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Effects of phorbol esters and secretagogues on nitrobenzylthioinosine binding to nucleoside transporters and nucleoside uptake in cultured chromaffin cells

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Secretagogues inhibited adenosine uptake in chromaffin cells without causing apparent changes in the uptake affinity. The inhibition caused by carbachol, nicotine and acetylcholine reached 50%. This inhibition was reproduced by the action of protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA; 100 nM), phorbol 12,13-dibutyrate (PDBu; 100 nM), dicaproin (10 μg/ml) and tricaprylin (10 μg/ml), with inhibitions of $V_{max.}$ of 18, 20, 37 and 47% respectively. No changes in the affinity of uptake were observed with these effectors. Down-regulation of protein kinase C by phorbol esters decreased the inhibitory effects of carbachol on adenosine uptake. Binding studies with nitrobenzylthioinosine (NBTI) showed a similar decrease in the number of transporters when chromaffin cells were treated with the same effectors used for the uptake studies. The high-affinity dissociation constants showed minor changes with respect to the control. The ratio between maximal uptake capacity and the transporter number per cell was not significantly modified by the action of secretagogues or direct effectors of protein kinase C. The number of high-affinity binding sites for NBTI was decreased in cellular homogenates by the direct action of protein kinase C activators, with staurosporine able to reverse this action. Protein kinase C from bovine brain in the presence of ATP and effectors, decreased the number of high-affinity NBTI-binding sites in purified chromaffin cell plasma membranes. These data suggest the possibility of a molecular modification at the transporter level.

INTRODUCTION

Adrenal chromaffin cells represent a good model in which to study the synthesis, storage, secretion and recovery of the vesicular content in neural tissues (Perrin & Aunis, 1985; Winkler, 1988). After the exocytotic release, ATP, one of the main stored components in the granules in addition to catecholamines, is degraded to adenosine by the sequential action of ectonucleotidases (Gordon *et al.*, 1986; Richardson *et al.*, 1987; Torres *et al.*, 1990*b*). The extracellular adenosine is effectively incorporated into the intracellular and granular nucleotide pool of this tissue (Rotllán & Miras-Portugal, 1985; Miras-Portugal *et al.*, 1986).

Transport, the first step in adenosine recovery, has been extensively studied in neural cells (Marangos *et al.*, 1982; Wu & Phillis, 1984; Miras-Portugal *et al.*, 1986). This step is of particular interest because it terminates the modulatory actions of adenosine through its membrane receptors (Williams, 1987). Potent inhibitors of adenosine transport have been employed pharmacologically to prolong the actions of extracellular adenosine, as occurs with dipyridamole, dilazep and nitrobenzylthioinosine (NBTI), and to characterize the type of adenosine transport (Marangos & Deckert, 1987; Lee & Jarvis, 1988). In chromaffin cells all adenosine transporters are very sensitive to inhibition by NBTI (Torres *et al.*, 1990*a*). In spite of recognition of the increasing importance of adenosine as a neuromodulator and vasoactive substance, there is only a little evidence available concerning the possible regulation of its transport. However, a stimulatory effect of nerve growth factor on adenosine transport has been described in cultured bovine chromaffin cells (Torres *et al.*, 1987). There is also recent evidence concerning the possible interaction between adenosine receptors and transporters (Delicado *et al.*, 1990). In the same model, the presence of activators of adenylate cyclase, such as forskolin, or direct activators of protein kinase A, such as the cyclic AMP analogue, chlorophenyl cyclic AMP, also affects adenosine transport (Sen *et al.*, 1990). In addition, the cyclic AMP-dependent protein kinases seem to modulate the exocytotic event (Burgoyne, 1984; Higgins & Berg, 1988). In this respect, the best known actions are those of calcium/calmodulin-dependent protein kinase and protein kinase C. Both kinases are activated by the action of secretagogues, with the calcium/calmodulin-dependent kinase having effects on cytoesqueletum dynamics (Hikita *et al.*, 1984; Bader *et al.*, 1985) and a significant redistribution of protein kinase C between the particulate and the cytosolic pools (Lee & Holz, 1986; Terbush *et al.*, 1988).

Such observations raise the principal question concerning the regulation of nucleoside transporters: is there any relationship between the secretory process and nucleoside uptake? The present paper attempts to show the existence of regulation of nucleoside transporters, and considers the key role played by protein kinase C effectors, directly and/or through membrane receptors.

EXPERIMENTAL

Materials

Collagenase (EC 3.4.24.3) and phenylmethanesulphonyl fluoride (PMSF) were supplied by Boehringer. Acetylcholine, ATP, Ca^{2+} ionophore A23187, carbachol, cytosine arabinofuranoside, dicaproin, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), dipyridamole, fluorodeoxyuridine, nicotine, NBTI, phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA), tricaprylin and staurosporine were from Sigma. Culture media, fetal calf serum and antibiotics were purchased

Abbreviations used: DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; NBTI, nitrobenzylthioinosine; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride.

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from Cultek (Flow Laboratories). Culture vessels were obtained from Cultek. [2,8-^{3}H]Adenosine (27 Ci/mmol) was from Amersham. [^{3}H]NBTI (26 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from DuPont–New England Nuclear. Ready Safe scintillation liquid for aqueous and non-aqueous samples was purchased from Beckman. All other reagents were supplied by Merck.

Cell preparation and culture

Primary collagenase-dissociated cells from bovine adrenal medulla were prepared and purified on a Percoll gradient as previously described (Miras-Portugal *et al.*, 1985). The cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum containing 10 μM-cytosine arabinofuranoside, 10 μM-fluorodeoxyuridine, penicillin (5 units/ml), streptomycin (5 μmg/ml), kanamycin (100 μg/ml) and amphotericin (2.5 μg/ml). These cells were plated in 24-well Costar cluster dishes (250000 cells/well) for adenosine transport and in Petri dishes [$(1.5–3) \times 10^6$ cells/35 mm dish] for binding studies. At least 16 h before the start of experiments, the culture medium was replaced with medium without serum. Experiments were performed 4–8 days after plating.

Adenosine uptake experiments

To measure adenosine uptake, cells were incubated with 0.5 μCi of [2,8-^{3}H]adenosine (27 Ci/mmol)/well in a volume of 200 μl of Locke's solution; non-labelled adenosine was added to give the required final concentrations. Uptake was determined during the linear period, which corresponded to the first 1 min from the start. Uptake was stopped by aspiration of the fluid and two washes with 1 ml of Locke's solution containing 10 μM-dipyridamole, as dipyridamole at this concentration inhibits completely adenosine efflux (Lee & Jarvis, 1988).

To study the effects of secretagogues and direct activators of protein kinase C on adenosine uptake, the cells were preincubated in the presence of acetylcholine (50 μM), carbachol (50 μM), nicotine (10 μM), DMPP (10 μM) or K^+ (60 mM) for 1 min at 37 °C, or in the presence of PDBu (100 nM), PMA (100 nM), dicaproin (10 μg/ml), tricaprylin (10 μg/ml) or A23187 (0.5 μM) for 10 min at 37 °C before starting the uptake experiments. The preincubation medium was aspirated and the incubation medium containing the corresponding effector and labelled adenosine was then added to measure the adenosine uptake as described above. Controls were carried out with the same preincubation period.

[^{3}H]NBTI binding to cultured chromaffin cells

NBTI binding studies were carried out as described by Torres *et al.* (1988). Petri dishes containing $(1.5–3) \times 10^6$ cells were incubated with a final volume of 1.5 ml of Locke's solution containing [^{3}H]NBTI to obtain a final concentration range of 0.02–5 nM-NBTI. After 30 min incubation at 37 °C the cells were quickly washed with 2×3 ml of cold Locke's solution containing 10 μM-NBTI. Under these conditions, binding displacement with the unlabelled ligand does not occur (Marangos & Deckert, 1987). Cells were then scraped out of the plastic dish and the radioactivity was counted in 10 ml of scintillation liquid. Controls for these experiments were assayed in the presence of [^{3}H]NBTI and 10 μM of the non-labelled compound, but without effectors. Non-specific binding values were subtracted from assay values. All experiments were carried out in the presence of 2 units of adenosine deaminase/ml to inhibit the effects of released endogenous adenosine.

To study the effects of secretagogues and activators of protein kinase C on NBTI binding, the cells were preincubated as described for transport experiments.

[^{3}H]NBTI binding to chromaffin tissue homogenate

To study NBTI binding to chromaffin tissue homogenates, adrenal glands were processed as follows. Non-frozen adrenal glands were dissected and homogenized in 0.32 M-sucrose/10 mM-Hepes (pH 7.2)/50 μM-PMSF (1:4, w/v). This homogenate was centrifuged at 800 *g* for 10 min and the supernatant was collected. To eliminate the high levels of purine bases, nucleosides and nucleotides present in this tissue, five cycles of dialysis with 10 mM-phosphate buffer (pH 7.4) containing 50 mM-KCl were carried out. The binding of [^{3}H]NBTI to the dialysed homogenate was performed by incubating 0.5 mg of protein in 500 μl of phosphate buffer and 10 mM-$MgCl_2$ containing graded concentrations of the labelled compound (0.02–5 nM). After 30 min incubation at 37 °C, membranes were collected on Whatman GF/F glass fibre filters. The filters were washed with 2×3 ml of ice-cold buffer containing 10 μM-NBTI and dried, and the radioactivity was then measured.

To study the effect of the protein kinase C activator tricaprylin and the protein kinase C inhibitor staurosporine, the cellular homogenates were preincubated for 10 min at 37 °C in the presence of 1 mM-ATP and 0.1 mM-$CaCl_2$. [^{3}H]NBTI binding was measured as described above.

Assay of protein kinase C

Protein kinase C was purified from bovine brain by DE52, phenyl-Sepharose and protamine-agarose chromatography as described elsewhere (Junco *et al.*, 1990).

Protein kinase C activity was assayed by the incorporation of [^{32}P]P_i into histone H1 in the absence or in the presence of Ca^{2+} and phospholipids. The assay (100 μl) consisted of (final concentrations): 20 μM-ATP (0.1–0.3 μCi), 1 mM-magnesium acetate, 5 mM-β-mercaptoethanol, 50 μg of histone (type IIIS from Sigma), 20 mM-Hepes, pH 7.5, and, except when otherwise indicated, 0.3 mM-$CaCl_2$, 10 μg of phosphatidylserine/ml and 1 μg of dioctanoylglycerol/ml, as previously described. The specific activity of protein kinase C was around 950 units/mg of protein (Díaz-Guerra *et al.*, 1988).

Plasma membranes were purified as described elsewhere (Delicado *et al.*, 1988; Torres *et al.*, 1988).

Values are the means ± S.D. of n experiments. P values were obtained according to Student's t test. Linear regression equations were calculated by the least-squares method using a linear regression program.

RESULTS

Effect of secretagogues and protein kinase C effectors on adenosine uptake

The stimulation of chromaffin cells with carbachol significantly decreased adenosine uptake by the same percentage at every adenosine concentration, as shown in the Michaelis–Menten representation (Fig. 1). Nevertheless, there were no apparent changes in the affinity of the transporters (Table 1). Similarly, other secretagogues such as acetylcholine, nicotine and DMPP had the same inhibitory effect on adenosine uptake. The kinetic parameters K_m and $V_{max.}$ for this uptake are summarized in Table 1. It is noteworthy that in bovine chromaffin cells the nicotinic receptors represent the majority of cholinergic receptors. When high K^+ concentrations were employed as a secretagogue (with corresponding increase in the cytosolic Ca^{2+}), inhibition of the adenosine uptake was also observed. This inhibition could be imitated by the Ca^{2+} ionophore A23187.

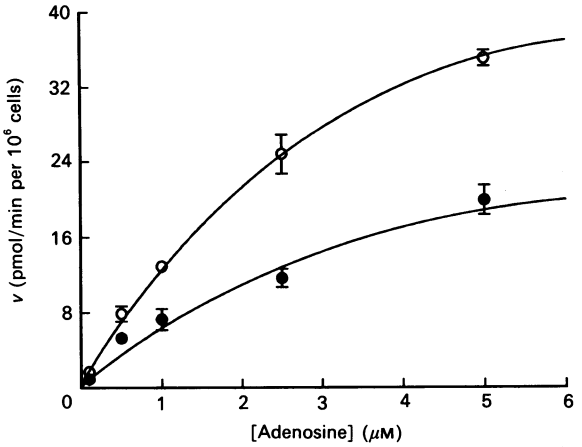

Fig. 1. Effect of carbachol on adenosine uptake in chromaffin cells

The concentration-dependence of adenosine uptake is shown. Cells were preincubated in the presence (●) or the absence (○) of carbachol (50 μM) for 1 min at 37 °C before starting the adenosine uptake experiments, as described in the Experimental section. Values are means ± S.D. of five experiments in quadruplicate.

Table 1. Inhibition of adenosine uptake by secretagogues and several effectors in cultured chromaffin cells

Cells were incubated in the presence or absence of acetylcholine (50 μM), carbachol (50 μM), nicotine (10 μM), DMPP (10 μM) or K^+ (60 mM) for 1 min, or with tricaprylin (10 μg/ml), PDBu (100 nM) or A23187 (0.5 μM) for 10 min at 37° C, before starting uptake experiments. Values are means ± S.D. of five experiments in quadruplicate. *$P \leqslant 0.001$.

Effector	K_m (μM)	$V_{max.}$ (pmol/min per 10^6 cells)	Inhibition (%)
Control	1.6±0.2	40±5.0	–
Acetylcholine	1.7±0.3	22±3.2*	45
Carbachol	2.0±0.3	20±5.0*	50
Nicotine	1.4±0.1	18±4.7*	55
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
A23187	1.9±0.3	30±7.3*	23
A23187+PDBu	1.7±0.3	15±4.0*	60
K^+	1.6±0.3	25±4.5*	37

It is well known that nicotinic stimulation of bovine chromaffin cells causes a rapid transient translocation of protein kinase C activity to membranes. This effect can be imitated by protein kinase C activators such as the tumour-promoting phorbol esters (Terbush *et al.*, 1988; Bittner & Holz, 1990). Therefore the effect of protein kinase C activation on adenosine uptake was studied using phorbol esters (PDBu, PMA) and other activators such as dicaproin and tricaprylin. These compounds significantly inhibited adenosine uptake, with minor changes noted in the affinity of the transporter (Table 1). In this cellular model, dicaproin and tricaprylin were more effective as adenosine uptake inhibitors than were phorbol esters, having a similar inhibitory capacity to that found with the secretagogues. Phorbol esters, even at higher concentrations than those reported in Table 1, were less effective inhibitors of adenosine uptake with a maximal inhibitory effect observed of about 25%. There were synergistic inhibitory actions on adenosine uptake when protein kinase C activation and Ca^{2+} mobilization were combined (PDBu+A23187).

Table 2. Effects of chronic phorbol ester treatment on adenosine uptake inhibition by secretagogues

Chromaffin cells were cultured in the presence of 400 nM-PMA for 24 h, at which time the PMA-containing medium was replaced with PMA-free medium. The effect of DMPP on adenosine uptake was measured immediately, 0 h and 6 h after PMA removal, as described in the Experimental section. Adenosine uptake was measured at a concentration of 1 μM. Values are the means ± S.D. of three experiments in triplicate. *$P \leqslant 0.001$ compared with the corresponding value in the absence of DMPP.

	Adenosine uptake (pmol/min per 10^6 cells)			
	0 h		6 h	
	−DMPP	+DMPP	−DMPP	+DMPP
Control	22.1±2.6	14.9±1.1*	21.1±1.2	16.3±0.6*
+PMA	20.5±3.4	20.85±1.2	19.3±1.3	13.4±0.8*

When cultured chromaffin cells were preincubated for 24 h in the presence of phorbol esters, down-regulation of protein kinase C occurred. In this situation, secretagogues had no effect on adenosine uptake (Table 2). The recovery of protein kinase C activity in these cells, 6 h after removing the phorbol esters from the culture medium, allowed the inhibitory effect of secretagogues on adenosine uptake to be expressed once again, with a percentage inhibition similar to that in controls (Table 2).

Effect of secretagogues and protein kinase C activators on [^{3}H]NBTI binding to cultured chromaffin cells

The decrease in transporter capacity caused by protein kinase C activation might be explained by a decrease in the number of transporters at the plasma membrane level. To investigate this hypothesis, nucleoside transporters in cultured chromaffin cells were quantified by using [^{3}H]NBTI, a ligand of high affinity and specificity. When [^{3}H]NBTI binding was measured in the presence of a secretagogue such as carbachol, the Scatchard plot showed a significant decrease in the number of high-affinity binding sites (Fig. 2). Similar results were obtained in the presence of the protein kinase C effectors PMA and PDBu. The K_d and $B_{max.}$ values are summarized in Table 3. The maximal binding capacities were clearly decreased in the presence of carbachol and phorbol esters, reaching close to 40–50% and 20–50% of control values respectively. The affinity dissociation constants showed only minor changes in comparison with controls.

The above results suggest that the decrease in the number of transporters in the plasma membrane could be due to a molecular modification of the transporter on treatment with secretagogues or PKC activators, which would hinder the recognition of NBTI by the transporters, or that internalization of these transporters into subcellular membrane fractions occurred.

[^{3}H]NBTI binding to chromaffin tissue homogenates in the presence of activators and inhibitors of protein kinase C

In order to identify whether molecular modification or retrieval from the plasma membranes of nucleoside transporters was occurring, the effects of protein kinase C activators and inhibitors on NBTI binding were studied in cellular homogenates. This model eliminates the possibility of subcellular redistribution of plasma membranes and contains all of the cellular membranes

Table 3. Effects of carbachol and phorbol esters on [^{3}H]NBTI binding in cultured chromaffin cells

Values are the means ± S.D. of five experiments performed in quadruplicate. *$P \leq 0.001$ compared with control.

Effector	K_d (nM)	$B_{max.}$ (sites/cell)
Control	0.54 ± 0.12	32068 ± 6965
Carbachol (50 μM)	0.60 ± 0.15	18792 ± 2782*
PDBu (100 nM)	0.44 ± 0.16	24024 ± 2087*
PMA (100 nM)	0.45 ± 0.10	23730 ± 1900*

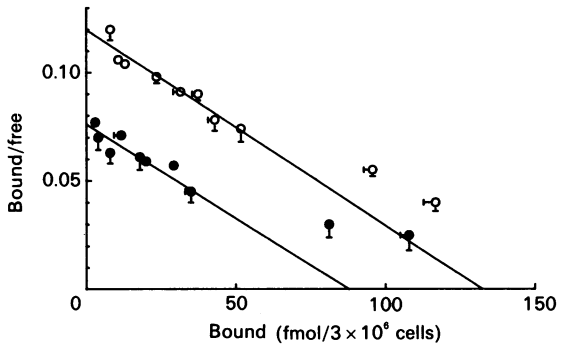

Fig. 2. Scatchard representation of [^{3}H]NBTI equilibrium of binding to cultured chromaffin cells

Cells (3×10^6) were preincubated in the presence (●) or the absence (○) of 50 μM-carbachol for 1 min at 37 °C as described in the text. This plot represents a typical experiment performed in quadruplicate.

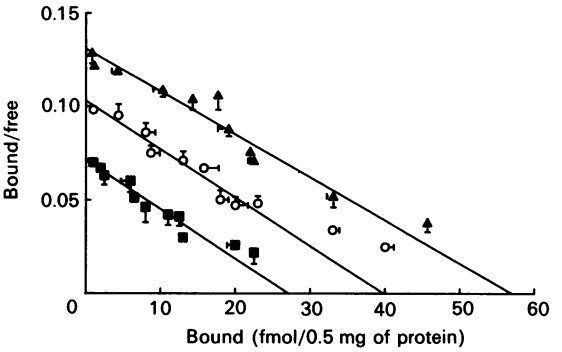

Fig. 3. Scatchard analysis of [^{3}H]NBTI equilibrium binding to chromaffin tissue homogenate

Protein (0.5 mg) was preincubated with tricaprylin (■, 10 μg/ml) or staurosporine (▲, 1 μg/ml) for 10 min at 37 °C before starting the binding experiments. ○, Control. All samples contained ATP (1 mM) and Ca^{2+} (0.1 mM). Values are the means ± S.D. of three experiments performed in quadruplicate.

and cytosolic components necessary for the possible action of protein kinase C, including the kinase itself. Furthermore, possible inaccessibility to internal cellular structures was eliminated. In the absence of an organized structure, the protein kinase C effectors modified the number of high-affinity binding sites (Fig. 3). Tricaprylin, a direct activator of protein kinase C, inhibited about 36% of the high-affinity binding sites compared with control. On the other hand, staurosporine, a protein kinase C inhibitor, increased the high-affinity binding sites by about 30%. The K_d values of the binding sites (0.65 ± 0.1 nM) was not modified by these effectors or inhibitors.

Table 4. Effect of purified brain protein kinase C on [^{3}H]NBTI binding to chromaffin cell plasma membranes

[^{3}H]NBTI binding experiments were carried out in the presence or absence of protein kinase C (PKC) (1 unit), Ca^{2+}, ATP and phospholipids as described by Díaz-Guerra *et al.* (1988). The concentration of [^{3}H]NBTI was 0.5 nM. [^{32}P]P_i indicates the incorporation of P_i from [γ^{32}P]ATP into plasma membrane proteins by purified PKC in the presence of the above-mentioned effectors. Results are means ± S.D. of five experiments. *$P \leq 0.01$ compared with result in the absence of PKC.

	−PKC	+PKC
[^{3}H]NBTI bound (fmol/mg of protein)	140 ± 10 (5)	75 ± 6*
[^{32}P]P_i (c.p.m./mg of protein)	4133 ± 670 (5)	64200 ± 4670*

Effect of purified protein kinase C on [^{3}H]NBTI binding

To confirm the possible molecular modification of transporters by activation of protein kinase C, highly purified plasma membranes from chromaffin tissue were directly treated with purified protein kinase C from bovine brain (Junco *et al.*, 1990) (Table 4). Protein kinase C in the presence of its effectors significantly decreased NBTI binding. Controls contained all activators of protein kinase C, but not the enzyme itself. The inhibition of NBTI binding was approx. 50%. These experiments provided additional evidence to support the hypothesis that a chemical modification of transporters occurs by phosphorylation through protein kinase C.

DISCUSSION

In cultured chromaffin cells either stimulation by secretagogues or activation of protein kinase C results in inhibition of adenosine uptake. The down-regulation of this enzyme prevents the inhibitory actions of secretagogues on adenosine uptake. This is the first report concerning the regulation of adenosine transporters by protein kinase C and its close relationship with the extracellular signals triggering exocytosis. In this neural model it is firmly established that secretagogues cause an intracellular translocation of protein kinase C. The association of this enzyme with cellular membranes can be reproduced with phorbol esters and Ca^{2+}, inducing the phosphorylation of a great number of proteins (Pocotte *et al.*, 1985; Lee & Holz, 1986; Terbush *et al.*, 1988; Bittner & Holz, 1990).

Protein kinase C activation also modifies another membrane protein, i.e. the Na^+-independent glucose transporter (Kitagawa *et al.*, 1989). The phosphorylation of these transporters *in vivo* and *in vitro* by protein kinase C has also been described in erythrocytes (Witters *et al.*, 1985). Since the nucleoside and glucose transporters mediate a functionally identical process with kinetic similarities (Plagemann *et al.*, 1988), it is not surprising that protein kinase C affects both membrane proteins. Another possible means of regulation of facilitated diffusion glucose transport is the translocation of transporters between the plasma membrane and the microsomal pool (Cushman & Wardzala, 1980; Simpson *et al.*, 1983), which has also been observed in chromaffin cells (Delicado & Miras-Portugal, 1987). For these studies it was necessary to quantify the number of

transporters present at the plasma membrane and in different subcellular membranes.

The number of nucleoside transporters can be quantified by binding experiments using a specific ligand, NBTI, for these transporters. Substances that modify the distribution of protein kinase C in cultured chromaffin cells significantly decreased the number of high-affinity binding sites for this ligand, with a parallel decrease in adenosine uptake capacity.

The experiments carried out with cellular homogenates in the presence of protein kinase C activators suggest that molecular changes to the transporters can take place. This hypothesis was supported by the direct action of purified brain protein kinase C on chromaffin cell plasma membranes, which decreased the high-affinity number of binding sites for NBTI. Taking into account the $V_{max.}$ and $B_{max.}$ values (Tables 1 and 3) for the adenosine transporters, there is a constant ratio for every effector. Thus a modified transporter loses its transport capacity and, at the same time, its capability to bind NBTI with high affinity.

The results reported here show that adenosine uptake is a highly regulated process. In chromaffin cells, one of the main functions of which is the exocytotic release of granular components, adenosine uptake is regulated by the same set of signals as the secretory event. The intracellular messages responsible for this tight co-ordination are triggered by secretagogues, protein kinase C being one of the most significant. It should be noted that chromaffin cells present purinergic P_{2y}-receptors on their plasma membranes. During exocytosis, ATP is released from the granular stores, but the extracellular levels reached in cultured cells after stimulation are below the micromolar range (Pintor *et al.*, 1991*a*,*b*). At this concentration, ATP and P_{2y}-agonists have no effect on adenosine uptake (Miras-Portugal *et al.*, 1991). In order to fully understand the regulation of adenosine uptake and its physiological significance, an extensive study of neural and non-neural tissues is necessary.

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