other tumoral models revealed that both 5-(SAENTA-x2)-fluorescein and 5-(SAENTA-x8)-fluorescein specifically interact with the NBTI-sensitive equilibrative nucleoside transporter (*es*-nucleoside transporter) [8,9].

In previous works it has been demonstrated that the *es*-nucleoside transporters present in neurochromaffin cells and other neural models are regulated by several mechanisms [10]. In this connection, a long-term regulation by thyroid hormones has been demonstrated in rat brain synaptosomes and in neurochromaffin cells [11,12]. In cultured chromaffin cells, the nucleoside transport is also modulated by extracellular signals coupled to the activation of protein kinases A and C, involving phosphorylation-dephosphorylation processes [13,14]. In the present work it has been demonstrated that the *es*-nucleoside transporters of neurochromaffin cells recognized 5-(SAENTA-x8)-fluorescein with high specificity. Moreover, the flow cytometric studies that distinguish different cell types showed that the nucleoside transport inhibition produced by secretagogues or protein kinase A activation also modified the 5-(SAENTA-x8)-fluorescein specific binding to individual chromaffin cells. This finding allows new approaches to the study of nucleoside transporters in heterogeneous populations of neural tissues, which so far have not been available because of the low number and heterogeneity of the cells obtained.

2. Materials and methods

2.1. Chromaffin cell culture

Primary dissociated adrenomedullary cells were obtained by collagenase digestion of bovine adrenal glands and the dissociated cells were then purified onto an urografin density gradient [15,16]. The purity of the cell cultures was routinely assayed by neutral red dye staining [17] and about 95–99% of them contained catecholamine cells. Finally, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum containing 10 μM cytosine arabinofuranoside, 10 μM fluorodeoxyuridine and antibiotics (penicillin 5 U/ml, streptomycin 5 mg/ml, kanamycin 100 μg/ml and amphotericin 2.5 μg/ml). The cells were plated in 24-well Costar dishes pretreated with collagen in order to make the cells more adherent and maintained at 37°C in a 5% CO_2/95% air humidified atmosphere.

The medium was routinely removed and replaced by Locke's solution (composition in mM: NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.01, HEPES 10 and glucose 5.5; pH 7.4) 1–2 h before the experiments were carried out.

For flow cytometry experiments the cells were kept in suspension at 4°C and used the following day.

2.2. Adenosine transport experiments

Cells plated at a density of 250 000 cells/well were washed in Locke's solution for 2 h and [³H]adenosine transport was measured during the first minute of incubation at 37°C as previously described [13].

For inhibition studies of adenosine transport, the cells were first preincubated for 10 min at 37°C with graded concentrations of 5-(SAENTA-x8)-fluorescein (0.1–100 nM) and [³H]adenosine transport was then measured in the presence of this compound.

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2.3. [³H]Nitrobenzylthioinosine binding experiments and its displacement by 5-(SAENTA-x8)-fluorescein

For binding studies the cultured chromaffin cells were plated at a density of $800\,000$–10^6 cells/well and washed with Locke's solution for 2 h prior to the binding experiments. The maximum number of [³H]NBTI binding sites per cell was determined by equilibrium binding analysis as previously described [14]. For displacement studies the cells were incubated for 20 min at 37°C with the required concentration of [³H]NBTI in the presence of graded concentrations of 5-(SAENTA-x8)-fluorescein.

In another set of experiments, [³H]NBTI equilibrium binding was carried out with chromaffin cells maintained in suspension, in order to quantify the number of [³H]NBTI binding sites in the same conditions as the flow cytometry binding studies. The cell suspension was centrifuged and washed, and the cells were finally resuspended in Locke's solution at a density of 2×10^6 cells/200 μl. The [³H]NBTI binding was initiated by the addition of 200 μl of a graded concentration of labeled ligand and incubated for 30 min at room temperature. After the incubation time, 100 μl of ice cold 50 μM NBTI in Locke's solution was added to each tube. The cells were then centrifuged at 15 000 rpm for 10 min. The supernatant was aspirated, the cell pellets were dissolved and the radioactivity counted.

The non-specific binding was calculated by the preincubation of the cells with 10 μM unlabeled NBTI for 10 min and by quantifying the [³H]NBTI binding sites in the presence of the unlabeled compound. This component was routinely subtracted from the total to obtain the specific binding.

The binding experiments were routinely carried out in the presence of adenosine deaminase (1 U/ml), to check the presence of extracellular adenosine released from the cells.

2.4. Flow cytometry binding studies

The cell suspension was centrifuged, washed twice in Locke's solution and finally resuspended at a density of 10^6 cells/ml. Chromaffin cells (10^6 cells) were incubated with a range of 5-(SAENTA-x8)-fluorescein concentrations between 0 and 100 nM at room temperature (25°C) for 10 min and the binding of the ligand was analyzed by flow cytometry.

Flow cytometry analysis was carried out using a FACScan flow cytometer (Becton-Dickinson, San José, CA). 10 000 cells were acquired per sample and fluorescence from 5-(SAENTA-x8)-fluorescein was measured as molecules of equivalent soluble fluorochromes (MESF), after analysis of fluorescence mean with *Quantum* 24 (FCS, Leiden) and QuickCal software (FCS, Leiden) following the manufacturer's instructions. Briefly, *Quantum* 24 is a kit that contains a set of calibrated fluorescent standards matching the emission and excitation spectra of 5-(SAENTA-x8)-fluorescein. It is used in the quantification of fluorescence intensities between 2000 and 50 000 MESF. In order to quantify fluorescence using a flow cytometer, the peak or mean channel must be expressed as relative channel number (RCN). The QuickCal program performs that conversion and linear regression to construct calibration plots.

During the cytometric analysis, propidium iodide was used (0.005%) in PBS medium in all samples to discriminate dead cells and neutral red staining to identify chromaffin cells.

In order to determine the specific binding of the fluorescent ligand to the nucleoside transporter, the cells were preincubated for 10 min with 10 μM of NBTI or dilazep, previous to the incubations with different concentrations of 5-(SAENTA-x8)-fluorescein.

In another set of experiments the cells were stimulated with several effectors previous to the incubations with the fluorescent ligand.

2.5. Data analysis

Most experiments were performed in quadruplicate. Values are means ± S.D. of n experiments.

3. Results

3.1. Effect of 5-(SAENTA-x8)-fluorescein on nucleoside transporter

The adenosine transport in chromaffin cells was efficiently inhibited by 5-(SAENTA-x8)-fluorescein as shown in Fig. 1A. The specific transport was completely abolished at 100 nM
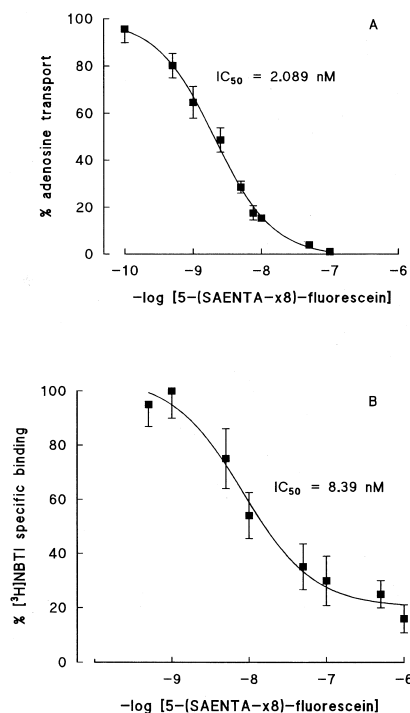

Fig. 1. A: Dose-dependent inhibition of adenosine transport by 5-(SAENTA-x8)-fluorescein in cultured chromaffin cells. Chromaffin cells were preincubated for 10 min at 37°C in the presence of graded concentrations of 5-(SAENTA-x8)-fluorescein (0–100 nM) and then adenosine transport was measured at 1 μM [³H]adenosine as described in Section 2. B: Inhibition of [³H]NBTI binding by increasing concentrations of 5-(SAENTA-x8)-fluorescein in cultured chromaffin cells. [³H]NBTI binding was measured for 20 min at 37°C by incubating the cells in the simultaneous presence of 1 nM of the labeled ligand and graded concentrations of 5-(SAENTA-x8)-fluorescein in a range of 0.5–1 μM. Only the specific binding is presented, calculated as described in Section 2. Values correspond to a typical experiment of four performed in quadruplicate.

concentration. The mean IC_{50} value calculated from the inhibition curves at 1 μM adenosine concentration was 2.063 ± 0.25 nM ($n=4$), indicating a high affinity of this fluorescent ligand for the nucleoside transporter.

In order to assess the specificity of 5-(SAENTA-x8)-fluorescein to the *es*-nucleoside transporter of chromaffin cells, we studied whether 5-(SAENTA-x8)-fluorescein was able to displace the binding of the most specific ligand, nitrobenzylthioinosine. Fig. 1B shows the displacement curve obtained at 1 nM [³H]NBTI by increasing concentrations of the fluorescent compound. Similar displacement curve profiles were obtained at different [³H]NBTI concentrations (0.5–10 nM) and the IC_{50} values calculated ranged from 4 to 15 nM. These IC_{50} values gave a first estimation of the K_i of 4.08 ± 1.3 nM ($n=4$). However, the [³H]NBTI specific binding to chromaffin cells was not completely inhibited by the highest concentrations of the fluorescent ligand used in these experiments (1 μM). This component represented about 12–20% of the total specific binding and was evident in all the displacement curves. The analysis of Harris and Winzor [18] was applied for the [³H]NBTI binding studies (Fig. 2). Briefly, this analysis uses all binding data obtained over a range of inhibitor concentrations and produces a linear plot in the case of competition between two ligands for one class of acceptor sites. The
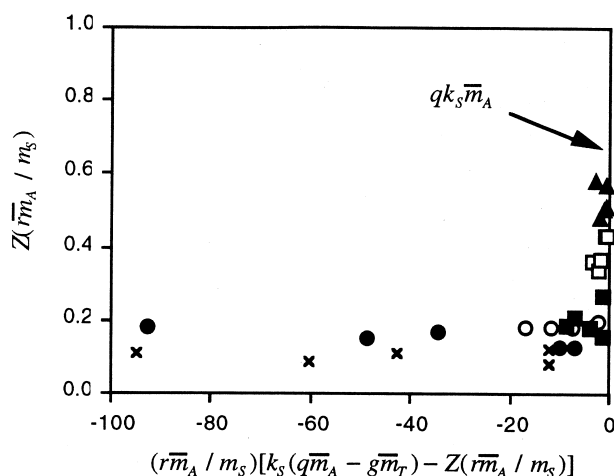
Fig. 2. Analysis of competition between [³H]NBTI and 5-(SAEN-TA-x8)-fluorescein for sites on chromaffin cells by the method of Harris and Winzor [18]. Total 5-(SAENTA-x8)-fluorescein concentrations (C_T) used were 5 nM (▲), 10 nM (□), 50 nM (■), 100 nM (○), 500 nM (●) and 1000 nM (×). The intercept ($qk_s m_A$) which should be obtained by linear squares fitting in the case of competitive inhibition is shown.

plot of the data for [³H]NBTI binding to chromaffin cells in the presence of 5-(SAENTA-x8)-fluorescein (Fig. 2) is a curve, indicating a pool of sites for [³H]NBTI which are inaccessible to the fluorescent compound. A method of quantitation of these 'cryptic' sites for [³H]NBTI has been established by Brocklebank et al. [19]. The analysis by this method (data not shown) predicts a 13% cryptic component ($\sim$ 25 000 sites/cell for 5-(SAENTA-x8)-fluorescein compared to 28 950 sites/cell for [³H]NBTI) and $K_D \approx 3$ nM for 5-(SAENTA-x8)-fluorescein.

3.2. Flow cytometry analysis of 5-(SAENTA-x8)-fluorescein binding to chromaffin cells

The chromaffin cells were incubated in the presence of increasing concentrations of 5-(SAENTA-x8)-fluorescein in the range of 1–100 nM and the intensity of cell associated fluorescence was measured within 10 min, which is the period in which equilibrium is reached. As can be seen in the fluorescence histograms in Fig. 3, the mean fluorescence intensity increased with increasing concentrations of 5-(SAENTA-x8)-fluorescein. The mean channel value obtained at 0 nM of the fluorescent probe represented the autofluorescence of chromaffin cells, and was routinely subtracted from each mean fluorescence channel. Due to the high level of autofluorescence in these cells, measurement of cell associated fluorescence intensity with 5-(SAENTA-x8)-fluorescein was not possible below a 1 nM concentration.

The intensity of cell associated fluorescence was considerably reduced when the cells were preincubated in the presence of the transport inhibitors 1 μM NBTI or dilazep. This represented the non-specific 5-(SAENTA-x8)-fluorescein binding and was subtracted from the total component to calculate the NBTI-sensitive specific binding. The displacement of the fluorescence histograms is shown in Fig. 4.

The mean channel value for each histogram was converted to MESF using standard fluorescein containing microbeads. When the specific binding of 5-(SAENTA-x8)-fluorescein in MESF was plotted against initial ligand concentration, a sat-

uration curve was obtained (Fig. 5), with a half maximal binding value of 6 ± 1.2 nM ($n = 9$). The maximal specific binding was 4400 ± 400 MESF/cell ($n = 9$).

3.3. Regulatory studies of 5-(SAENTA-x8)-fluorescein binding by several effectors in chromaffin cells

In previous studies we demonstrated that the adenosine transport in cultured chromaffin cells is significantly inhibited by stimulation of the cells with secretagogues, such as carbachol and DMPP. The [³H]NBTI binding was also affected in the same fashion. When the cells kept in suspension were treated with the same secretagogues and the 5-(SAENTA-x8)-fluorescein binding was analyzed by flow cytometry, a significant reduction in the fluorescence signal was observed, which was close to 50% inhibition (Fig. 4). Similar results were obtained when cells were stimulated with forskolin, a direct activator of adenylate cyclase that is able to increase intracellular cAMP levels.

4. Discussion

This paper clearly shows that the *es*-nucleoside transporter present in neurochromaffin cells specifically recognized the fluorescent ligand, 5-(SAENTA-x8)-fluorescein. The fluorescent compound completely inhibited the NBTI-sensitive adenosine transport with high affinity, to the same extent as the specific inhibitors, NBTI and dilazep. 5-(SAENTA-x8)-fluorescein also displaced the [³H]NBTI binding to the cells, indicating that the two ligands interact with a common site. The analysis of the equilibrium binding of [³H]NBTI and 5-(SAENTA-x8)-fluorescein to chromaffin cells by the method of Harris and Winzor [18] shows concave curvilinearity, indicating a component of [³H]NBTI binding that is inaccessible to 5-(SAENTA-x8)-fluorescein. These findings are consistent with studies of the binding of a similar fluorescent ligand, 5-(SAENTA-x2)-fluorescein, to leukemic RC2A cells [7]. The
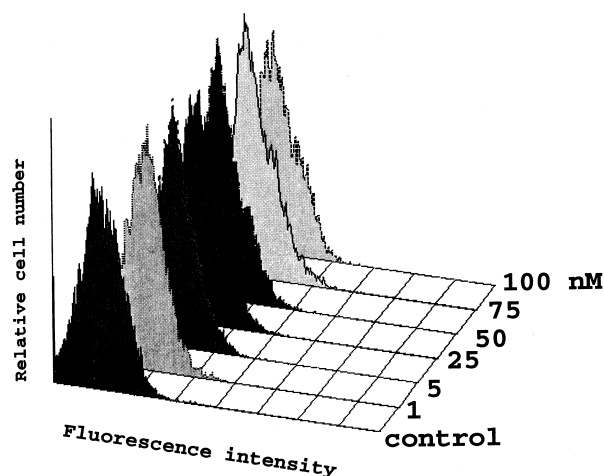

Fig. 3. Fluorescence histograms of 5-(SAENTA-x8)-fluorescein binding to chromaffin cells. Cells were incubated for 10 min at room temperature with different concentrations of 5-(SAENTA-x8)-fluorescein from 0 to 100 nM. The cell-bound fluorescence was analyzed by flow cytometry as described in Section 2. The fluorescence histogram designated 'control' was done at 0 nM of 5-(SAENTA-x8)-fluorescein, and represents the cell autofluorescence. The fluorescence signals were slightly increased with increasing 5-(SAENTA-x8)-fluorescein concentration and were stabilized at 25–30 nM.
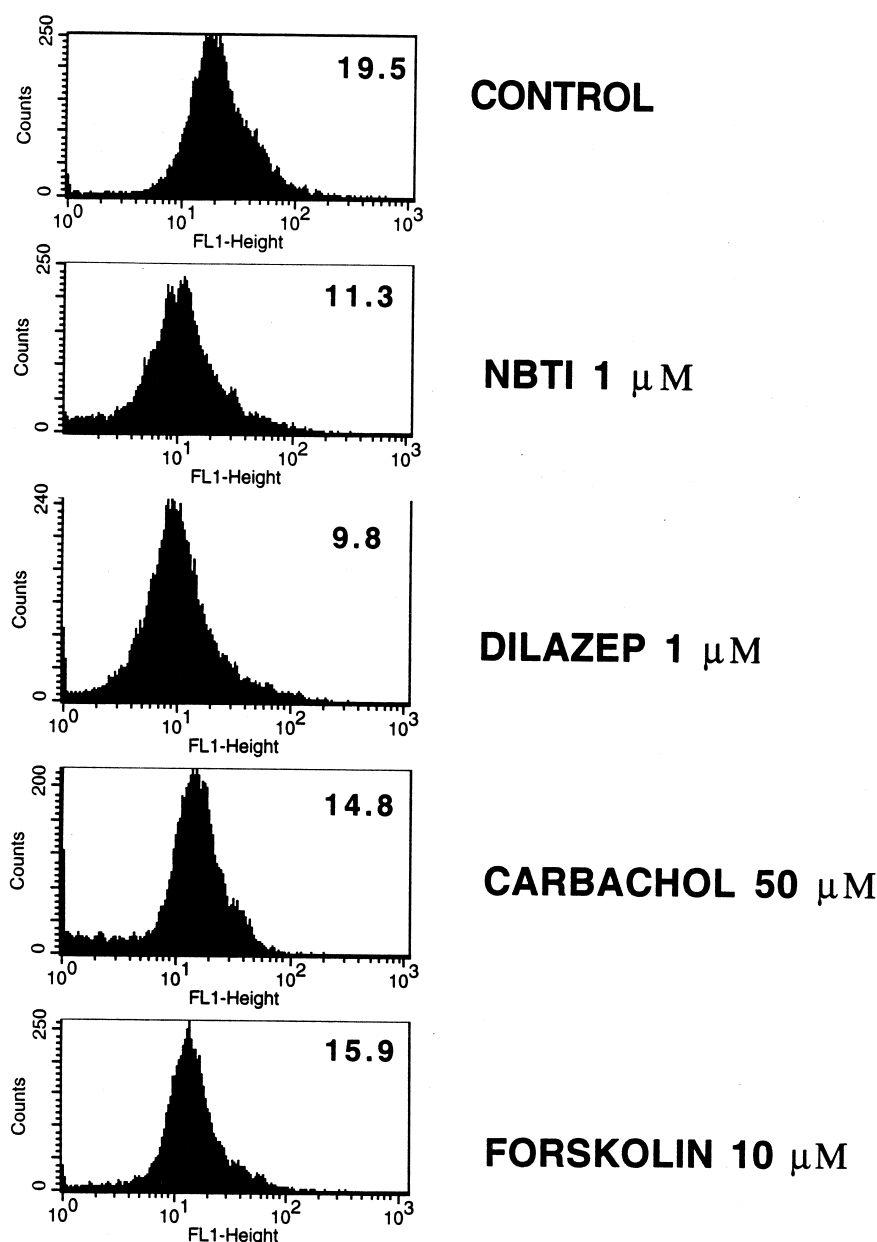
Fig. 4. Inhibition of 5-(SAENTA-x8)-fluorescein binding by several effectors. Chromaffin cells kept in suspension were preincubated for 10 min at room temperature with 1 μM of the transport inhibitors NBTI and dilazep. Then, the fluorescence probe was added and the cells were further incubated for 10 min. The cell-bound fluorescence obtained in these conditions represents the blank value of the flow cytometric binding experiments. In another set of experiments, chromaffin cells were stimulated at room temperature with 50 μM carbachol and 10 μM DMPP (data not shown) for 5 min, and with 1 μM forskolin for 10 min. Then, the cell-bound fluorescence intensity was measured for a further 10 min incubation period with 25 nM of 5-(SAENTA-x8)-fluorescein. The differences in the histogram displacement are evidenced by the mean channel values located in the upper left corner of each panel. These histograms correspond to a typical experiment out of nine performed at 25 nM of 5-(SAENTA-x8)-fluorescein.

nature of the 'cryptic component' is uncertain. It could represent either (i) an additional class of sites on the *es*-nucleoside transporter or (ii) an intracellular pool of nucleoside transporters, assuming that the lipophilic NBTI molecule diffuses across the plasma membrane. Although in chromaffin cells the nucleoside transporters are also located at intracellular structures, they are not accessible to NBTI binding, excluding the second possibility. Comparative studies of [3H]NBTI binding to intact cells or digitonin permeabilized chromaffin cells show the same [3H]NBTI binding sites. Access of NBTI to the intracellular binding sites is achieved when cells are lysed in hypoosmotic medium and the cellular structure and organelles are destroyed [20,21].

Flow cytometric experiments showed that binding of 5-(SAENTA-x8)-fluorescein to chromaffin cells was inhibited by NBTI, which confirms that the two ligands interact with a common site. The K_D for the binding of 5-(SAENTA-x8)-fluorescein is similar to [3H]NBTI and that obtained in leukemic RC2a cells [7]. The number of binding sites expressed as MESF per cell was lower than described in leukemic cells. Similar differences were obtained in NBTI binding sites for the two cellular types.
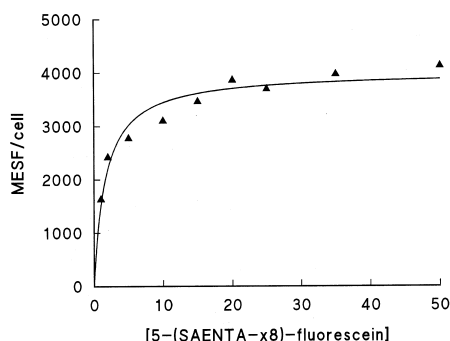
Fig. 5. Scatchard representation of the 5-(SAENTA-x8)-fluorescein binding to chromaffin cells. The mean channel values of the fluorescence histograms obtained at each 5-(SAENTA-x8)-fluorescein concentration were corrected for cell autofluorescence and then converted to MESF as described in Section 2. Blank values were obtained in the presence of 1 μM of NBTI or dilazep and were subtracted from the total and only the NBTI-sensitive 5-(SAENTA-x8)-fluorescein specific binding is represented. Values correspond to a representative experiment out of nine.

It is well known that the stimulation of chromaffin cells with carbachol, or specific agonists of nicotinic receptors, induces catecholamine secretion with a parallel activation of protein kinase C [14]. The stimulation of chromaffin cells with forskolin increases intracellular cAMP levels and activates protein kinase A [13]. In our experimental conditions forskolin had no effect on catecholamine secretion. It is clearly shown that chromaffin cells stimulated with secretagogues or forskolin exhibited a significant decrease in specific 5-(SAENTA-x8)-fluorescein binding. This finding in single chromaffin cells correlates well with the [3H]NBTI binding modification carried out in chromaffin cell populations and reveals that chromaffin cells represent a very homogeneous cell population with respect to the regulation of nucleoside transport by these extracellular signals. Moreover, this study indicates that the fluorescent compound, 5-(SAENTA-x8)-fluorescein, is as selective and specific as NBTI not only in leukemic or tumoral cells but also in a neural model, neurochromaffin cells. It will allow future approaches to the study of nucleoside transporter in neural cell preparations. The availability of specific fluorescent ligands for nucleoside transporters, together with specific markers for different neuronal types, can provide new methods for studying the distribution and possible regulation of nucleoside transporters in the nervous system.

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