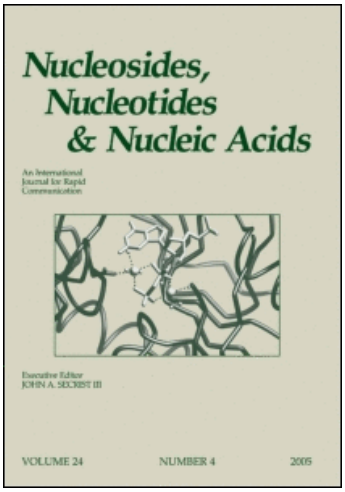

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Nucleoside Transport in Neurons. Regulation by Secretagogues and Effectors of Protein Kinases A and C

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NUCLEOSIDE TRANSPORT IN NEURONS. REGULATION BY SECRETAGOGUES AND EFFECTORS OF PROTEIN KINASES A AND C.

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ABSTRACT. In chromaffin cells, secretagogues and direct activators of protein kinase C and protein kinase A inhibited the nucleoside transport with a parallel decrease in the high affinity binding sites.

Adenosine modulates a variety of physiological functions. In recent years, this nucleoside has been widely accepted as an important neuromodulator, mediated by several subtypes of membrane receptors (1,2). The modulatory effects of adenosine on the nervous system are terminated by adenosine uptake through a nucleoside transporter system. The nucleoside transport has been largely studied in brain preparations and two main types seem to exist according to the sensitivity to nitrobenzylthioinosine (NBTI) (3-5). The presence of a sodium-dependent adenosine transport has been described in renal cortex and enterocytes, but its presence is not clear in the nervous system (6,7). Most adenosine transport studies in neural tissues have been made with sinaptosomes, which are very useful preparations. However, they present the disadvantages of being from an heterogenous cell population and where the cellular integrity is lost.

Since bovine adrenal chromaffin cells are peripheral postganglionic sympathetic neurons and are considered cellular model for neurosecretion, it is therefore interesting to study nucleoside transport and its regulation in these cells (8-10). On the other hand, in chromaffin cells the enzymes responsible for the cycle ATP-adenosine have been characterized, not only the intracellular salvage enzymes but the ectonucleotidases present in the plasma membrane (11-13).

TABLE I.KINETIC PARAMETERS OF NUCLEOSIDE TRANSPORT IN CHROMAFFIN CELLS.

Nucleoside	Km (uM)	Vmax (pmol/10^6 cells. min)	
Adenosine	1.6 ± 0.2	40 ± 5	(15)
Inosine	125.0 ± 15.0	420 ± 20	(16)
Uridine	300.0 ± 50.0	1,400 ± 60	**
Inhibitor	**Ki (nM)**	**mechanism**	
Dilazep	10.00 ± 2	non-competitive	**
Dipyridamole	5.00 ± 0.5	non-competitive	(15)
NBTI	0.01 ± 0.003	non-competitive	(17)

Transport experiments were carried out with cultured cells, plated in 24-well-Costar cluster dishes (250,000 cells/well). The kinetic parameters were determined measuring the transport during the first minute of incubation at 37°C. Ten minutes preincubation with inhibitors were done before the adenosine transport was determined.
** Manuscript in preparation.

CHARACTERIZATION OF NUCLEOSIDE TRANSPORT IN CHROMAFFIN CELLS. Nucleoside transport has been studied in cultured chromaffin cells. Primary collagenase-dissociated cultured chromaffin cells from bovine adrenal medulla were prepared on self-generating Percoll gradient and maintained in culture, as previously described (14). The kinetic parameters for diverse nucleosides are summarized in Table I.

As occurs in all of the studied mammalian systems, the nucleoside transport presented the highest affinity for adenosine (15,16). The Km value obtained for this substract was two orders of magnitude lower than that described for uridine. The three inhibitors reported in Table I were able to inhibit completely the adenosine transport in chromaffin cells, NBTI being the most effective. NBTI caused 100% inhibition on adenosine transport at the nanomolar concentration range, indicating that a great majority if not all of adenosine transporters in this system were highly sensitive to NBTI, and were mediated by a facilitated diffusion mechanism. Furthermore, in this cellular model sodium could be replaced by lithium or choline without significant effects on adenosine transport.

An alternative approach studying the adenosine transporters involves the use of [^{3}H]-NBTI, the most potent inhibitor, as a binding probe, allowing their molecular characterization. The inhibition of adenosine transport by NBTI was associated with high affinity binding of this compound to chromaffin cells (Kd = 0.54 $\pm$ 0.12 nM). The binding of [^{3}H]-NBTI quantified a number of 32,068 $\pm$ 6,965 high affinity sites per cell. The molecular type that covalently binds to NBTI after photolabelling showed a molecular weight of 42,000 $\pm$ 5,000 Da.

MODULATION OF ADENOSINE TRANSPORT IN CULTURED CHROMAFFIN CELLS. The neurosecretory event in chromaffin cells is normally triggered by activation of cholinergic receptors, inducing a rise in cytosolic calcium (18,19). More recently, it has been demonstrated that the exocytotic process can be also modulated by another cascade of intracellular second messengers, which are displayed following chromaffin cell stimulation. The possibility was studied that adenosine transport in chromaffin cells could be modulated by the same extracellular and intracellular signals involved in catecholamine secretion. The effects of these membrane signals and/or the corresponding second messengers on adenosine transport are summarized in Table II. The stimulation of chromaffin cells with carbachol or DMPP (1,1-dimethyl-4-phenylpiperazinium iodide), considerably decreased the adenosine transport capacity, without significant changes in the affinity constant. When high K^+ was employed as a secretagogue, with the corresponding increase in the cytosolic calcium, an inhibition of the adenosine transport was also observed. This inhibition could be imitated by a Ca^{2+} ionophore, A-23187.

In chromaffin cells it has been described that secretagogues and other substances increasing the cytosolic calcium, cause a translocation of protein kinase C to membrane and activation of the enzyme (20). Direct activators of protein kinase C significantly decreased the adenosine transport. When the protein kinase C activation and the calcium mobilization were combined (PDBu, phorbol, 12,13 dibutyrate plus A-23187), an additive response was observed. The inhibition percentage was similar to that obtained with secretagogues.

Other membrane receptors are coupled to adenylate cyclase/protein kinase A system. In this way, the direct activator of adenylate cyclase, forskolin, was an inhibitor of adenosine transport. In our system, with

TABLE II. INHIBITION OF ADENOSINE TRANSPORT BY SECRETAGOGUES AND SEVERAL EFFECTORS IN CULTURED CHROMAFFIN CELLS.

Effector	Km (uM)	Vmax	% I
Control	1.6 ± 0.2	40 ± 5.0	--
Carbachol	2.0 ± 0.3	20 ± 5.0 **	50
DMPP	1.4 ± 0.3	20 ± 6.3 **	50
Tricaprylin	1.7 ± 0.3	21 ± 3.4 **	47
PDBu	1.6 ± 0.2	32 ± 4.0 **	20
A-23187	1.9 ± 0.3	30 ± 7.3 **	23
A-23187 + PDBu	1.7 ± 0.3	15 ± 4.0 **	60
K+	1.6 ± 0.3	25 ± 4.5 **	37
Forskolin	2.2 ± 0.3	18 ± 3.0 **	55
ClPhcAMP	2.0 ± 0.1	21 ± 4.0 **	47

Vmax are expressed as pmol/10^6 cells. min.

Cells were incubated in the presence or absence of carbachol (50 uM), DMPP (10 uM) or K^+ (60 mM) for 1 min before starting the transport experiments, and with Tricaprylin (10 ug/ml), PDBu (100 nM), A-23187 (0.5 uM), forskolin (0.5 uM) or ClPhcAMP (100 uM) for 10 min at 37°C.

Values are means ± SD of five experiments in quadruplicate.

** $p \leq 0.001$.

0.5 uM forskolin the cAMP levels rise four times with respect to initial values. Similar results were obtained when cells were preincubated with a cAMP analog, ClPhcAMP (8-[4-chlorophenylthio] adenosine -3:5-monophosphate cyclic), which enters the cells (21).

For all these effectors, only minor changes in the Km values were observed.

The synergism between protein kinase C and protein kinase A effectors was difficult to observe at concentrations which gave the maximal inhibition on adenosine transport separately. Only when submaximal concentrations were tested, the combinations of ClPhcAMP plus DMPP or ClPhcAMP plus K^+ showed an additive inhibitory effect of about 70% on adenosine transport.

TABLE III. EFFECTS OF CARBACHOL AND OTHER EFFECTORS ON [^{3}H]-NBTI BINDING IN CULTURED CHROMAFFIN CELLS.

Effector	Kd (nM)	Bmax	Vmax/Bmax
Control	0.54 ± 0.12	32,068 ± 6,965	12
Carbachol	0.60 ± 0.15	18,792 ± 2,782 **	11
PDBu	0.44 ± 0.16	24,024 ± 2,087 **	13
PMA	0.45 ± 0.10	23,730 ± 1,900 **	14
Forskolin	0.49 ± 0.05	21,300 ± 2,000 **	9
ClPhcAMP	0.42 ± 0.03	12,000 ± 2,000 **	17

Bmax are expressed as binding sites per cell.

The binding experiments were accomplished with 3 x 10^6 cells. Cells were preincubated with carbachol and other effectors as described in Table II for transport experiments.

To calculated the Vmax/Bmax quotient, the values from Table II, were expressed as adenosine molecules transported per cell and per second.

Values are means ± SD of three experiments in quadruplicate.

** $p \leq 0.001$.

[^{3}H]-NITROBENZYLTHIOINOSINE BINDING TO CHROMAFFIN CELLS. The above results in adenosine transport inhibition by secretagogues and direct activation of protein kinase C or A suggested that changes might have occurred at the transporter level. Thus, the NBTI binding experiments in the presence of several effectors were carried out. Table III shows that carbachol, phorbol esters and an increase in the cAMP levels remarkably decreased the number of high affinity binding sites (Bmax) in the plasma membrane of cultured chromaffin cells. Moreover, only minor changes at the dissociation constant (Kd) were observed.

Taking into account the Vmax values of adenosine transport and Bmax values of binding experiments, the efficiency of the nucleoside transport system was roughly 12 adenosine molecules internalized per second and per transporter. The efficiency of adenosine transporters was similar in stimulated cells by carbachol or treated with phorbol esters. However, when chromaffin cells were preincubated with forskolin or ClPhcAMP, their efficiency values varied. On the other hand, the physiological relevance

of protein kinase A activation, related to exocytosis, remains unclear in chromaffin cells.

[^{3}H]-NITROBENZYLTHIOINOSINE BINDING TO CHROMAFFIN TISSUE MEMBRANES. The decrease in the number of adenosine transporters in stimulated chromaffin cells could be due to an internalization of transporters and their translocation to intracellular membranes, as occurs with the glucose transporters, or to a molecular modification of the transporter itself.

In order to test these possibilities the number of adenosine transporters was quantified in chromaffin tissue homogenates (800 x g supernatant), in the presence or absence of several effectors. The 800 x g supernatant presented the advantage of containing all cellular membranes and also the cytosolic components necessary for protein kinases action.

Table IV shows that activation of the protein kinase C or protein kinase A also decreased the high affinity binding sites for NBTI in chromaffin tissue homogenates. But no significant changes in the affinity of these transporters (Kd) were observed. These results indicate that in the absence of cellular integrity, where the possibility of membrane internalization was excluded, the decrease in the NBTI binding sites by activation of protein kinases C or A could be due to a molecular modification at the membrane transporter level that would hinder the recognition of NBTI by the transporter.

The most potent inhibition of [^{3}H]-NBTI binding to chromaffin tissue homogenates was obtained with the cAMP analog, ClPhcAMP,in the presence of ATP or its non-hydrolyzable analog, ATP-γ-S, that can be employed as a substract of protein kinase A but is not a substract for protein phosphatases. The decrease in the number of high affinity binding sites was higher with ATP-γ-S. Controls with ATP-γ-S, instead of ATP, did not show significant differences.

***EFFECT OF PURIFIED PROTEIN KINASE C ON* [^{3}H]-NBTI BINDING.** To support the possible molecular modification of transporters by activation of protein kinase C, highly purified plasma membranes of chromaffin tissue were directly treated with purified protein kinase C from bovine brain (22). Results are summarized in Table V. Protein kinase C in the presence of its effectors significantly decreased the NBTI binding sites. Controls were made in the presence of all activators of this enzyme. The inhibition of NBTI was approximately to 50%. This is an additional test to support the

TABLE IV. INHIBITION OF [^{3}H]-NBTI BINDING TO CHROMAFFIN TISSUE MEMBRANES BY SEVERAL EFFECTORS.

Effector	Kd (nM)	Bmax (fmol/mg protein)
Control	0.40 ± 0.15	87 ± 10
Carbachol	0.35 ± 0.05	57 ± 6 **
Tricaprylin	0.32 ± 0.04	68 ± 7 **
Forskolin	0.31 ± 0.03	32 ± 1 **
ClPhcAMP	0.30 ± 0.06	44 ± 2 **
ClPhcAMP + ATP-γ-S	0.25 ± 0.05	20 ± 3 **

The binding experiments were accomplished with 1 mg protein/assay. The membranes were preincubated with the effector as described previously in Table II for transport experiments. Controls were made in the presence of 1mM ATP and 0.1 mM EGTA, to avoid the actions of calcium. To study the effect of carbachol, 100 uM GTP, 1 mM $CaCl_2$ were added to the preincubation medium. All the preincubations were made in the presence of 1 mM ATP.

Values are means ± SD of three experiments in quadruplicate.

** $p \leq 0.001$.

TABLE V. EFFECT OF PURIFIED BRAIN PROTEIN KINASE C ON [^{3}H]-NBTI BINDING TO CHROMAFFIN CELL PLASMA MEMBRANE.

Bound	- PKC	+ PKC
[^{3}H]-NBTI (fmol/mg protein)	140 ± 10 (5)	75 ± 6 **
^{32}Phosphate (cpm)	620 ± 100 (5)	9,630 ± 700 **

[^{3}H]-NBTI binding experiments were made in the presence or absence of protein kinase C (PKC), calcium, ATP and phospholipids as described Díaz-Guerra et al (1988) (23). [^{3}H-NBTI] = 0.5 nM.

Protein kinase C activity was assayed by the incorporation of [^{32}P]Pi into histone H1.

** $p \leq 0.001$.

(n) number of experiments.

hypothesis that a chemical modification of transporters by phosphorylation through protein kinase C takes place. Nevertheless, additional studies with purified transporters will be necessary in order to confirm these results.

DISCUSSION. Adenosine transport seems to be a highy regulated process in cultured chromaffin cells. Extracellular signals, related to secretagogues, trigger exocytosis and control the adenosine transport in these neurosecretory cells at the same time. Thus, the extracellular adenosine, derived from ATP exocytotically released, can remain in contact with its membrane receptors for longer periods of time (13, 24).

Moreover, the same second messengers that both originate or increase exocytosis inhibit the adenosine transport. An increase in the intracellular Ca^{2+} or cAMP levels, and direct activation of protein kinases A or C, always correspond to a decrease in the transport capacity and in the high affinity NBTI binding sites. The efficiency of transporters is maintained with all the effectors previously described.

The decrease in NBTI binding sites can be imitated in cellular homogenates in the presence of protein kinases A and protein kinase C effectors and also in highly purified plasma membranes treated with PKC. These results support a chemical modification of transporters that interferes with the NBTI binding.

The adenosine transport control reported here is a short-term regulation. Besides, chromaffin cells may be related to other extracellular factors. One of the most important is the Nerve Growth Factor (NGF), that increased the adenosine transport capacity when it was added to culture medium, although its molecular mechanisms are still unknown (25). It seems probable that short and long-term regulation of adenosine transport occurs in chromaffin cells in order to integrate the extracellular signals and the cellular metabolic state.

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