Phosphorylation of adenosine in renal brush-border membrane vesicles by an exchange reaction catalysed by adenosine kinase

Joan SAYÓS,* Carles SOLSONA,† Josefa MALLOL,* Carmen LLUIS* and Rafael FRANCO*‡

*Departament de Bioquímica i Fisiologia, Unitat de Bioquímica i Biologia Molecular A, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1, Barcelona 08028, Catalunya, Spain, and †Laboratori de Neurobiologia Cellular i Molecular, Departament de Biologia Cellular i Anatomia Patològica, Facultat de Medicina, Hospital de Bellvitge, Universitat de Barcelona, Barcelona 08071, Catalunya, Spain

Uptake of [^{3}H]adenosine in brush-border membrane (BBM) vesicles from either rat or pig kidney leads to an accumulation of intravesicular [^{3}H]AMP. The lack of significant levels of ATP and the presence of AMP in BBM indicated that a phosphotransfer between [^{3}H]adenosine and AMP occurs. The phosphotransfer activity is inhibited by iodotubercidin, which suggests that it is performed by adenosine kinase acting in an ATP-independent manner. The existence of a similar phosphotransferase activity was demonstrated in membrane-free extracts from pig kidney. From the compounds tested it was shown that a variety of mononucleotides could act as phosphate donors. The results suggest that phosphotransfer reactions may be physiologically relevant in kidney.

INTRODUCTION

Cell adenine nucleotides and their metabolites, mainly adenosine, are involved in the regulation of numerous intracellular processes in kidney (Spielman and Arend, 1991). Part of the intrarenal adenosine production comes from cellular adenosine release. In kidney (Siegel et al., 1983), as in other tissues (Meghji et al., 1988; Van den Berghe et al., 1989; Bontemps et al., 1993a), production of adenosine, inosine and hypoxanthine increases when catabolism of intracellular ATP is accelerated, for instance in hypoxia and during enhanced energy consumption. The extracellular production of adenosine is also important, particularly in renal tubules, where enzymes involved in adenosine production outside the plasma membrane act on extracellular nucleotides (Culic et al., 1990). The origin of extracellular nucleotides is unclear, though some come from the blood through glomerular filtration. Since proximal-tubular exocytic vesicles may discharge nucleotides into the luminal fluid, a significant amount may come from tubular cells by exocytosis (Culic et al., 1990). Extracellular adenosine acts on adenosine receptors (Wu and Churchill, 1985; Murray and Churchill, 1985; Palacios et al., 1987; Freissmuth et al., 1987a,b; Weber et al., 1988; Arend et al., 1988; Spielman and Arend, 1991; Blanco et al., 1992; Schwiebert et al., 1992; Olivera and López-Novoa, 1992; Olivera et al., 1992) and is then metabolized or transported into cells by means of nucleoside-specific carriers (Le Hir and Dubach, 1984; Van Waarde et al., 1992). Recently, the presence of a new type of adenosine receptor has been demonstrated in brush-border membranes (BBM) (Blanco et al., 1992). BBM from kidney proximal tubule have specific adenosine carriers, one Na^+-dependent and another Na^+-independent (Le Hir and Dubach, 1984; Franco et al., 1990).

Adenosine uptake is important not only to maintain suitable extracellular nucleoside concentrations but also in the recovery of intracellular nucleotide levels after their depletion, since recovery from hypoxia requires the restoration of intracellular ATP concentration (Weinberg and Humes, 1986; Mandel et al., 1988; Weinberg et al., 1988; Blanco et al., 1990; Cadnapaphornchai et al., 1991). Two different pathways are able to restore nucleotides. One starts from adenosine, which is phosphorylated to AMP by adenosine kinase, and the other starts from hypoxanthine and adenine, which are converted into IMP or AMP by their respective phosphoribosyltransferases. In both cases, availability of extracellular nucleosides and intracellular substrates, as ATP and phosphoriboxyl pyrophosphate, is required.

Culic et al. (1990) demonstrated the presence of ectoenzymes in rat renal-cortical BBM vesicles that produce adenosine as a final product using as substrate ATP, ADP or AMP, all of which are capable of regulating adenine nucleotide catabolism. Working with pig kidney BBM, we have previously described that exogenous ATP was rapidly degraded mainly to inosine. The presence of ecto-(adenosine deaminase) and ecto-(AMP nucleotidase) was shown, the latter being due to alkaline phosphatase activity, since it was inhibited by levamisole and orthovanadate. The absence of inhibition of the nucleotidase activity by adenosine 5′-[$\alpha\beta$-methylene]diphosphate demonstrated the absence of 5′-nucleotidase from these vesicles (Blanco et al., 1993).

The aim of this study was to investigate the uptake of adenosine in BBM vesicles of kidney proximal tubules, which have specific transport systems for the nucleoside. In a preliminary study we reported the appearance of [^{3}H]AMP from [^{3}H]adenosine in isolated BBM vesicles of proximal tubules (Sayós et al., 1991). The phosphorylation can be due to the action of adenosine kinase in the presence of ATP, or might take place in an ATP-independent manner due to a phosphotransfer exchange between intravesicular AMP and [^{3}H]adenosine. Phosphotransfer reactions between nucleosides and mononucleotides were first reported to be catalysed by 5′-nucleotidase. Recently a novel enzyme described by Garvey and Krenitsky (1992), as well as adenosine kinase itself (Bontemps et al., 1993b), have been described to be able to catalyse such a reaction. We have further studied the activity responsible for this phosphoexchange between adenosine and AMP in kidney BBM and demonstrated that the enzyme responsible is also adenosine kinase, as reported by Bontemps et al. (1993b) in liver.

Abbreviation used: BBM, brush-border membrane.

‡ To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

[^{3}H]Adenosine and Atomflow liquid-scintillation cocktail were purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). Adenosine, inosine, ATP, ADP, AMP, IMP, GMP, UMP, dAMP, tetrabutylammonium phosphate and deoxycoformycin were purchased from Sigma. For assays with 2,3-bisphosphoglycerate, different reagents were employed with identical results; they were the pentasodium salt (Sigma lot 12H7065) and the pentacyclohexylammonium salt (Sigma, Boehringer Mannheim). 5-Iodotubercidin was from Research Biochemicals Inc. Hypoxanthine, xanthine and 1,1,2-trichloroethane (Freon) were from Merck. Heptane and h.p.l.c.-grade acetonitrile were from Carlo Erba. Other reagents were the best grade available. Deionized water further purified with a Millipore Milli-Q System was used throughout.

Preparation of BBM vesicles and membrane-free extracts

BBM vesicles were isolated, from pig or Wistar male rat kidneys, by a Mg^{2+} precipitation method as described by Lin et al. (1981). Purity of preparation was assessed by the enrichment of alkaline phosphatase (EC 3.1.3.1), compared with the starting homogenate. Values of 11–15-fold enrichment were obtained. The purified vesicles were suspended in a medium containing 100 mM mannitol and 20 mM Hepes/Tris (pH 7.4). They were stored at −80 °C and, before use, were rapidly thawed in a 37 °C water bath and homogenized five times through a 23-gauge needle.

To prepare membrane-free extracts, fresh pig kidneys were obtained from the local slaughterhouse immediately after the death of the animals. Cortices were removed after kidney perfusion and dissection. Perfusion buffer (pH 7.4) contained (mM) 10 Tris, 140 NaCl, 4 KCl, 1 $CaCl_2$. Tissue was homogenized in 2 vol. of 25 mM Hepes buffer (pH 7.1) containing 20 mM KCl and 1 mM dithiothreitol (buffer A), with a Potter–Elvehjem homogenizer (500 rev./min, 5 strokes, 4 °C). Crude homogenates were centrifuged at 4 °C, 80000 *g*, 75 min. The supernatant was collected and dialysed overnight against 100 vol. of buffer A and filtered on a Sephadex G-25M column to remove all the nucleotides and other small molecules.

H.p.l.c. analysis of purine compounds

In all cases, before processing, samples were deproteinized by incubation with 1.2 M $HClO_4$ for 10 min at 0 °C. Samples were then centrifuged and the supernatants were delipidized by addition of 1 vol. of a mixture of heptane/Freon (1:4, v/v), and neutralized with 1.2 M KOH solution. The aqueous phase was removed and frozen for analysis. H.p.l.c. analysis was performed with a Shimadzu LC-6A system with a u.v. spectrophotometric detector SPD-6A and a Radiomatic radio-chromatography detector A-250X in parallel. ^{3}H-labelled purine compounds were quantified in 40 μl portions of a mixture of sample and standard solution containing ATP, ADP, AMP, IMP, adenosine, inosine, hypoxanthine and xanthine (100 μM of each), by using a 5 μm Spherisorb ODS-2 (Phase Sep) 250 mm × 4.6 mm reverse-phase column. Elution was performed in a binary gradient, composed of buffer A (30 mM KH_2PO_4, 7.5 mM tetrabutylammonium phosphate, pH 5.45) and buffer B [30 mM KH_2PO_4, 7.5 mM tetrabutylammonium phosphate, pH 7.0, and 50% (v/v) acetonitrile]. The elution was a modification of the method described by Hammer et al. (1988) and consisted of three steps. Step 1 was a 7 min linear gradient from 5% to 23% buffer B. Step 2 was an isocratic 7 min period at 23% buffer B. Step 3 was a 16 min linear gradient from 23% to 80% buffer B. Re-equilibration was carried out in two steps, a 1 min decrease to 5% buffer B and an isocratic 15 min period at 5% buffer B. The flow rate was 1 ml/min.

Determination of unlabelled purine compounds was performed in 1 ml samples of BBM (20 mg of protein/ml), which were centrifuged at 100000 *g* for 48 min at 4 °C. Pellets were extracted with 1 ml of NH_4OH containing 0.1 μM deoxycoformycin, a transition-state inhibitor of adenosine deaminase (Henderson et al., 1972). The solvent was removed by freeze-drying and the residue was dissolved in 200 μl of 1.2 M $HClO_4$. Samples were delipidized and neutralized (see above) before the chromatographic procedure, which was performed in a Kontron 450-MT h.p.l.c. system with a Kontron 430 double-wavelength detector. Purity of the peaks was assessed by measuring the ratio of absorbance at 254 and 280 nm.

[^{3}H]Adenosine uptake in BBM

This was measured in triplicate by a rapid-filtration technique, as described elsewhere (Franco et al., 1990). After filtration, vesicles were extracted with 2 M NH_4OH containing 0.1 μM deoxycoformycin. The solvent was removed by freeze-drying, and the residue was dissolved in 100 μl of 1.2 M $HClO_4$ and then delipidized and neutralized as described above. The composition of the radioactive label within the vesicles after [^{3}H]adenosine uptake was measured by h.p.l.c.

Determination of the phosphotransfer activity

Phospho-exchange activity between adenosine and a variety of phosphate donors was assayed as described by Bontemps et al. (1991). Filtered cytosolic fractions (final concn. 3 mg of protein/ml) were incubated at 37 °C in 50 mM Hepes buffer (pH 7.2) containing (unless otherwise indicated) 10 mM $MgCl_2$, 10 mM P_i and 0.1 μM deoxycoformycin, with different nucleotides (ATP, AMP, IMP, GMP, UMP and dAMP) as phosphate donors and 2 μM [^{3}H]adenosine as phosphate acceptor. The reaction was initiated by addition of 10 μl of cytosolic fraction in final volume of 50 μl and was terminated by addition of 200 μl of 1.2 M $HClO_4$. The samples were deproteinized and delipidized, and purine compounds were analysed by h.p.l.c. as described above. At the lowest substrate concentrations indicated in Figures 2 and 3, the [^{3}H]AMP formation was linear between 0 and 30 min.

Protein and ATP determinations

Protein was measured by the bicinchoninic acid (BCA) method (Pierce), as described by Sorensen and Brodbeck (1986). ATP was assayed by the luciferin–luciferase method, using a photomultiplier-recorder system as described elsewhere (Solsona et al., 1991). The buffer solution, containing 50 mM H_2PO_4 and 5 mM $MgSO_4$ was adjusted with NaOH to pH 7.4. A firefly vial extract (Sigma) was diluted with 2 ml of double-distilled water, D-luciferin (Sigma) was added to the suspension at a final concentration of 2.5 mg/ml, and centrifuged for 5 min in a bench microfuge. A portion (10–20 μl) of the luciferin–luciferase suspension was added to a haemolysis tube containing 0.5 ml of 2% Triton X-100 in buffer solution. The tube was placed into the recording chamber and, when a baseline was reached, the vesicles suspension was added. If there is ATP entrapped in the vesicles, a immediate emission of light is produced. Portions (1 pmol and 5 pmol) of standard ATP solution were added and the amount of

Table 1 Metabolism of [^{3}H]adenosine in rat and pig BBM

BBM (4 mg of protein/ml) were incubated (37 °C, 20 min) with 2.4 μM [^{3}H]adenosine in 20 mM Hepes/100 mM mannitol buffer, pH 7.4, in the absence (Control) or presence of 1 μM deoxycoformycin (DCF), 5 μM iodotubercidin (ITu) or both (DCF + ITu). Samples were processed as described in the Materials and methods section and the tritiated compounds were separated and quantified. Abbreviations: Hyp, hypoxanthine; Ino, inosine; Ado, adenosine. Values are means ± S.D. of three separate experiments (n.d., not detectable).

		[^{3}H]Metabolites formed (pmol/mg of protein)			
		Hyp	Ino	Ado	AMP
Rat	Control	8.3 ± 0.2	2.9 ± 0.1	n.d.	19.6 ± 0.8
	DCF (1 μM)	5.7 ± 0.2	1.7 ± 0.1	3.8 ± 0.4	33 ± 1
	ITu (5 μM)	11.2 ± 0.5	1.5 ± 0.1	n.d.	0.10 ± 0.01
	DCF (1 μM) + ITu (5 μM)	7.7 ± 0.2	1.9 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
Pig	Control	2.6 ± 0.7	27.2 ± 0.2	n.d.	2.1 ± 0.1
	DCF (1 μM)	4.5 ± 0.2	2.7 ± 0.2	8.1 ± 0.4	30 ± 2
	ITu (5 μM)	25 ± 2	2.7 ± 0.1	n.d.	0.5 ± 0.1
	DCF (1 μM) + ITu (5 μM)	5.3 ± 0.1	3.1 ± 0.2	8.7 ± 0.6	1.5 ± 0.2

ATP entrapped in vesicles was calculated by linear regression (the amplitude of the peak of light emission is proportional to the ATP content). This method led us to measure on-line the presence of ATP in the vesicles suspension instantly, so minimizing the possible breakdown by extravesicular ATPases.

Analysis of kinetic data

Kinetic data were analysed by non-linear regression with the ENZFITTER program (Elsevier Biosoft) or other programs developed in our laboratory (Canela, 1984; López-Cabrera et al., 1988), by using the equations corresponding to either Michaelis–Menten or co-operative behaviour. In the latter case the equation employed was:

$$v = (VS^h)/(S_{0.5} + S^h)$$

where v is the initial reaction rate, V the maximum rate, h the Hill coefficient and $S_{0.5}$ the substrate concentration at which half of the maximum velocity is attained.

Five replicates of each point were performed. Goodness of fit was tested according to reduced χ^2 or S.D. values given by the programs. A modified F test was used to analyse whether a co-operative model significantly improved on the fit to the Michaelis–Menten model.

RESULTS

Intravesicular metabolism of adenosine was assessed by incubating (35 min) rat renal BBM vesicles (3–4 mg/ml) with 2.4 μM [^{3}H]adenosine. The components of the mixture were isolated and quantified by h.p.l.c. (see the Materials and methods section). The results are presented in Table 1. The intravesicular metabolism of adenosine in rat BBM isolated and incubated in the absence of metabolic inhibitors indicates an accumulation of [^{3}H]AMP as well as the presence of [^{3}H]inosine and [^{3}H]hypoxanthine. When rat BBM were preincubated with deoxycoformycin as a potent adenosine deaminase inhibitor, the accumulation of [^{3}H]AMP was greatly enhanced and intravesicular [^{3}H]adenosine was detected. In this case the remaining purine compounds were found at the same level as in control BBM. To test the participation of adenosine kinase in the phosphorylation of adenosine to AMP, the same set of experiments were repeated in the presence of an inhibitor of the enzyme, iodotubercidin. As indicated in Table 1, the presence of iodotubercidin, either alone or in combination with deoxycoformycin, completely prevented the formation of intravesicular [^{3}H]AMP. This indicated that adenosine kinase is the enzyme responsible for the appearance of this [^{3}H]AMP coming from extravesicular [^{3}H]adenosine. Similar experiments were performed with BBM isolated from pig cortical membranes (Table 1). In pig BBM the production of [^{3}H]AMP from [^{3}H]adenosine was lower than the production obtained with rat BBM. This could be due to the higher amount of adenosine deaminase present in pig BBM, compared with rat BBM (Blanco et al., 1993). It is likely that a large amount of [^{3}H]adenosine is converted into [^{3}H]inosine before it can be taken up by the vesicles. In the presence of deoxycoformycin the level of [^{3}H]AMP formed is relatively high and comparable with that found in rat BBM. Since the level of [^{3}H]AMP was severely decreased in the presence of iodotubercidin, the enzyme responsible for the phosphorylation of adenosine in pig BBM appeared to be adenosine kinase (Table 1).

The kinase activity of adenosine kinase requires ATP as energy source. To address the participation of intravesicular ATP as the energy source for the synthesis of [^{3}H]AMP, the level of nucleosides and nucleotides was measured by h.p.l.c. in BBM from pig kidney. The most abundant compounds were (pmol/mg of protein) AMP (450 ± 30), inosine (160 ± 20) and adenosine (24 ± 3). The level of ATP was less than 5 pmol/mg of protein. To confirm this low level of ATP in BBM, a more sensitive bioluminescence technique, using the luciferin/luciferase assay, was used (Table 2). The low level of ATP was confirmed (2.3–3.1 pmol/mg of protein; Table 2), which cannot account for the amount of [^{3}H]AMP formed in the experiments described above (more than 30 pmol/mg of protein). Moreover, this low level did not change upon incubation of BBM with unlabelled adenosine, which further confirms that ATP was not the direct source of phosphate (Table 2). It seems that the appearance of [^{3}H]AMP as a consequence of [^{3}H]adenosine uptake in BBM is due to a phospho-exchange reaction in which the intravesicular AMP acts as phosphate donor.

In order to determine whether this activity was exclusive to BBM, the formation of [^{3}H]AMP from [^{3}H]adenosine and unlabelled AMP was investigated in membrane-free preparations. Thus, membrane-free cytosolic extracts from pig renal cortex were extensively dialysed and filtered. In these extracts the level of ATP was undetectable, and metabolism of either adenosine or AMP was not significant in the conditions of the assays. Extracts were incubated with [^{3}H]adenosine in the presence of unlabelled AMP. The formation of [^{3}H]AMP (Figure 1a) and the dis-

Table 2 ATP content in pig BBM incubated with adenosine

BBM (4 mg of protein/ml) were incubated in the absence (0 min) or in the presence of 2.4 μM adenosine for two time intervals (15 and 30 min). ATP was determined in the incubations by the luciferin–luciferase assay as indicated in the Materials and methods section. Results, in pmol of ATP/mg of protein, are means ± S.E.M. of three separate experiments. Abbreviation: DCF, deoxycoformycin.

	ATP (pmol/mg)		
Time of incubation (min)...	0	15	30
Control BBM	3.1 ± 0.5	2.6 ± 0.4	2.5 ± 0.7
BBM obtained in presence of 0.1 μM DCF	2.3 ± 0.8	1.9 ± 0.2	1.3 ± 0.3

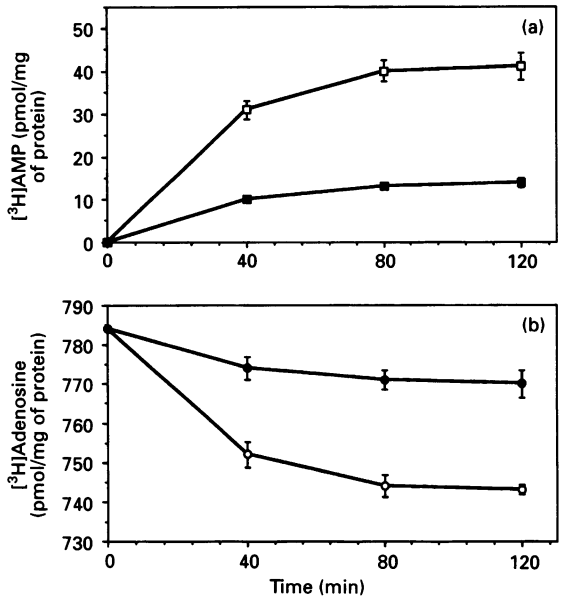

Figure 1 Time-dependence of the phospho-exchange reaction between [^{3}H]adenosine and AMP in membrane-free extracts from pig kidney

Membrane-free cytosolic extracts (3 mg of protein/ml) from renal cortex were dialysed and filtered, and then incubated (37 °C) with 1 mM AMP and 2 μM [^{3}H]adenosine in 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM P_i and 0.1 μM deoxycoformycin. The formation of [^{3}H]AMP (**a**) and the disappearance of [^{3}H]adenosine (**b**) in the absence (white symbols) or presence (black symbols) of 10 μM iodotubercidin are displayed. Values are means ± S.D. of three separate experiments.

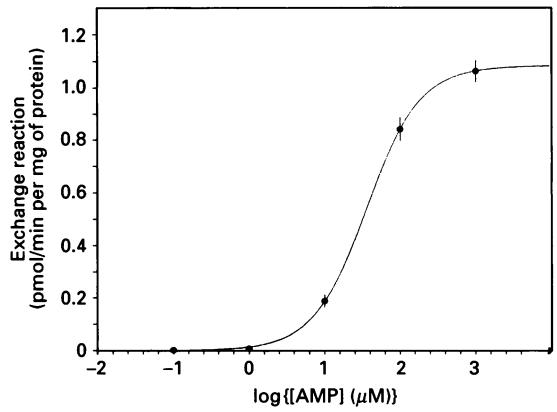

Figure 2 AMP saturation curve for the phospho-exchange reaction between [^{3}H]adenosine and AMP

Extracts (3 mg of protein/ml) extensively dialysed and filtered were incubated (37 °C, 20 min) with 2 μM [^{3}H]adenosine in 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM P_i, 0.1 μM deoxycoformycin and variable concentrations of AMP. Values are means ± S.D. of single experiments in triplicate.

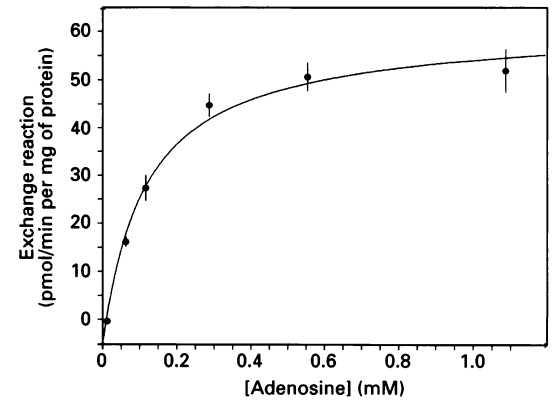

Figure 3 Adenosine saturation curve for the phospho-exchange reaction between [^{3}H]adenosine and AMP

Extracts (3 mg of protein/ml) were dialysed and filtered, and then incubated (37 °C, 20 min) with variable concentrations of [^{3}H]adenosine in 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM P_i, 0.1 μM deoxycoformycin and 1 mM AMP. Values are means ± S.D. of single experiments in triplicate.

appearance of [^{3}H]adenosine (Figure 1a) were measured by h.p.l.c. As shown in Figure 1(b), the appearance of [^{3}H]AMP was analogous to the disappearance of [^{3}H]adenosine (Figure 1b), which indicates that the phosphotransfer reaction between AMP and adenosine took place. The inhibition of such transphosphorylation between AMP and adenosine by iodotubercidin (Figure 1) indicates that adenosine kinase is the enzyme responsible for such activity in kidney homogenates. Since the homogenates were extensively dialysed, adenosine kinase was acting in an ATP-independent manner, as in BBM. Upon variation of the concentration of the phosphate donor there was an increase in the appearance of the radiolabelled mononucleotide (Figure 2). The curve shown in Figure 2 is sigmoid, with an apparent $s_{0.5}$ value of 82 ± 5 μM and a Hill coefficient (h) 1.23 ± 0.02. The concentration-dependence of adenosine in the presence of 1 mM AMP gave a Michaelis–Menten curve, with an apparent K_m for adenosine of 120 ± 20 μM (Figure 3).

To address the question of whether such transphosphorylation, which in appearance is a futile cycle (AMP + adenosine = adenosine + AMP), has a physiologically relevant role, experiments were performed in which dAMP, IMP, GMP and UMP replaced AMP as possible phosphate donors. As indicated in Table 3, all of them but UMP acted as phosphate donors. Since 2,3-bisphosphoglycerate affects a number of phosphohydrolytic and phosphotransfer reactions (Worku and Newby, 1982; Bontemps et al., 1988, 1989, 1991; Tozzi et al., 1991; Garvey and Krenitsky, 1992), the effect of the compound was analysed when assaying the phosphotransfer reaction between AMP and

Table 3 Mononucleotides as phosphate donors for the phosphotransfer reaction

Extracts (3 mg of protein/ml), extensively dialysed and filtered, were incubated (37 °C, 20 min) with 2 μM [^{3}H]adenosine in 50 mM Hepes buffer, pH 7.2, containing 10 mM $MgCl_2$, 10 mM P_i, 0.1 μM deoxycoformycin and 1 mM of the mononucleotides indicated. Samples were processed as described in the Materials and methods section and [^{3}H]AMP was measured. Values are means ± S.D. of three separate experiments.

	[^{3}H]AMP formed (pmol/mg of protein)
AMP	143 ± 10
IMP	42 ± 3
GMP	161 ± 13
dAMP	30 ± 2
UMP	2.0 ± 0.1

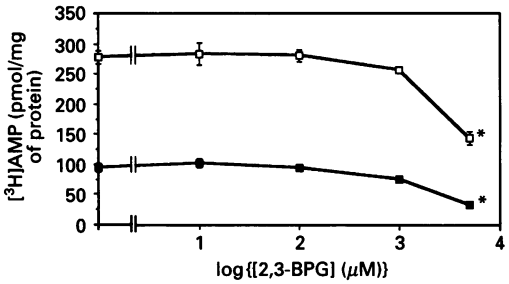

Figure 4 Effect of 2,3-bisphosphoglycerate on the phospho-exchange reaction between [^{3}H]adenosine and AMP

Extracts (3 mg of protein/ml) were extensively dialysed and filtered, and then incubated, in 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM P_i and 0.1 μM deoxycoformycin, with 2 μM [^{3}H]adenosine, AMP [10 (■) and 100 (□) μM] and variable amounts of 2,3-bisphosphoglycerate (2,3-BPG). Values are means ± S.D. of three separate experiments (*$P < 0.001$).

Table 4 Production of [^{3}H]AMP, [^{3}H]ADP and [^{3}H]ATP in renal extracts incubated with [^{3}H]adenosine and ATP

Extensively dialysed and filtered extracts (3 mg of protein/ml) were incubated (37 °C, 60 min) with 2 μM [^{3}H]adenosine in 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM P_i, 0.1 μM deoxycoformycin and 1 mM ATP. Samples were processed as described in the Materials and methods section and ^{3}H-labelled adenosine, AMP, ADP and ATP were separated and quantified. Values are means ± S.D. of three separate experiments.

	Content (pmol/mg protein)
[^{3}H]ATP	419 ± 12
[^{3}H]ADP	234 ± 17
[^{3}H]AMP	166 ± 10
[^{3}H]Adenosine	31 ± 2

[^{3}H]adenosine. The compound when assayed in the absence of AMP did not produce [^{3}H]AMP from [^{3}H]adenosine, indicating that 2,3-bisphosphoglycerate was not acting as a substrate. Moreover, when assayed in the presence of AMP (10 and 100 μM), 2,3-bisphosphoglycerate (100 μM–1 mM) did not appreciably modify the quantity of [^{3}H]AMP formed from [^{3}H]adenosine. At higher concentrations (5 mM) 2,3-bisphosphoglycerate inhibited the phosphotransfer reaction (Figure 4).

The metabolism of [^{3}H]adenosine in cytosolic extracts was measured in the presence of ATP. In such conditions the pattern of formation of [^{3}H]adenine nucleotides was completely different from that obtained in the presence of AMP. Thus a high proportion of [^{3}H]ADP and [^{3}H]ATP species were formed (Table 4), which did not appreciably change when Mg^{2+} was omitted from the assays. Thus, metabolism in the presence and the absence of ATP differs in that added Mg^{2+} did not affect the ATP-dependent reaction(s), whereas it was necessary for the iodotubercidin-sensitive phosphotransferase activity. In fact, the absence of Mg^{2+} from the incubation medium did not lead to detectable levels of [^{3}H]AMP when extracts were incubated with [^{3}H]adenosine and unlabelled AMP under the conditions described in Figure 1.

DISCUSSION

The study of [^{3}H]adenosine metabolism upon incorporation by BBM from renal cortex led to the finding of a high proportion of intravesicular [^{3}H]AMP. This high proportion of AMP was more pronounced when adenosine deaminase, present on the outer surface of BBM (Blanco et al., 1993), was inhibited.

The enzyme responsible for the conversion of [^{3}H]adenosine into [^{3}H]AMP appears to be adenosine kinase, since it is inhibited by the specific inhibitor iodotubercidin. However, adenosine kinase does not act through the phosphorylation of adenosine by means of ATP. In intact BBM the low level of ATP, which did not change on incubation of BBM with adenosine, could not account for the production of [^{3}H]AMP via kinase activity. In contrast, BBM had high levels of AMP (450 pmol/mg of protein), which could account for a phosphotransfer activity. Furthermore, this phospho-exchange activity was demonstrated in the membrane-free cytosolic fraction which, due to the extensive dialysis performed plus the filtration through Sephadex G-25, did not contain ATP. The different pattern of nucleotide formation obtained when ATP was present in the assays is further proof that the appearance of [^{3}H]AMP from adenosine (in the absence of ATP) was different from a kinase activity of adenosine kinase.

A phosphotransferase activity due to 5′-nucleotidase has been described in different systems (Tozzi et al., 1991; Worku and Newby, 1982). In our case, 5′-nucleotidase cannot be the enzyme responsible for the phosphotransferase activity found in renal BBM, since the enzyme is not present in these vesicles. Extravesicular mononucleotides may be hydrolysed in BBM, but this is due to the presence of unspecific alkaline phosphatases (Blanco et al., 1993). On the other hand, a cocktail of alkaline phosphatase inhibitors (levamisole and ortovanadate) did not modify the levels of intravesicular [^{3}H]AMP obtained from extravesicular [^{3}H]adenosine (results not shown). The novel activity described by Garvey and Krenitsky (1992) in placenta appears to be different from adenosine kinase, but, interestingly, it has several common features with the phosphotransfer activity reported here for kidney. Thus both activities (i) showed similar K_m (adenosine) values in the range 120–200 mM, (ii) were dependent on Mg^{2+} and (iii) were not due to soluble 5′-nucleotidase. Apart from the inhibition by iodotubercidin and the lack of stimulation by 2,3-bisphosphoglycerate, they differ in the hydrolysis of AMP due to the novel enzyme in the absence of adenosine (Garvey and

Krenitsky, 1992), which is not appreciable in the kidney-cortex cytosolic fraction.

The comparison of our data in kidney with those reported by Bontemps et al. (1991) in liver suggests that the phosphotransfer activity is similar in both tissues. In a recent study which complements their previous report, Bontemps et al. (1993b) demonstrate that adenosine is phosphorylated in anoxic hepatocytes which are depleted of ATP. The same activity was found in rat liver cytosol extracts in which ATP was carefully and completely removed. Analysing the inhibitory effect of both iodotubercidin, an inhibitor of adenosine kinase, and diadenosine tetraphosphate, a potent stimulator of 5′-nucleotidase, the authors conclude that adenosine kinase is in fact responsible for both reactions: the kinase activity in the presence of ATP, and the phosphotransfer between AMP and adenosine in the absence of ATP. The presence in kidney extracts of high amounts of hydrolytic activity towards diadenosine tetraphosphate (Blanco et al., 1992), did not allow us to test the compound as a modulator of the phospho-exchange activity. It should be noted that 2,3-bisphosphoglycerate, which so far has ben reported to be a potent stimulator of phosphotransfer activities reported in human colon carcinoma (Tozzi et al., 1991) and human placenta (Garvey and Krentisky, 1992), also activated the phospho-exchange reaction catalysed by adenosine kinase in rat liver (Bontemps et al., 1993b). This compound had no effect at concentrations up to 1 mM, whereas at 5 mM it inhibited the phosphotransfer reaction in pig kidney extracts (Figure 4). This suggests that, in phospho-exchange activities due to adenosine kinase, there are either species differences or tissue-specific forms which lead to enzyme molecules that differ in their sensitivity towards 2,3-bisphosphoglycerate.

As Garvey and Krenitsky (1992) report, adenosine phosphotransferases have K_m values far above the intracellular concentration of their substrates (adenosine and AMP), at least in normoxia. Thus the physiological significance of such a reaction is unclear. One possible role, derived from the fact that IMP and GMP are phosphate donors, is that phosphotransfers may be important in hypoxia for recovery of adenine nucleotide levels by the capture by adenosine of the phosphate present in a variety of mononucleotides. This opens new perspectives for understanding the role of these phosphotransferase activities, which could handle a wide range of phosphorylated compounds as phosphate donors. In fact, as Bontemps et al. (1993b) point out, the incorporation of labelled adenosine into adenine nucleotides should not be considered to be proof of kinase activity of adenosine kinase in anoxia.

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