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The stepwise hydrolysis of adenine nucleotides by ectoenzymes of rat renal brush-border membranes

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Evidence is presented for the existence of ectoenzymes in rat renal cortical brush-border membrane vesicles that produce adenosine as a final product using either ATP, ADP or AMP as substrate. The enzymes are insensitive to levamisole, ouabain, oligomycin and *N*-ethylmaleimide, and have absolute requirement for divalent cations with following order of activation $Mg^{2+} > Ca^{2+} > Mn^{2+} > Ba^{2+} > Zn^{2+}$. At least two separate enzymes can be distinguished. One is capable of hydrolyzing ATP, other nucleoside triphosphates and ADP, but not AMP. The enzyme is insensitive to concanavalin A. The other enzyme hydrolyzes AMP and is strongly inhibited by this lectin. Mg^{2+}-stimulated ATP hydrolysis displays saturation kinetics which is not of the simple Michaelis-Menten type, but is biphasic with a high-affinity ($K'_m = 0.16$ mM) and low-affinity site ($K'_m = 9.0$ mM), respectively. The low-affinity site hydrolyzes ATP, ITP and GTP to a similar extent, whereas CTP and UTP with about 40% lower rate. The high-affinity site splits ATP much better than other nucleoside triphosphates. Hydrolysis of ADP follows simple Michaelis-Menten saturation kinetic with apparent $K_m = 0.38 \pm 0.06$ mM. Inhibition, activation and substrate specificity studies indicate that nucleoside triphosphatase and nucleoside diphosphatase may reside on the same protein. Kinetics of the AMP hydrolysis is hyperbolic with apparent $K_m = 76 \pm 9$ μM. The cascade of ectonucleotidases in the brush-border membrane of the proximal tubule may catalyze the degradation of filtered nucleotides into adenosine and phosphate, the compounds which are thereafter probably reabsorbed by separate transport systems.

Introduction

The diverse biological activities of adenosine have been recognized for many years. It is accepted that adenosine and adenine nucleotides influence various biological activities such as: rate of blood flow in heart [1], vascular tonus, neurotransmitter release in cholinergic synapses [2], platelet aggregation [3] and lipolysis in adipose tissue [4].

Little information exists about the regulation of adenosine production in kidney, although it is known that adenosine affects various renal physiological functions including blood flow, glomerular filtration rate, release of renin and transport of electrolytes [5,6]. The intrarenal production of adenosine increases under circumstances when catabolism of ATP is accelerated, i.e., in hypoxia and during enhanced energy consumption [7]. Enzymes involved in adenosine production are located on the external side of the plasma membrane and their principal substrates are extracellular nucleotides.

Sequential degradation of extracellular ATP through stepwise reactions ATP → ADP → AMP → adenosine, by separate ectoenzymes, namely by nucleoside triphosphatase (EC 3.6.1.15), nucleoside diphosphatase (EC 3.6.1.6) and 5′-nucleotidase (EC 3.1.3.5), has been extensively studied in cholinergic synapses [2], endothelial and smooth muscle cells [8,9].

However, in kidney successive order of hydrolytic reactions is hardly operative since renal 5′-nucleotidase is strongly inhibited by ATP and ADP, as found by Le Hir et al. [10,11]. Since the degree of inhibition is

Abbreviations: Con A, concanavalin A; DCCD, *N,N′*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; DEP, diethyl pyrocarbonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide.

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influenced by the concentration of ATP and ADP, a different rate of adenosine production could be observed depending on the condition studied.

It is supposed that ectoenzyme responsible for ATP and ADP catabolism in kidney is Mg^{2+}- or Ca^{2+}-stimulated nucleotidase. Similar enzyme activities have been reported in plasma membranes, mitochondria, lysosomes, endoplasmic reticulum from skeletal, cardiac, and smooth muscle, liver lung, placenta and pancreas [12].

There has been a grat deal of uncertainty about the exact location of the Ca^{2+}/Mg^{2+}-ATPase in the membranes of proximal tubular cells. Such an enzyme has been located in either basolateral membranes from rat [13], or in brush border membranes of rat kidney cortex from pig [12] and rabbit [14]. However, the evidence has been presented for both basolateral and brush-border membrane location, for pig [15] and rat kidney cortex [16]. Studies with isolated rabbit kidney tubules indicate that Ca^{2+}- or Mg^{2+}-ATPase activity is present along the entire nephron, with maximal activity in the proximal and collecting tubules [17]. Although the exact function of these ATPases is not known, their role in transmembrane transport of divalent cations has been proposed in some studies [13,17]. The activities of ecto-ADPase and ecto-5′-nucleotidase in mammalian brush border membranes have also been demonstrated [10,14] but less extensively studied.

In order to evaluate the possible function of membrane bound enzymes involved in the metabolism of adenine nucleotides, we have studied the ectonucleotidase activities in isolated rat renal cortical brush border membranes and examined their properties in detail. Estimates of kinetic constants of ecto-ATPase, ecto-ADPase and ecto-5′-nucleotidase are compared. Our data indicate that brush-border membranes exhibit three separate ectoenzyme activities which sequentially degrade extracellular ATP and produce adenosine as a final product.

Materials and Methods

Materials

Nucleotides, (all disodium salts), phospho*enol*pyruvate (tricyclohexyl ammonium salt), lactate dehydrogenase, pyruvate kinase (both from rabbit muscle in glycerol solution) were purchased from Boehringer (Mannheim, F.R.G.), oligomycin, NEM and DES from Serva (Heidelberg, F.R.G.). DCCD from Calbiochem (Los Angeles, CA, U.S.A.), ouabain, sodium orthovanadate from Merck (Darmstadt, F.R.G.), and levamisole from Sigma (St. Louis, MO, U.S.A.). [8-^{14}C]ATP (spec. act. 54.3 mCi/mmol), [8-^{14}C]ADP (spec. act. 54.5 mCi/mmol), and [8-^{14}C]AMP (spec. act. 53.8 mCi/mmol) were from Amersham (Buckinghamshire, U.K.). Other chemicals were of analytical grade.

Preparation of brush-border membrane vesicles

Brush-border membrane vesicles were isolated from the kidney cortex of the Wistar male rats (150–200 g) according to the Mg^{2+}/EGTA precipitation method of Biber et al. [18]. Isolated and purified vesicle preparations were resuspended in 5 mM Hepes-Tris buffer (pH 7.0), 100 mM KCl and 300 mM mannitol to the protein concentration of 10–20 mg/ml. Vesicles were either used for enzyme activity determination immediately following isolation, or were kept in liquid nitrogen until further use.

Purity of brush border membrane vesicles was checked by measuring activity of specific marker enzymes alkaline phosphatase (EC 3.1.3.1) and leucine arylamidase (EC 3.4.11.2) which were enriched, as compared to homogenate, 6.9 ± 0.5 ($n = 7$) and 13.7 ± 0.9 ($n = 7$) fold, respectively. Contamination with basal lateral membranes was negligible as measured by Na^+/K^+-ATPase (EC 3.6.1.3) activity, which was enriched 0.48 ± 0.07 ($n = 7$) fold.

Enzyme assays and protein determinations

Determination of ATPase activity by the P_i-liberation method. Assay was performed in 1060 μl of reaction medium containing (final concentrations) 50 mM Hepes-Tris (pH 7.4), 100 mM KCl, 50 mM mannitol, 1 mM levamisole to inhibit alkaline phosphatase, 1 mM ouabain to inhibit Na^+/K^+-ATPase, 5 μg/ml oligomycin to inhibit mitochondrial H^+-ATPase, 1 mM *N*-ethylmaleimide (NEM) to inhibit NEM-sensitive endo-ATPase in brush-border membrane vesicles, 5 mM $MgCl_2$ and 20 μg of vesicle protein. The reaction mixture was preincubated at 37°C for 10 min and the ATPase reaction was initiated by adding ATP-final concentration 5 mM (unless otherwise specified). The reaction was carried out at 37°C for 15 min and terminated by adding 0.1 ml of ice-cold 1.12 M trichloroacetic acid. The liberated inorganic phosphate was measured by the method of Fiske and SubbaRow [19]. The ATPase activity was linear up to 75 μg protein per assay.

For the studies of substrate specificity ATP, ITP, GTP, CTP, and UTP in either 5 mM or 0.1 mM (final concentration) of each were used. When specified, inhibitors or divalent cations were added from water (vanadate, NEM, $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$) or ethanol stock (DEP, DCCD, DES) before addition of vesicles. The final concentration of these compounds are indicated in the legends of the figures and tables. Controls contained an equivalent amount of 1% ethanol.

Determination of ATPase activity by the coupled enzymatic assay. The assay was performed according to the slightly modified method of Sabolić and Burckhard [20]. Vesicles (20 μg) were added to 2.00 ml of the reaction medium containing (final concentrations): 50 mM Hepes-Tris (pH 7.4), 300 mM mannitol, 100 mM

KCl, 2.0 mM ouabain, 1.0 mM levamisole, 5.0 μg/ml oligomycin, 12 U/ml pyruvate kinase, 24 U/ml lactate dehydrogenase, 0.30 mM NADH and 0.53 mM phospho*enol*pyruvate. The reaction was initiated by adding 200 μl of equimolar solution of ATP and $MgCl_2$ to the final concentration range from 0.033 to 7.7 mM . The decrease in NADH absorbance was continuously monitored at 340 nm at 37°C. During the measurement, the vesicles were constantly stirred. For the calculation of the ATPase activity the rates recorded between the 1st and 3rd minute were used. The ATPase activity obtained was linear up to 75 μg of vesicle protein.

ADPase activity. ADPase activity was measured by the P_i-liberation assay. Brush-border membrane vesicles (20 μg protein) were added to 1.0 ml prewarmed ADPase reaction medium containing 100 mM Hepes-Tris (pH 7.4), 5 mM $CaCl_2$, 56 μg/l concanavalin A (to inhibit 5′-nucleotidase), 1 mM levamisole, 1 mM ouabain, and 5 μg/ml oligomycin. Reaction was initiated by adding ADP to the final concentration between 0.05 and 3.00 mM. The reaction was stopped after 15 min by adding 0.1 ml of 1.12 mM trichloroacetic acid and liberated P_i assayed by the colorimetric reaction [19].

5′-Nucleotidase activity. The 5′-nucleotidase activity was measured by the P_i-liberation assay according to the same procedure as described above for determination of the ATPase activity, and by using AMP as substrate in the concentration range between 0.25 and 3.00 mM .

Measurements of adenosine production

The products of [8-^{14}C]adenine nucleotide metabolism by brush-border membrane vesicles were separated by TLC in the solvent system of Pull and McIlwain [21] on the silica-gel coated plastic sheets impregnated with fluorescent indicator. Enzymatic hydrolysis of [8-^{14}C]ATP, [8-^{14}C]ADP and [8-^{14}C]AMP (spec. act. 0.2608 mCi/mmol each), was carried out, as stated above, for corresponding nucleotidase activities. At the end of the incubation period the reaction was stopped by adding trichloroacetic acid. Aliquots (10 μl) from incubation samples were chromatographed directly with 10 μl of standard marker mixture at room temperature. In the solvent system used: butanol/ethylacetate/methanol/NH_4OH (7:4:3:4, v/v), the R_f values of ATP, ADP and AMP were 0.00; and for adenosine R_f was 0.70. Spots were located under UV light, cut out and eluted in 1.2 ml 0.1 mM HCl for 12 h. The radioactivity in the eluate was determined in a Rackabeta-LKB liquid scintillation counter with the external standardization. The radioactivity in each spot was expressed as a fraction of the total on each TLC plate and converted into nmol of adenosine produced per min per mg protein. Recovery of radioactivity from TLC plates was on average over 90%.

Miscellaneous

Leucine arylamidase was measured by using commercial kit (Boehringer, Mannheim, F.R.G.), and Na^+/K^+-ATPase according to Berner and Kinne [22] and alkaline phosphatase according to King and Armstrong [23].

Protein concentration was measured by the method of Bradford [24] using bovine serum albumin as a standard. Pyrophosphate concentration was measured according to method of O'Brien [25], using a commercial kit (Sigma, U.S.A.).

Results

Pattern of adenine nucleotide catabolism

The rates of catabolism of ATP, ADP and AMP were initially studied by incubating 3 mM substrate with brush-border membrane vesicles. Considerable rate of hydrolysis of terminal phosphate group was observed with all the substrates used. Initial velocities obtained were as follows: for ATPase 0.94 ± 0.06, for ADPase 0.62 ± 0.12 and for 5′-nucleotidase 0.39 ± 0.09 μmol P_i/min per mg protein, respectively. Since the isolated brush-border membrane vesicles are oriented right-side out [16,18] and since the inhibitors of mitochondrial ATPase, Na^+/K^+-ATPase, alkaline phosphatase and vacuolar type ATPase were present in the assay, the measured nucleotide phosphohydrolase activities were assigned to the activity of the enzymes present at the external surface of the membrane.

In order to test the possibility that brush-border membrane vesicles contain enzymes capable of producing pyrophosphate (PP_i) from the nucleotide substrates we measured the PP_i production from 3 mM ATP or 3 mM ADP. By the method we used the pyrophosphate concentration was hardly detectable, indicating that catabolism of nucleotides proceeds by sequential hydrolysis of terminal phosphate group. After consecutive

TABLE I

Adenosine production by the brush-border membrane vesicles

Brush-border membrane vesicles were incubated with indicated concentrations of [8-^{14}C]ATP, [8-^{14}C]ADP, and [8-^{14}C]AMP in the standard incubation medium as described in Methods. [8-^{14}C]Adenosine production was assayed after 30 min of incubation by means of tlc and liquid scintilation counting. Data are means ± S.D. from 3–6 membrane preparations.

Nucleotide	Concentration (mM)	Adenosine production (nmol/min per mg protein)
ATP	3.0	29.6 ± 3.84
ADP	3.0	25.5 ± 14.0
AMP	3.0	472 ± 85
ATP	0.1	36.0 ± 5.0
ADP	0.1	42.1 ± 5.3
AMP	0.1	61.2 ± 2.1

hydrolytic steps nucleotides are finally degraded to adenosine and P_i.

We measured the rate of [8-^{14}C]adenosine production from [8-^{14}C]ATP, [8-^{14}C]ADP and [8-^{14}C] AMP and we found that brush-border membrane vesicles produced adenosine at the considerable rate from the given substrates in both 3 mM and 0.1 mM nucleotide concentrations (Table I). At 0.1 mM ATP or ADP almost no phosphorylated nucleoside remained in the reaction medium after 30 min of incubation. Results indicate that virtually all ATP and ADP were broken down to adenosine. The rates of adenosine production from 3 mM ATP or 3 mM ADP were lower than the rates from 0.1 mM of corresponding substrate, most probably due to stronger inhibition of 5′-nucleotidase by high concentration of nucleotides [10,11]. Each substrate was unchanged when incubated in the absence of membrane vesicles, indicating that nonenzymatic hydrolysis was not influencing the results.

Further experiments were designed to examine the individual properties of the ATP, ADP and AMP hydrolyzing enzymes.

Kinetic properties of ecto-ATPase in brush-border membrane vesicles

Approximate kinetic constants of ATP hydrolysis were derived from the experiments where ATPase activity was followed as a function of Mg^{2+}-ATP concentration as shown in Fig. 1.

Analysis of the data presented in a form of Lineweaver-Burk plot suggested presence of two kinetic forms with a high-affinity and with a low-affinity site. In the concentration range tested (0.033–7.7 mM) it was not possible to achieve the enzyme saturation. Estimates

TABLE II

Nucleotide specificity of Mg^{2+}-ATPase in brush-border membrane vesicles

Nucleoside triphosphatase activity in brush-border membrane vesicles was vollowed at 5 mM or at 0.1 mM nucleoside triphosphate concentration. ATPas activity was measured by the P_i-liberation method. The results are shown as percentages of ATP hydrolyzing activity. Data are means obtained from three or four membrane preparations.

Substrate	Relative ATPase activity [%]	
	5 mM	0.1 mM
ATP	100	100
ITP	109.8	56.3
GTP	102.3	56.1
CTP	56.3	34.9
UTP	65.4	43.9

of the K_{m1} and K_{m2} as well as of v_{max1} and v_{max2} derived from the Lineweaver-Burk plot, were used for the calculation of the individual curves (representing ATP hydrolysis) corresponding to low- and high-affinity site, respectively. The initial velocity observed may be presented as a sum of individual contributions:

$$v_0 = \frac{v_{max_1}[ATP]}{K_{m_1}+[ATP]} + \frac{v_{max_2}[ATP]}{K_{m_2}+[ATP]}$$

The calculated curves represent the best fit of the experimental data.

Substrate specificity

In order to provide more information about the high- and the low-affinity catalytic sites nucleoside triphosphate specificity was tested at high (5 mM) and at low

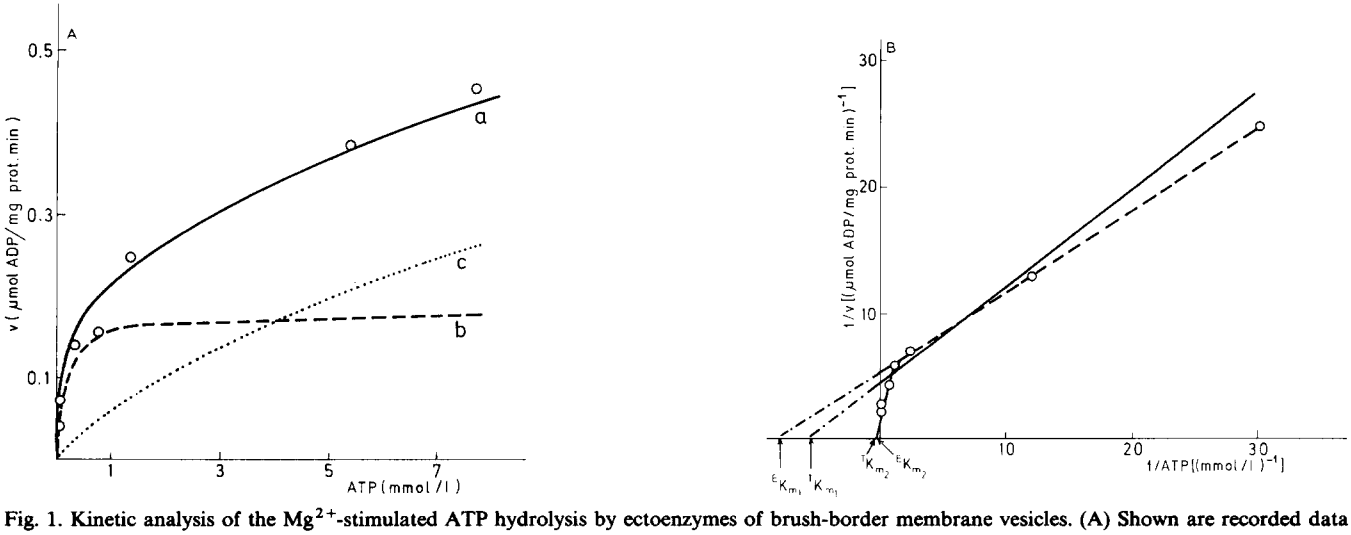

Fig. 1. Kinetic analysis of the Mg^{2+}-stimulated ATP hydrolysis by ectoenzymes of brush-border membrane vesicles. (A) Shown are recorded data and calculated curve for ATP hydrolysis according to the proposed model with high- and low-affinity sites (a), and individual contribution of the high-affinity (b) and low-affinity site (c). ATPase activity (20 µg of membrane protein) was determined in the concentration range of Mg^{2+}-ATP 0.033–7 mM by coupled enzymatic assay. Each point represents mean from 10 determinations out of 5 different membrane preparations. (B) Lineweaver-Burk plot of the same data; $^{E}K_{m1}$, $^{E}K_{m2}$ represent experimentaly observed values, whereas $^{T}K_{m1}$ (0.16 mM) $^{T}K_{m2}$ (9 mM) are apparent values derived from the proposed model.

(0.1 mM) substrate concentration. As shown in Table II hydrolytic activity was higher with purine than with pyrimidine nucleotides, both at 5 mM and at 0.1 mM substrate concentration. At 5 mM nucleotide GTP and ITP were hydrolyzed at approximately the same rate as ATP, whereas CTP and UTP were hydrolyzed with lower rates. At 0.1 mM substrate concentration ATP was hydrolyzed much better than any other nucleoside triphosphate used.

Effects of inhibitors

In the preliminary experiments we found that Mg^{2+}-ATPase activity from brush border membrane vesicles was insensitive to a number of inhibitors which diminish activity of other ATP hydrolyzing enzymes. For ouabain (1 mM), oligomycin (5 μg/ml), levamisole (1 mM), NEM (1 mM) and vanadate (0.5 mM) no effect on the ecto-ATPase activity was observed (results not shown). These inhibitors were therefore regularly present in the assay system in order to decrease the effects of other ATPases present in the membranes.

To find out possible differences between the high affinity and low affinity catalytic sites, we tested series of putative inhibitors in high and low substrate concentrations. Table III represents results obtained with DCCD, DES and DEP. DCCD is known as a chemical modifier of the free carboxyl group, producing significant decrease in the activity of Na^+/K^+-ATPase and mitochondrial H^+-ATPase [26]. Among all inhibitor tested only DCCD produced a different degree of inhibition at 3 mM and 0.1 mM ATP concentration. We also tested the inhibitory action of DCCD on hydrolysis of other nucleoside triphosphates at high and low substrate concentration. At 3mM substrate concentration the rate of hydrolysis of ITP,GTP,CTP and UTP was reduced by 63–65% regardless of the nucleotide tested. At 0.1 mM substrate concentration 72–84% of the activity was measured with CTP, ITP and GTP, and no inhibition with UTP was observed.

TABLE III

Effects of various inhibitors on ecto-ATPase activity in brush-border membrane vesicles

Brush-border membrane vesicles were preincubated with the inhibitor at the concentration indicated, during 10 min. Data are expressed as percentages (mean ± S.D.) of the control ATPase activity obtained from three membrane preparations, and measured by the P_i-liberation method.

Addition	Relative ATPase activity [%]	
	3 mM ATP	0.1 mM ATP
None	100	100
DCCD (1 mM)	23.2 ± 2.7	86.4 ± 25.1
DES (0.1 mM)	46.8 ± 3.7	51.4 ± 1.6
DEP (5 μM)	61.1 ± 20.0	63.0 ± 20.2
DEP (50 μM)	59.2 ± 18.3	60.1 ± 10.3

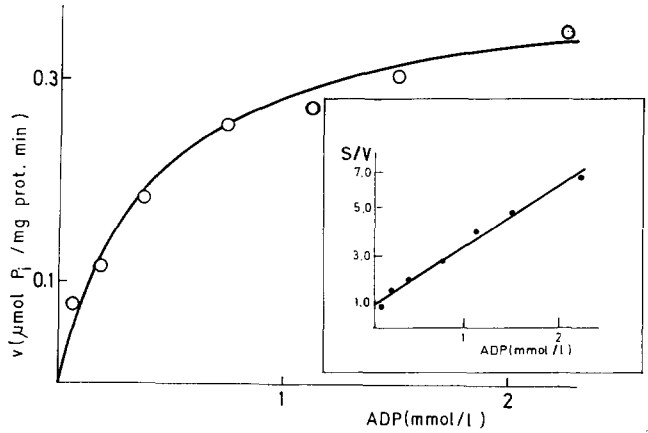

Fig. 2. Kinetics of the ADPase activity in brush-border membrane vesicles. Calculated curve for ADPhydrolysis according to apparent $K_m = (3.82 \pm 0.55) \cdot 10^{-4}$ M and apparent $v_{max} = 0.404 \pm 0.018$ μmol/min per mg protein derived from the Hanes plot of the experimental data. ADPase activity (20 μg of membrane protein) was determined in the concentration range of Ca^{2+}-ADP 0.05–3.00 mM Concanavalin A (56 mg/l) was included in the reaction medium. Each point represents mean obatined from four vesicle preparations. Inset: Hanes plot of the experimental data.

Although DES is a potent inhibitor of H^+ ATPase in mitochondria and endocytotic vesicles [27], and DEP specifically inhibits enzymes were histidine is involved in the catalytic act [28], they could not be used to discriminate between high- and low-affinity site, since both activities were decreased to the same extent in the presence of these inhibitors (Table III).

Kinetic properties of ADP hydrolysis catalyzed by ectonucleotidase from brush-border membrane vesicles

Estimates for the of K_m and v_{max} values for the ADP hydrolysis were derived from the experiments where ADPase activity was measured as a function of ADP concentration (Fig. 2). In order to prevent any further hydrolysis of AMP and production of P_i due to AMP degradation concanavalin A (56 mg/l) was added to the reaction medium. We found that Con A was a potent inhibitor of kidney 5′-nucleotidase, but without any influence on the ADP hydrolysis. Fig. 2 shows that ADP hydrolysis follows a simple hyperbolic kinetics. A Hanes plot of ADPase activity (concentration range of ADP 0.05–3.00 mM) was linear with apparent $K_m = 0.38 \pm 0.06$ mM, and apparent $v_{max} = 0.41 \pm 0.02$ μmol/min per mg protein (Fig. 2).

Kinetic properties of the 5′-ectonucleotidase from brush-border membrane vesicles

5′-Ectonucleotidase from brush-border membrane vesicles hydrolyzed AMP according to the simple Michaelis-Menten kinetics (Fig. 3). Apparent value for K_m was 76.0 ± 9.4 μM and for v_{max} 0.41 ± 0.03 μmol/min per mg protein.

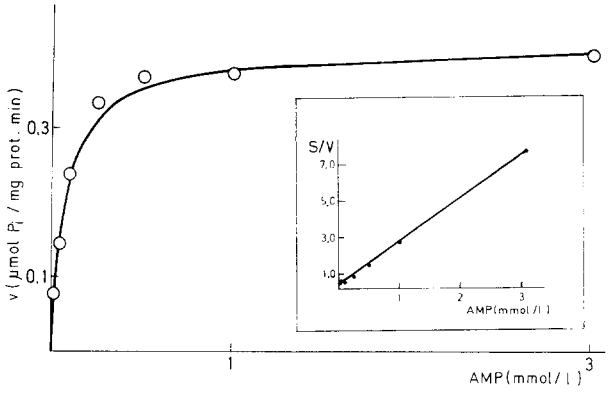

Fig. 3. Effect of AMP concentration on activity of 5′-ectonucleotidase from brush-border membrane vesicles. Calculated curve for AMP hydrolysis for the concentration range 0.025–3.00 mM of AMP. Data are means out of four membrane preparations. Inset: Hanes plot of the experimental data.

TABLE IV

Effect of various inhibitors on ectonucleotidase activities in brush-border membrane vesicles

Inhibitors at the concentrations indicated were added from ethanol (DCCD and DES) or water (Con A) stock solution and preincubated with brush-border membrane vesicles for 10 min at 37 °C. Concentration of nucleotides was 3 mM. Enzyme activities were determined by the P_i-liberation method. Data are means ± S.D. from three membrane preparations. The DCCD and DES effects on ATPase activities are taken from TABLE III.

Addition	Relative nucleotidase activity [%]		
	ATP	ADP	AMP
None	100	100	100
DCCD (1 mM)	23.2 ± 2.7	28.8 ± 2.3	96.0 ± 12.0
DES (0.1 mM)	46.8 ± 3.7	49.5 ± 13.8	102.1 ± 4.4
Con A (15 µg/ml)	100.3 ± 5.1	109.3 ± 19.3	15.5 ± 6.3
Con A (150 µg/ml)	83.0 ± 24.2	96.4 ± 5.9	7.1 ± 3.0

Comparison of the ectonucleotidases from brush-border membrane vesicles

Effect of divalent cations. Fig. 4 demonstrates that ecto-ATPase and ecto-ADPase have the same requirement for divalent cations. No detectable activity was observed in the absence of divalent cations or with EGTA present in the reaction medium. Both enzymes exhibit maximal activity with Mg^{2+} ion present in equimolar solution with the substrate. When 3 mM $CaCl_2$ was used instead of 3 mM $MgCl_2$ ADPase activity did not change significantly whereas ATPase activity was reduced by 25%. Significant activities were recorded with 3 mM $MnCl_2$ but the activity of ATPase was slightly higher than the ADPase activity. With $ZnCl_2$ and $BaCl_2$ both enzymes were activated to a similar extent, ranging from 25 to 43%. In these experiments, however, the actual concentration of free cation was not calculated.

Effect of inhibitors. Comparative studies of the inhibitory effects of DCCD, DES and concanavalin A upon ectonucleotidases from brush-border membrane vesicles are presented in Table IV. Results revealed that ATPase and ADPase activities exhibited similar sensitivity to DCCD and DES and resistance to Con A. On the contrary, 5′-nucleotidase was completely insensitive to the inhibitory action of DCCD and DES, but Con A in concentration of 150 µg/ml was able to inhibit as much as 93% of the 5′-nucleotidase activity.

Results obtained indicated that rates of hydrolysis of ATP and ADP by the enzymes in brush border membrane vesicles share a certain degree of similarity. In Fig. 5 comparison between two enzyme activities is drawn as a correlation line based on different criteria:

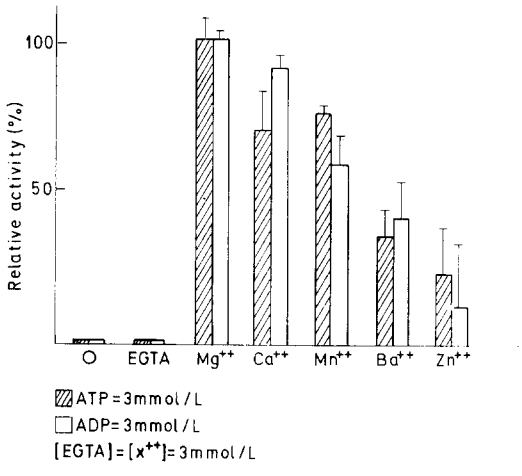

Fig. 4. Effect of divalent cations on the ecto-ATPase and exto-ADPase activities in brush-border membrane vesicles. The enzyme activities were estimated by the P_i-liberation assay in the presence of either 3 mM ATP or 3 mM ADP and indicated divalent cation in the form of the chloride salt (3 mM). The results are percentages of the activity with $MgCl_2$, and are means ± S.D. obtained from three vesicle preparations.

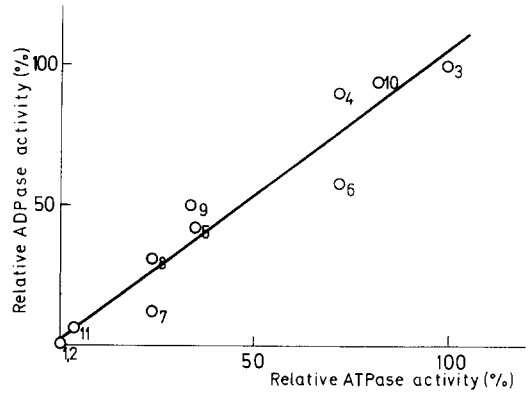

Fig. 5. Correlation between ecto-ATPase and ecto-ADPase activities in brush-border membrane vesicles. 1, no cation added; 2, 3 mM EGTA; 3, 3 mM $MgCl_2$; 4, 3 mM $CaCl_2$; 5, 3 mM $BaCl_2$; 6, 3 mM $MnCl_2$; 7, 3 mM $ZnCl_2$; 8, 1 mM DCCD; 9, 0.1 mM DES; 10, 150 µg/ml Con A; 11, pyrophosphate production. The data of relative enzyme activities were taken from the experiments shown in Table IV and Fig. 4.

(i) interaction with inhibitors: DCCD, DES, Con A, EGTA; (ii) divalent cation activation: Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Zn^{2+}, and (iii) pyrophosphate production. According to the calculated coefficient of correlation, $r = 0.964$, the indication that ATP and ADP are split by the same enzyme could not be excluded.

Discussion

In this paper we demonstrated that brush-border membrane vesicles purified from homogenates of rat kidney cortex posses ectoenzymes that are capable of producing adenosine as a final product using either ATP, ADP or AMP as a substrate. The enzymes which carry out the reaction sequence ATP → ADP → AMP → adenosine are insensitive to levamisole, ouabain, oligomycin, vanadate, and NEM and therefore are different from alkaline phosphatase, Na^+/K^+-ATPase, mitochondrial H^+-ATPase, and vacuolar type H^+-ATPases, respectively.

We found no evidence that ATP and ADP were degraded by hydrolysis of terminal pyrophosphate group. Thus, it is evident that brush border membrane vesicles contain no ecto ATP/ADP pyrophosphatase (EC 3.6.1.8). The initial rate of adenosine production was inversely related to the starting concentrations of ATP or ADP used, while rate of AMP hydrolysis was proportional to the substrate concentration. These results are consistent with the assumption that the adenosine producing enzyme (5′-nucleotidase) is inhibited by the high concentration of ATP and ADP. In other words, the higher the concentration of ATP or ADP used, higher degree of inhibition of 5′-nucleotidase is produced.

There are several published results which support this suggestion. Gordon et al. have clearly demonstrated that ADP and/or ATP exert a feed-forward inhibition of AMP hydrolysis in the endothelial cells [29]. Le Hir et al. found that ecto-5′-nucleotidase of the renal brush border is inhibited by ATP and ADP in a concentration dependent manner [10]. Similar findings are presented for the nucleotidase from rat heart [30] and crude extracts of rat kidney [31].

By simultaneously generating AMP, the substrate needed for adenosine production and, by influencing the concentration of ATP and ADP (inhibitors of 5′-nucleotidase), ecto-ATPase and ecto-ADPase play major role in the regulation of the relative amounts of adenosine and adenine nucleotides.

Kinetic analysis

Individual kinetic analysis and characterization of ecto-ATPase, ecto-ADPase and ecto-5′-nucleotidase may provide a better insight into dynamics of adenosine metabolism. Mg^{2+}-stimulated ATP hydrolysis failed to follow a simple Michaelis-Menten kinetics. The experimental data were fitted adequately only when model with two independent catalytic sites had been assumed: $K_{m1} = 0.160$ mM and $K_{m2} = 9.00$ mM, respectively. The physiological implications of the low affinity site are probably insignificant, since concentration of ATP in the environment of the proximal tubule is expected to be in the low micromolar range [32]. Similar kinetics, with two apparent K_m values for Mg^{2+}-ATP hydrolysis (0.24 mM and 1.15 mM), has been observed for Ca^{2+}- or Mg^{2+}-ATPase activity in isolated pancreatic plasma membranes [33]. However, these results are in contradiction with previous findings for Ca^{2+}- or Mg^{2+}-ATPase. Namely, in pig [12] and rabbit renal brush-border membranes [14, 34], and rat kidney homogenate [31], indication for only one affinity site for ATP hydrolysis with the apparent K_m for Mg-ATP of 0.14–0.33 mM was demonstrated.

In our experiments different substrate specificity and sensitivity to inhibitors were observed, at high and at low substrate concentrations. At 0.1 mM substrates, i.e., at concentration which should mostly reflect the activity of the high-affinity site, the rate of ATP hydrolysis was much higher than the rate of hydrolysis of other nucleotide triphosphates. These findings are in accordance with results obtained by other laboratories [9,16]. Furthermore, this reaction was partially inhibited by DES and DEP, but weakly by DCCD. At high (3–5 mM) concentration of substrates, all purine nucleotides were split with similar efficiency. This reaction was also partially inhibited by DES and DEP, and strongly by DCCD. At present, it is not clear whether two catalytic sites with different affinity for nucleotides and with different sensitivity to inhibitors, reside on the same protein, or two kinetically different ecto-nucleotidases are present in our preparations of brush-border membranes.

Once produced by the action of ecto-ATPase (either high- or low-affinity catalytic site), ADP is further hydrolyzed. According to our results Ca^{2+}/Mg^{2+}-stimulated ADP hydrolysis followed simple Michaelis-Menten kinetics with an apparent $K_m = 0.38 \pm 0.06$ mM. In cultured pig endothelial and smooth muscle cells, the kinetics with ADP was also hyperbolic with a K_m of 0.1–0.15 mM [9,35]. The enzyme catalyzed hydrolysis of ADP to AMP, only in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+}. The actual substrate seemed to be the divalent cation-ADP complex, rather than sole ADP. Inhibition and divalent cation activation studies revealed the striking similarity between ecto-ADPase and ecto-ATPase. Such findings indicated that these two enzymes, could not be distinguished one from another. The same was found for the partially purified ATPase from rabbit kidney brush-border membranes [14], and for the enzyme in plasma membranes from rat liver [36]. ATP-diphosphohydrolase (EC 3.6.1.5) from bovine aorta microsomes was purified to homogeneity and was found

that enzyme hydrolyzes nucleoside di- and triphosphates [37]. However, some other studies in rabbit kidney cortex brush-border membranes [33], in cultured endothelial and smooth muscle cells and in rat kidney cortex homogenates suggest, that ecto-ATPase and ecto-ADPase are two different enzymes [9,35,38].

In the experiments, where possible competition between ATP and ADP was tested, lowering of the rate of 3 mM ATP hydrolysis was found if 3 mM ADP was added to the reaction medium (results not shown). This observation, however, is not conclusive evidence that ATP and ADP are hydrolyzed at the same catalytic site. The results might be assigned to negative cooperative effect of ADP upon ATP catabolism, or to a simple product inhibition.

The third enzyme in the sequence of events leading from ATP to adenosine is 5′-nucleotidase. Being insensitive to DCCD, DES and DEP but almost completely inhibited by Con A, this enzyme is evidently different from ectoenzymes that hydrolyze ATP and ADP. The AMP hydrolysis followed simple Michaelis-Menten saturation kinetics, with apparent $K_m = 76.0 \pm 9.4$ μM. These results are inconsistent with findings of Le Hir et al. [12] who reported a much lower value for K_m (5.77 μM). The discrepancies may arise from the different assay systems used: P_i-liberation method instead of adenosine production measuring, or from the range of the AMP concentrations studied: 25–3000 μM instead of 1.7–83 μM. Our additional experiments with ^{14}C-labeled AMP hydrolysis confirmed previously obtained results (i.e., $K_m = 76.0 \pm 9.4$ μM).

In conclusion, our present findings demonstrate that system of ectonucleotidases present in brush-border membranes is capable of regulating the adenine nucleotide catabolism. Two (or three) ectonucleotidases are forming a cascade involved in the production of adenosine from ATP. Further studies are indicated in order to establish whether the system that we have characterized in brush border membrane vesicles is really effective in vivo.

While rationalizing the physiological role of ectoenzymes described the major source of ambiguity is a significantly lower extracellular ATP concentration (0.2 μM rat and rabbit; and 2 μM human) [39], when compared to the K_m values obtained. The concentrations are too low even for the high-affinity site of ecto-ATPase. The origin of the substrates for brush-border ectonucleotidases is unclear. Nucleotides from blood normally reach tubular lumen via glomerular filtration. However, the concentrations of plasma nucleotides up to 100-times higher than normal are found following extensive platelet degranulation, injury, and traumatic shock [40].The selective release of ATP upon stimulation may contribute to the transient increase of nucleotide concentration.

Finally, one can not exclude the possibility that even in normal conditions a significant amount of substrates comes from tubular cells by exocytosis. Proximal tubular cells contain a huge number of endosomes and exhibit a very high constitutive recycling of the luminal membrane [41]. Per analogy with the mechanism of ATP release in other cells [40], proximal tubular exocytotic vesicles may discharge nucleotides in the luminal fluid. Thereafter they are sequentially hydrolyzed by the brush-border ectoenzymes. According to the hypothesis of Le Hir et al. [11] energy deficit produced by the high rate of glomerular filtration is manifested by the high concentration of adenosine, derived from the catabolism of ATP. Adenosine in turn, is capable of producing a decrease in glomerular filtration rate, thus balancing transport work and the energy status of kidney.

If freely filtered, ATP and other nucleotides can be readily degraded by series of brush-border ectonucleotidases to adenosine and P_i, as demonstrated in this paper. Both, adenosine and P_i, are thereafter reabsorbed by separate transport systems [42–44], or adenosine could stimulate the specific receptors on brush-border membrane.

Receptors for adenosine and ATP have been well described in various cell types (for review, see Ref. 40). Moreover, they have been distinguished one from another by relative substrate affinities.

Existence of A1 or A2 adenosine receptors has been reported in some parts of the kidney [45–48]. Our preliminary studies with ^{3}H-NECA binding failed to confirm presence of A_2 receptors in the whole range of NECA concentration tested (from 2.5 nM up to 50μM.

Certain similarities between P_2 purinergic receptor and ecto-ATPase activity in hepatocytes indicate that ecto-ATPase may be considered as the P_2 receptor itself [36,49,50]. It was suggested that ecto-ATPase may be involved in termination of the effect of ATP upon the cell, via its phosphatase activity, but further studies are necessary to gain better insight into this subject.

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