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Further characterization of an adenosine transport system in the mitochondrial fraction of rat testis

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Abstract

Previous work from our laboratory has demonstrated the presence of high-affinity binding sites for [^{3}H]nitrobenzylthioinosine ([^{3}H]NBTI), a marker of adenosine uptake systems, in the mitochondrial fraction of rat testis. Here, we characterize this system functionally through [^{3}H]adenosine uptake assays. This system ($K_m = 2 \pm 1.3$ μM; $V_{max} = 86.2 \pm 15.5$ pmol/mg protein/min) was found to be saturable, non sodium-dependent and sensitive to temperature, pH and osmolarity. [^{3}H]Adenosine incorporation was potently inhibited by hydroxynitrobenzylthioguanosine (HNBTG, $IC_{50} = 3$ nM) although NBTI inhibited this uptake weakly ($IC_{50} = 72.7 \pm 37.1$ μM). Dilazep > dipyridamole ≥ hexobendine inhibited [^{3}H]adenosine incorporation at low micromolar concentrations. The nucleosides inosine and uridine were weak inhibitors of this system. The adenosine receptor ligands N^6-phenylisopropyladenosine (PIA) and 2-chloroadenosine inhibited the uptake only at micromolar concentrations. Neither 5′-(*N*-ethylcarboxamido)-adenosine (NECA) nor theophylline inhibited adenosine uptake by more than 60% but the mitochodrial benzodiazepine receptor ligands 4′-chloro-diazepam (Ro 5-4864) and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl) isoquinoline carboxamide (PK 11195) were able to inhibit it. The lack of inhibition by the blockers of the mitochondrial adenine-nucleotide carrier, atractyloside and α,β-methylene-ATP, indicates that [^{3}H]adenosine uptake occurs via a transporter other than this carrier. All these results support the existence of an equilibrative adenosine transport system, which might mediate the passage of adenosine formed in the mitochondria to the cytoplasm.

Keywords: [^{3}H]Adenosine; Adenosine uptake; Mitochondrion; Testis, rat; Benzodiazepine receptor ligand, mitochondrial

1. Introduction

Adenosine is involved in the regulation of a variety of physiological processes in multicellular organisms. In the central nervous system (CNS), adenosine has been established as an important modulator of neuronal activity (see Fredholm, 1995 for a review). Adenosine and adenosine analogues have well-known neuroprotective (reviewed by Rudolphi et al., 1992), cardioprotective (Ely and Berne, 1992; Poucher et al., 1994) and vasodilator (Tucker and Linden, 1993) actions. In addition, adenosine inhibits platelet aggregation (Hourani, 1996) and is involved in the regulation of metabolism (Oetjen et al., 1990) in asthmatic broncoconstriction (Driver et al., 1993), in pain (Burnstock, 1996; Ledent et al, 1997), in activation of steroidogenesis (Wolff and Cook, 1977) and in sperm motility (Vijayaraghavan and Hoskins, 1986; Shen et al., 1993).

Central and peripheral effects of adenosine acting extracellularly are mediated through its interaction with membrane P_1 purinoceptors. These receptors were initially classified into A_1 and A_2 (A_{2A} and A_{2B}) receptors, which inhibit and stimulate adenylate cyclase, respectively. More recently, another subtype of adenosine receptor (A_3), coupled negatively to adenylate cyclase, was cloned (Zhou et al., 1992) and, although this receptor was first hypothesized to be involved in reproduction, it was later found to induce mast cell degranulation (Ramkumar et al., 1993) and therefore, is likely to have a role in allergic processes rather than in reproduction. The use of antisense oligonucleotides and gene-knockout approaches has recently helped to determine the function of each subtype of receptor (Nyce, 1999).

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Among the variety of tissues in which they are present, A_1 adenosine receptors have been cloned (Nakata, 1990) and characterized in rat (Murphy et al., 1983; Rivkees, 1994) and bovine testis (Cushing et al., 1988), where they show a preferential localization in the Sertoli cells of the seminiferous tubules (Monaco and Conti, 1986). Also, a high level of expression of adenosine A_3 receptors has been found in rat testis (Zhou et al., 1992).

The actions of released adenosine are widely considered to be primarily terminated by a rapid, avid removal mechanism for adenosine, namely, the nucleoside transport system, and therefore, inhibition of nucleoside transport may potentiate the beneficial actions of adenosine. There is a heterogeneity of nucleoside transporters in mammalian cells and a classification into equilibrative and concentrative has been established (see Jennings et al., 1998 for a review). Also, equilibrative transporters can be distinguished by their sensitivity to nitrobenzylthioinosine (NBTI) (Belt, 1983; Jennings et al., 1998) and sub-classified into equilibrative-sensitive and equilibrative-insentitive transporters. The NBTI-sensitivity of adenosine transport correlates well with high-affinity [^{3}H]NBTI binding (Jarvis and Young, 1982). Therefore, NBTI binding can be used as a tool to study functional NBTI-sensitive nucleoside transporters (Marangos et al., 1982).

Previous studies from our group (Camins et al., 1994) demonstrated that binding of mitochondrial benzodiazepine receptor ligands such as [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 was displaced by NBTI in the mitochondrial fraction of rat and human prostate. These results suggested the presence of NBTI binding sites near the mitochondrial benzodiazepine receptor and led us to perform [^{3}H]NBTI binding experiments in the mitochondrial fraction of rat testis, a tissue with a high density of mitochondrial benzodiazepine receptors (De Souza et al., 1985) . From these experiments, we characterized high-affinity [^{3}H]NBTI binding sites in this preparation (Camins et al., 1996), suggesting that there is a nucleoside transport system in rat testis mitochondria.

Here, we attempt to further characterize the adenosine transporter system in the mitochondrial membrane of rat testis using a direct parameter ([^{3}H]adenosine uptake) and also, to determine pharmacologically how adenosine transporter inhibitors and other drugs affect this system. The presence of an adenosine transporter at this level could explain some of the effects of adenosine in germ cells, such as the maintenance of ATP levels (Monaco and Conti, 1986).

2. Materials and methods

2.1. Chemicals

[^{3}H]Adenosine (2,5′,8-[^{3}H]adenosine, 60.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Ro 5-4864 (4′-chloro-diazepam) was from Fluka Chemie (Buchs, Switzerland). PK 11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl) isoquinoline carboxamide) was purchased from Pharmuka Laboratories (Asnières, France). *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI), 5′-(*N*-ethylcarboxamido)-adenosine (NECA), N^6-phenylisopropyladenosine (PIA) and 6-amino-2-chloropurine riboside (2-chloroadenosine), hydroxynitrobenzylthioguanosine (HNBTG), dipyridamole, adenosine, inosine, uridine, theophylline, α,β-Me-ATP, atractyloside and choline chloride were obtained from Sigma Chemical (St. Louis, MO, USA). Dilazep was supplied by Prodesfarma (BCN, Spain) and hexobendine by Lacer (BCN, Spain). All other chemicals were purchased from commercial sources. Hexobendine, theophylline, PK-11195 and Ro 5-4864 solutions were prepared in ethanol. Dipyridamole was dissolved in ethanol/polyethylene-glycol/bidistilled water. The other compounds were dissolved in bidistilled water. All these solutions were diluted with the buffer solution to the desired concentrations. The final concentration of ethanol was maintained at $< 0.1\%$. Ethanol at this concentration had no effect on the parameters measured.

2.2. Mitochondrial preparation

The protocol concerning the use of experimental animals carried out in this work has been approved by the ethics commitee of the Universitat de Barcelona, according to the European Community guidelines. Adult male Sprague–Dawley rats weighing 250–275 g were obtained from C.E.R.J. (Le Génest, France). They were maintained under a 12-h light/dark cycle and fasted 24 h before the experiments. Animals were killed by decapitation and the testes were removed, washed and placed in ice-cold homogenization buffer (0.32 M sucrose and 0.01 M HEPES, pH 7.4). Mitochondrial preparation was performed as described elsewhere (Camins et al., 1996). Briefly, the organs were homogenized with a Potter–Elvejheim Teflon-glass homogenizer at setting 4. The homogenate was centrifuged at $800 \times g$ for 10 min at 4°C and the resulting supernatant was re-centrifuged at $7000 \times g$ for 10 min. The pellet was resuspended in buffer and incubated for 5 min at 37°C to minimize the endogenous adenosine content. Finally, the homogenate was washed by centrifugation at $7000 \times g$ for 10 min twice.

The final pellet was resuspended in the assay buffer (composition, in mM: NaCl, 140; KH_2PO_4, 1.5; $CaCl_2$, 1.3; Na^+-HEPES, 10; D-glucose, 5 and Mg_2Cl, 2) in a Potter–Elvejheim Teflon-glass homogenizer. Protein content was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

The enrichment and purity of the mitochondrial fraction was assessed by enzymatic assays and electronic microscopy. Briefly, in the final pellet, the activity of succinate dehydrogenase, a mitochondrial marker, was determined by the method of Earl and Korner (1965). This

activity (101.95 ± 8.05 nmol/min/mg of protein) was fourfold greater than that of the postnuclear supernatant. In the same pellet, 5′-nucleotidase activity (203 ± 32 mg P_i/h), an enzyme that occurs mainly in the cytoplasmic membrane, was only 1.5% that of the postnuclear supernatant. Electron microscopy (20,000 ×) of the final pellet was performed according to the method of Hayat (1972). This demonstrated a mitochondrial purity higher than 95% and confirmed the absence of cytoplasmic membrane fractions. Although some sperm tails were observed in this preparation, no [^{3}H]adenosine uptake was detected in a preparation of rat sperm, thus, ruling out any interference by these impurities (data not shown). This observation is consistent with those of Brown and Casillas (1984) and Shen et al. (1993), who found no significant adenosine uptake in mammalian spermatozoa.

2.3. Uptake experiments

0.1 ml of the mitochondrial preparation (containing approximately 0.4 mg of protein) was added to 0.8 ml of assay buffer. The mixture was preincubated for 30 min at 37°C, and the uptake was initiated by the addition of 0.1 ml of a solution containing non-labeled adenosine plus [^{3}H]adenosine up to a final specific activity of 1 Ci/mmol. The total (labeled plus non-labeled) adenosine concentration range in saturation uptake experiments was from 0.25 to 20 μM.

Uptake was allowed to proceed for 30 s at 37°C and was stopped by adding a ''mixture'' of the nucleoside uptake inhibitors dilazep and dipyridamole at final concentrations of 1 and 0.1 mM, respectively. The samples were immediately filtered under vacuum over Whatman GF/B glass fiber filters, pre-soaked in 0.5% polyethyleneimine. Filters were rapidly washed three times with 4 ml of ice-cold buffer and placed in vials containing 10 ml of Cocktail Biogreen 1 (Scharlau). Radioactivity was measured by liquid scintillation spectroscopy in a Beckman LS-1800 counter with an efficiency of 37%. Non-specific incorporation was determined in the presence of 10 μM unlabeled adenosine by incubation in ice water.

Na^+-dependence was estimated using an incubation medium in which NaCl and Na^+-HEPES had been isoosmotically replaced by choline chloride and Tris–HCl, respectively.

The effect of temperature on uptake was measured at a [^{3}H]adenosine concentration of 0.25 μM. In kinetic studies, the uptake reactions (containing 0.25 μM [^{3}H]adenosine) were stopped at different times. To study the effect of pH variations, uptake was performed in assay buffer whose pH had been adjusted with HCl to a value of 6, 7.4 and 8, respectively. In order to differentiate the uptake into mitochondria from binding, the osmotically active intracellular space was reduced by the addition of sucrose (up to an osmolarity of 1100 mosM) to the incubation medium (Franco et al., 1990).

2.4. Inhibition of [^{3}H]adenosine uptake

0.8 ml of increasing concentrations of the test drugs in incubation buffer was added to 100 μl of the mitochondrial suspension (containing 0.4 mg of protein) and preincubated for 30 min at 37°C. After preincubation, 100 μl of a [^{3}H]adenosine solution was added to a final concentration of 0.25 μM. For nucleoside competition (adenosine, uridine and inosine), no preincubation was performed and uptake was initiated by the addition of mitochondria to tubes containing [^{3}H]adenosine and the inhibitors at the appropriate concentration. Incubation was carried out for a further 30 s at 37°C and thereafter, the samples were processed as described in Section 2.3.

2.5. Data analysis

K_m, V_{max} and IC_{50} values were determined by non-linear regression analysis using the GraphPAD Prism program (GraphPAD software, San Diego, CA). The same software was used to determine the best fitting curve (1 or 2 components). All results are expressed as means ± standard error of the mean (S.E.M.) from three to six different experiments carried out in duplicate. Differences between values were estimated using one-way analysis of variance (ANOVA); a P-value < 0.05 was considered as significant.

3. Results

3.1. Time course of [^{3}H]adenosine uptake

Optimal incubation time was determined by measuring the time course of [^{3}H]adenosine uptake at a concentration of 0.25 μM. Uptake was linear ($r = 0.993$, $n = 5$) for the first 8 min (Fig. 1). For subsequent the experiments, an incubation time of 30 s was used.

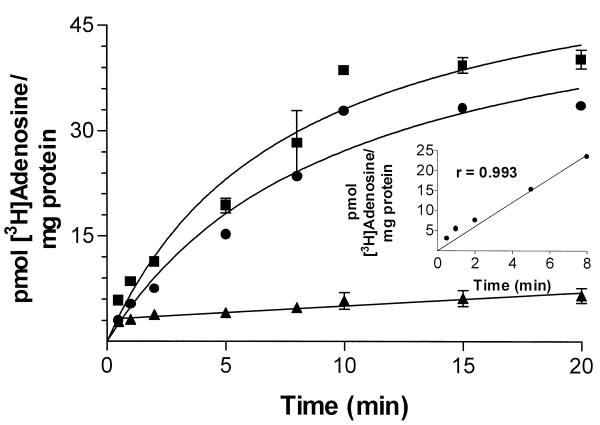

Fig. 1. [^{3}H]Adenosine accumulation by rat testis mitochondria as a function of incubation time. 0.4 mg of protein was added to 0.25 μM [^{3}H]adenosine and uptake was allowed for different incubation periods ranging from 30 s to 20 min. (■) Total uptake, (●) specific uptake, (▲) non-specific uptake. The inset shows the linear regression of the specifically incorporated [^{3}H]adenosine during the first 8 min.

3.2. Saturation uptake of [^{3}H]adenosine into rat testis mitochondria

Non-linear regression of saturation curves (Fig. 2A) gave a K_m value of 2 ± 1.3 μM and a V_{max} of 86.2 ± 15.5 pmol/mg protein/min. These experiments demonstrated that [^{3}H]adenosine uptake in this preparation has a single ($P > 0.05$ vs. a two-component fit) and a saturable component which follows the Michaelis–Menten kinetics. The linear Lineweaver–Burk plot (Fig. 2B) corroborated the presence of a single-component high-affinity transport system.

3.3. [^{3}H]adenosine uptake in the absence of sodium

The incorporation of [^{3}H]adenosine in rat testis mitochondria in an incubation medium where NaCl and Na^+-HEPES had been iso-osmotically replaced by choline chloride and Tris–HCl, respectively, did not differ significantly from that obtained in the presence of Na^+ (Fig. 2A). The K_m and V_{max} values were 2.7 ± 0.5 μM and 83.6 ± 12.7 pmol/mg protein/min, respectively ($P > 0.05$ compared to the values in the presence of Na^+).

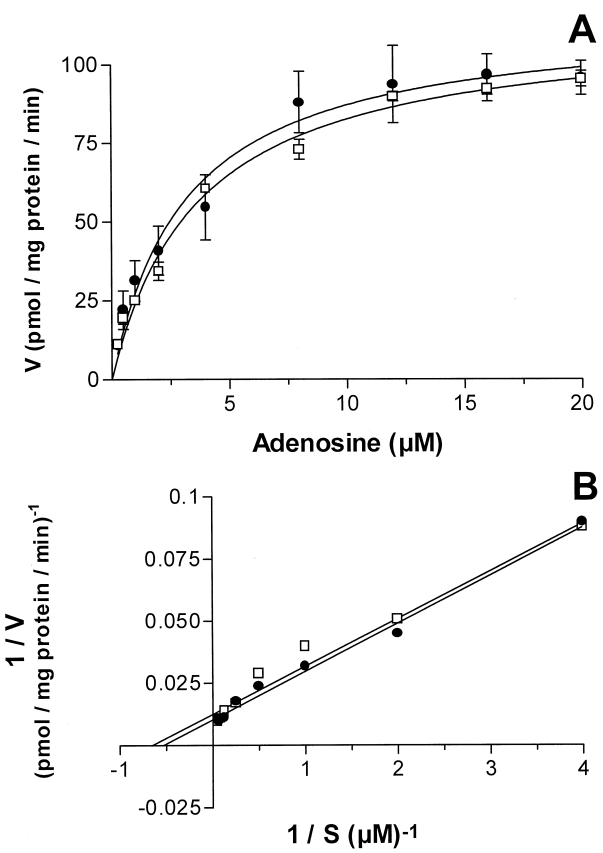

Fig. 2. Saturation uptake of [^{3}H]adenosine into rat testis mitochondria in the presence and in the absence of sodium. 0.4 mg of protein was incubated for 30 s at 37°C with increasing concentrations of [^{3}H]adenosine (0.25–20 μM). Saturation experiments (panel A) were performed in a standard incubation medium containing sodium (●) (see Section 2) or in a sodium-free medium (□). Panel B shows the Lineweaver–Burk plots of data from these experiments.

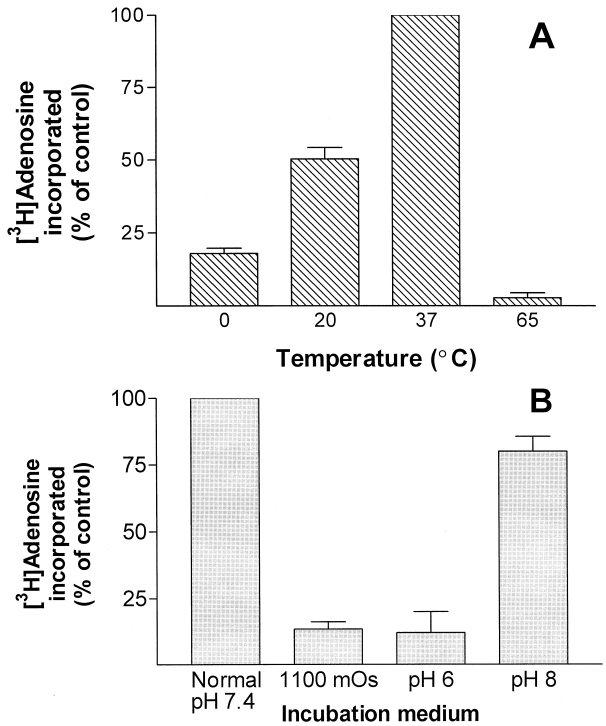

Fig. 3. Effect of temperature (A), pH and osmolarity (B) on [^{3}H]adenosine uptake in the mitochondrial fraction of rat testis. Mitchondria were incubated with 0.25 μM [^{3}H]adenosine as described in Section 2 at different temperatures, pH or in a high-osmolarity medium, respectively. Values are the mean of three separate experiments performed each in duplicate and are expressed as a percentage of the uptake at 37°C.

3.4. Temperature dependence

[^{3}H]adenosine uptake in the mitochondrial fraction of rat testis was reduced by 82% when the incubation was carried out at 0°C. The uptake increased gradually up to 37°C. A further increase in the incubation temperature to 65°C decreased adenosine uptake, which was almost completely abolished (Fig. 3A).

3.5. Effect of osmolarity and pH

The osmolarity of the medium was increased by the addition of sucrose. At the highest osmolarity tested (1100 mosM), the transport was only 13% of that obtained in the normal assay conditions (Fig. 3B). As far as changes in pH is concerned, uptake was maximum at pH 7.4 (100%), slightly decreased at pH 8 (80% of control) and minimum at pH 6 (12% of control) (Fig. 3B).

3.6. Inhibition of adenosine uptake

Several compounds that affect adenosine bioavailability were used to characterize pharmacologically the adenosine

uptake in rat testis mitochondria. Inhibition curves are represented in Fig. 4 and the inhibition constants (IC_{50}) and maximal inhibition values are given in Table 1.

As potent inhibitors of certain nucleoside transporters, NBTI and HNBTG were tested on [^{3}H]adenosine uptake (Fig. 4A). Surprisingly, of these two compounds, only HNBTG inhibited [^{3}H]adenosine uptake potently (IC_{50} around 3 nM), while inhibition by NBTI was at micromolar concentrations (IC_{50} around 73 μM).

Other putative inhibitors of adenosine uptake were tested in a concentration range from 10 nM to 100 μM (Fig. 4B). According to their IC_{50} values, the rank order of potency was: dilazep > dipyridamole ≥ hexobendine.

Inosine and uridine, which are substrates for several nucleoside transport systems, were also tested. Inhibition curves for [^{3}H]adenosine uptake (Fig. 4C) revealed that inosine was a better inhibitor than uridine, which only had a maximal inhibition of about 65%. As expected, adenosine completely inhibited [^{3}H]adenosine uptake, with an IC_{50} value of 1.04 ± 0.32 μM, which is in agreement with its K_m value.

Of the specific adenosine A_1 and A_2 receptor ligands tested in our experiments, PIA (adenosine A_1 receptor agonist) and 2-chloroadenosine (adenosine A_1/A_2 receptor agonist), but not NECA (adenosine A_1/A_2 receptor agonist), inhibited adenosine uptake only at micromolar

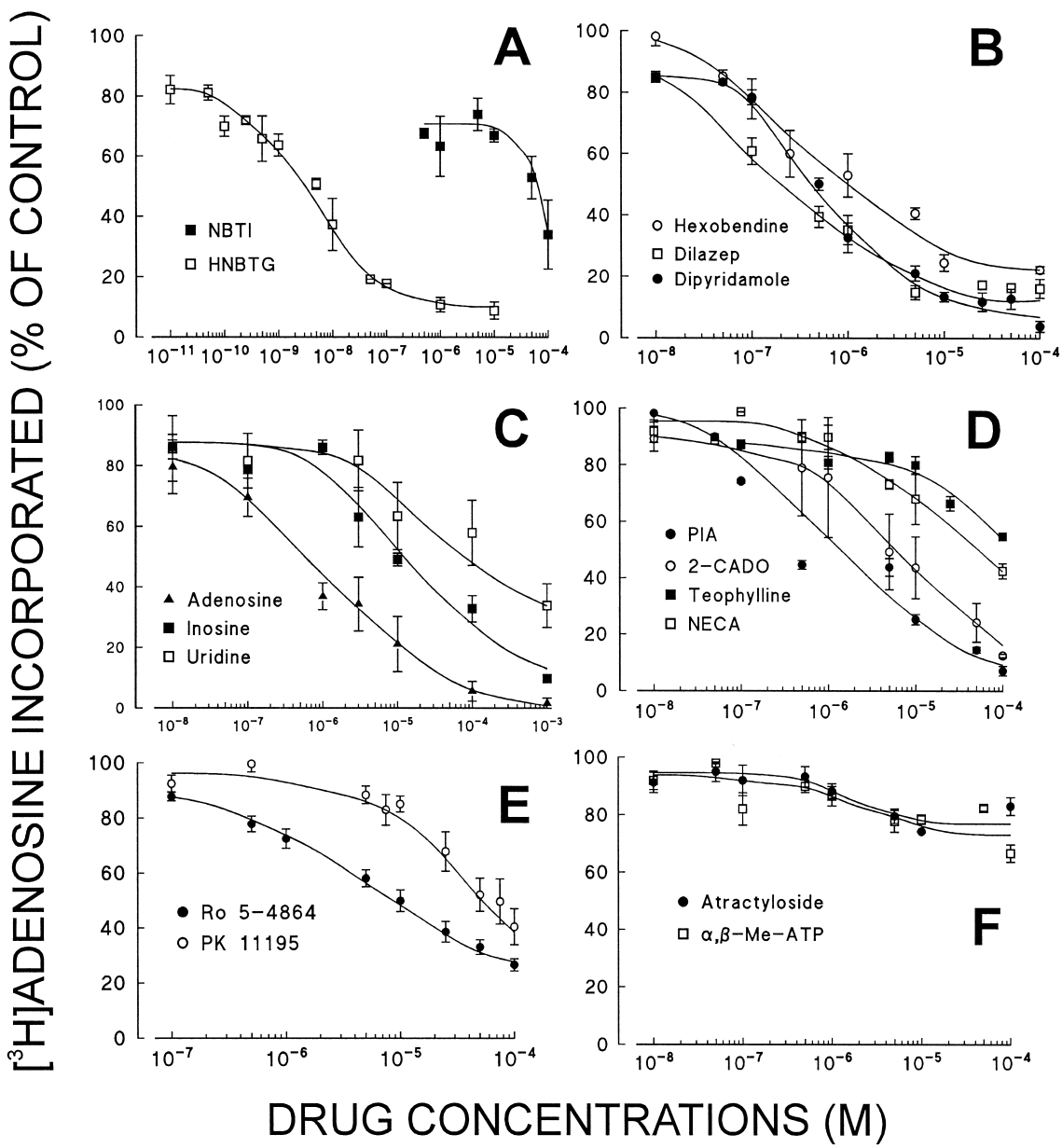

Fig. 4. Inhibition curves of the different compounds tested on [^{3}H]adenosine uptake in the mitochondrial fraction of rat testis. 0.4 mg of protein was preincubated 30 min at 37°C with a range of concentrations of the inhibitor. After preincubation, 0.25 μM [^{3}H]adenosine was added to each tube and uptake was carried out for a further 30 s before being stopped and the reaction mixture filtered and measured. Points are the mean of three or more experiments carried out in duplicate ± S.E.M. (2-CADO = 2-chloroadenosine).

Table 1
Inhibition of [^{3}H]adenosine uptake in the mitochondrial fraction of rat testis
Data are the means ± S.E.M. of three or more experiments carried out in duplicate. Maximal inhibition is that obtained at the highest concentration tested. n.d.: not determined.

Compound	IC_{50} (μM)	Maximal inhibition (%)
NBTI	72.7 ± 37.1	71
HNBTG	0.003 ± 0.0009	91
Dilazep	0.2 ± 0.1	84
Dipyridamole	0.6 ± 0.01	96
Hexobendine	0.7 ± 0.2	78
Adenosine	1 ± 0.3	100
Inosine	7.3 ± 2.9	90
Uridine	89.7 ± 38.3	66
2-chloroadenosine	7.6 ± 6.1	88
NECA	78.5 ± 12	58
PIA	2.1 ± 0.5	93
Theophylline	622 ± 20	46
PK 11195	58.3 ± 12.7	60
Ro 5-4864	12.4 ± 3.1	73
α,β-methylene-ATP	n.d.	23
Atractyloside	n.d.	18

concentrations (Fig. 4D), PIA being the most potent. Theophylline (adenosine A_1/A_2 receptor antagonist) only inhibited about 45% of adenosine uptake at the highest concentration tested (100 μM).

The inhibition of the accumulation of adenosine by the mitochondrial benzodiazepine receptor ligands Ro 5-4864 and PK 11195 was apparently non-competitive, Ro 5-4864 being about fivefold more potent than PK 11195 (Fig. 4E).

α,β-Methylene-ATP and atractyloside (two inhibitors of the ADP/ATP mitochondrial carrier) did not inhibit adenosine uptake at concentrations up to 100 μM (Fig. 4F).

4. Discussion

Nucleoside transport systems are widely distributed in living organisms but, although adenosine receptors have been characterized in rat (Murphy et al., 1983; Rivkees, 1994) and bovine (Cushing et al., 1988) testis, the presence of a mitochondrial adenosine transporter had not been reported. To our knowledge, the present study represents the first characterization of an adenosine uptake system in rat testis mitochondria. However, several works demonstrate the formation of adenosine by mitochondria from other organs and, therefore, the existence of a nucleoside transporter in the mitochondrial membrane with a homeostatic role would not be surprising. For example, it has been described that rat kidney mitochondria are able to form and degrade extraordinary amounts of adenosine (Henke et al., 1989) and part of this adenosine has been found to cross the mitochondrial membranes (Ziegler et al., 1990). In our experiments, [^{3}H]adenosine uptake was not modified by atractyloside, ruling out the possibility that transport takes place through the mitochondrial ADP/ATP carrier and supporting the existence of another transport system that is labeled by [^{3}H]NBTI.

The [^{3}H]adenosine uptake system that we have characterized in rat testis mitochondria bears a single and saturable component which follows Michaelis–Menten kinetics and has the characteristics of a high-affinity uptake system with an apparent K_m around 2 μM. This value is consistent with those obtained in other preparations, such as rat cerebral cortical synaptosomes (Bender et al., 1981) or cultured bovine chromaffin cells (Miras-Portugal et al., 1986).

The adenosine uptake in rat testis mitochondria was a temperature-dependent process. The sudden drop in the uptake rate above 37°C indicates possible denaturation of the carrier protein. These properties are similar to those reported in human erythrocyte ghosts (Schrader et al., 1972), rabbit polymorphonuclear leukocytes (Berlin, 1973) and rat brain cortical synaptosomes (Bender et al., 1981).

The experiments carried out in the absence of sodium reveal that the adenosine uptake system that we have characterized in rat testis mitochondria is independent of the sodium concentration and, therefore, it could be classified as the equilibrative type (Jennings et al., 1998). The uptake was sensitive to changes in the osmolarity of the medium, indicating that most of the radioactivity measured in the mitochondria represents transport rather than binding to the membrane surface. Also, it was maximal at pH 7.4 and was inhibited at pH 6, suggesting that this transporter works better in the normal intramitochondrial pH range than in acidotic conditions.

In order to characterize this transporter pharmacologically, several compounds were tested for [^{3}H]adenosine uptake inhibition in rat testis mitochondria. The different pattern between inhibition of mitochondrial transport and binding to adenosine receptors was assessed by the results obtained from experiments with adenosine receptor ligands. These results suggest that the identified site does not resemble adenosine receptor binding sites, because PIA and 2-chloroadenosine had significantly lower affinity (in the micromolar range) than that reported (in the nanomolar range) for their binding to adenosine receptors in rat testis (Monaco and Conti, 1986). Moreover, neither NECA nor theophylline (at least, at sub-micromolar concentrations) inhibited adenosine incorporation in our preparation, whereas they have high affinity for adenosine receptors. In addition, previous reports that NECA does not seem to be an inhibitor of the adenosine transporter in cytoplasmic membrane (Ehringer and Pérez, 1984; Wu and Phillis, 1984) are similar to our results in which this compound did not inhibit adenosine incorporation in rat testis mitochondria. Nevertheless, it has been demonstrated that adenosine receptor ligands interact with adenosine transporters at micromolar concentrations, inhibiting [^{3}H]NBTI binding in rat (Geiger et al., 1985) and guinea pig (Marangos and Deckert, 1987) brain. Also, 2-chloroadenosine (Jarvis et al., 1985) and PIA (Blazynski, 1991)

have been found to be substrates for NBTI-sensitive nucleoside transporters. In all these cases, the reported affinity and order of potency for these compounds are similar to those obtained in our preparation, pointing to the occurrence of an equilibrative NBTI-sensitive nucleoside transport system in rat testis mitochondria.

Atractyloside and α,β-methylene-ATP, two specific inhibitors of the ADP/ATP mitochondrial carrier, did not inhibit adenosine uptake in our preparation, indicating a specificity for nucleoside and not for nucleotide substrates. This observation rules out the possibility that the adenosine transport we measure takes place through the mitochondrial ADP/ATP carrier. This specificity was also observed among the nucleosides: after testing whether the uptake of adenosine occurred in preference to that of other nucleosides, such as inosine or uridine, we found that inosine and uridine, at the highest concentration tested, only inhibited adenosine uptake by 90% and 65%, respectively, with lower affinity than adenosine. These results indicate that adenosine was taken up in preference to other endogenous nucleosides. Similar results suggesting that adenosine is the preferred substrate for nucleoside transport have been described for rat brain synaptosomes (Wu and Phillis, 1984) and cultured chick neurones (Thampy and Barnes, 1983).

Several coronary vasodilators, which are believed to produce relaxation of vascular smooth muscle through inhibition of nucleoside transport and thus, potentiate endogenous adenosine, were effective inhibitors of the adenosine uptake in rat testis mitochondria, with an order of potency of: dilazep > dipyridamole ≥ hexobendine. This order coincides with that described in both peripheral (Kolassa et al., 1978; Mustafa, 1979) and CNS membranes (Davies and Hambley, 1983).

There is an extensive evidence that NBTI is a useful high-affinity probe for studying nucleoside transport. In human erythrocytes, there is a strict proportionality between the amount of NBTI bound to high-affinity sites and the degree of transport inhibition, suggesting that high-affinity binding may represent a specific interaction with the nucleoside transport system. Jarvis and Young (1982) observed that, in nucleoside impermeable-type sheep erythrocytes, there was an absence of NBTI binding sites, whereas they were present in nucleoside-permeable erythrocytes. This observation provides strong evidence that NBTI binds specifically to a functional nucleoside transport system. Similar observations were made by Cass et al. (1981), who indicated the absence of binding sites for NBTI in nucleoside transport-deficient mouse lymphoma cells. But, on the other hand, NBTI-insensitive uptake systems have been described, showing an absence of high-affinity [^{3}H]NBTI binding sites (Belt, 1983; Geiger et al., 1985).

We have previously described [^{3}H]NBTI high-affinity binding sites in rat testis mitochondria (Camins et al., 1996) and, therefore, we would expect inhibition of [^{3}H]adenosine incorporation by this compound. Surprisingly, in the same preparation, NBTI was unable to inhibit adenosine uptake at low concentrations but HNBTG did so with nanomolar affinity ($IC_{50} = 3$ nM). HNBTG also showed nanomolar affinity at these binding studies (K_i around 2.5 nM). From these results, it could be hypothesized that there is a transporter whose function is not inhibited by binding of NBTI. Similar observations were made by Gati et al. (1986) in a line of Novikoff hepatoma cells showing high [^{3}H]NBTI binding affinity but very low sensitivity to NBTI inhibition. Nevertheless, Sayós et al. (1994) found that, in brush border membranes from pig renal cortices, there was a high-affinity population of [^{3}H]NBTI binding sites, but also, that [^{3}H]adenosine uptake was NBTI insensitive. These authors attributed this paradox to a change in the structure of the transporter during membrane preparation, which would lead it to retain the NBTI binding but to lose the NBTI-sensitive adenosine uptake. A similar explanation could be given to our case, where sensitivity to NBTI was considerably decreased while [^{3}H]NBTI binding was conserved. This change in the structure of the transporter may be slight enough to conserve the inhibiting properties of HNBTG.

Since in previous studies we described a possible relationship between the mitochondrial benzodiazepine receptor and an adenosine uptake system (Camins et al., 1994), the effects of Ro 5-4864 and PK 11195 were tested. In our study, the inhibition of adenosine uptake by Ro 5-4864 was apparently not competitive, with an IC_{50} value in the micromolar range. This is in agreement with the results obtained by Hammond et al. (1983) in human erythrocytes. PK 11195 only inhibited 50% of adenosine uptake at a concentration of 100 μM. These results agree with those previously reported in [^{3}H]NBTI binding studies (Camins et al., 1996). The different interaction between Ro 5-4864 and PK 11195 with this uptake system could be explained by differences in their structure (Ro 5-4864 is a benzodiazepine compound and PK 11195 is an isoquinoline derivative) and also because these two drugs interact with the mitochondrial benzodiazepine receptor at distinct sites (Skowronski et al., 1987).

To sum up, the present work describes the characterization of a new and specific adenosine transport system in rat testis mitochondria. This system is apparently insensitive to NBTI although it possesses high-affinity [^{3}H]NBTI binding sites, but is highly sensitive to HNBTG, and its rate is influenced by other nucleoside transport inhibitors and peripheral benzodiazepine receptor ligands. Although further studies need to confirm the physiological role of this transporter, it can be hypothesized that it mediates the passage of the adenosine formed in the mitochondria across the mitochondrial membrane.

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