# ATP-diphosphohydrolase Activity in Rat Renal Microvillar Membranes and Vascular Tissue

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**Ecto-nucleotidases may have a role in the regulation of purinoceptor-mediated responses. ATP-diphosphohydrolase or apyrase has been described as an ecto-nucleotidase, which is characterized by a low specificity for its substrates and bivalent cations. The aim of this work was to demonstrate the presence of apyrase as an ecto-enzyme in the rat kidney. ATPase–ADPase activities of the renal microvillar membrane preparation, which correspond to 'right side out' membranes, were characterized. The detection of ATP–diphosphohydrolase in the renal vasculature was done through perfusion of isolated rat kidney. ATPase–ADPase activities of the microvillar membrane preparation and apyrase share similar kinetic properties. These include: low substrate and bivalent metal specificities and insensitivity towards inhibitors like: oligomycin, ouabain, verapamil, levamisole and $Ap_5A$. The $M_r$ of native ATPase and ADPase activities was determined by the $^{60}Co$ irradiation–inactivation technique being around 65 kDa for both hydrolytic activities. Immunowestern blot analysis also supports the presence of apyrase in microvilli. Perfusion of isolated rat kidney with ATP and ADP, in the presence or absence of different inhibitors or apyrase antibodies indicated the existence of this enzyme in the vascular endothelium. The identification of ATP-diphosphohydrolase as an ecto-enzyme both in microvilli and vasculature support the proposal that the enzyme may have an important role in the extracellular metabolism of nucleotides.** **Copyright © 1996 Elsevier Science Ltd**

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## INTRODUCTION

Many cell types release nucleotides into the extracellular environment, which can interact with specific cell-surface receptors and affect many biological processes (Gordon, 1986). These nucleotides are rapidly metabolized locally by ecto-nucleotidases, which may have a role in the regulation of purinoceptor-mediated responses. In addition, adenosine is a significant product of hydrolysis of extracellular nucleotides (Stanley *et al.*, 1982).

Extracellular nucleotides and adenosine have a number of effects on renal tissue. Gutiérrez *et al.* (1992) have shown that ATP and ADP stimulate release of $PGE_2$ and Inscho *et al.* (1994) have proposed that ATP acting at $P_{2y}$ receptors on endothelial cells stimulates liberation of NO. Both $PGE_2$ and NO are vasodilators and cause increases in renal flow and urine filtration. Extracellular ATP also influences the tubuloglomerular feedback mechanism (Mitchell and Navar, 1993). While both ATP and adenosine stimulate ion uptake, ATP has a vasoconstrictive effect on afferent but not efferent arterioles, whereas adenosine constricts both vascular segments (Inscho *et al.*, 1994).

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In addition, extracellular adenosine decreases renin liberation.

A $Ca^{2+}/Mg^{2+}$-ATPase has been reported in rabbit kidney brush border or microvillar membranes (Mörtl *et al.*, 1984 and references therein) and in pig renal cortex (Van Erum *et al.*, 1988,1995 and references therein). This enzyme is different from the $Ca^{2+}$-dependent transport ATPase, which is located in the basolateral membrane. The so-called $Ca^{2+}/Mg^{2+}$-ATPase has a low specificity for its substrates and cations, requires cation concentrations in the millimolar range, and is insensitive to oligomycin. Mörtl *et al.* (1984) provided evidence for the occurrence of this enzyme in microvilli. This has recently been confirmed by ultrastructural immunocytochemistry (Čulić *et al.*, 1990; Sabolić *et al.*, 1992) with antibodies directed against rat liver ecto-ATPase.

Rat glomeruli contain a nucleoside diphosphatase activity, which is associated with the plasma membranes of endothelial cells and glomerular basal membranes (Poelstra *et al.*, 1991). This ADPase activity may be significant in preventing platelet aggregation and subsequent thrombus formation in rat kidney *in vivo*, and may also have a role in inflammatory processes (Poelstra *et al.*, 1992). This activity has not yet been biochemically characterized.

In this paper, we present kinetic, physicochemical and immunological evidence that the nucleotide phosphohydrolase activities in renal microvillar and endothelial membranes are attributable to a single enzyme, ATP-diphosphohydrolase (EC 3.6.1.5) or apyrase. This has important implications in the knowledge of ecto-nucleotidases responsible for extracellular ATP and ADP hydrolysis. Ecto-apyrase together with 5′-ecto-nucleotidase could sequentially dephosphorylate ATP to adenosine: ATP→ADP→AMP→adenosine. These ecto-enzymes might have a role in the modulation of extracellular ATP-induced functional changes and in scavenging the purine ring, ensuring the incorporation of adenosine into the cell (Plesner, 1995).

## MATERIALS AND METHODS

### *Materials*

All the chemicals, of analytical grade or the purest grade available, were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

### *Microvillar membranes from rat kidney cortex*

The membrane fraction was prepared by layering 8 ml of homogenate prepared from the renal cortex (in 0.5 M sucrose with 0.1 mM phenyl methylsulfonyl fluoride, PMSF) on a discontinuous sucrose gradient (8 ml each of 1.7 M and 1.4 M sucrose) and centrifugation (60 min, 90,000 ***g***, Beckman SW 20.1 rotor) as described by Berger and Sacktor (1970). Layers enriched with membranes were washed twice with 0.25 M sucrose.

### *Enzyme assays*

ATPase–ADPase activities were assayed by measurement of the liberation of Pi from ATP or ADP. The Pi was determined according to Fiske and SubbaRow (1925) or Ernster *et al.* (1950) (in electrofocusing experiments). The standard assay conditions were: 2 mM nucleotide with 5 mM bivalent cation in 100 mM Tris–HCl, pH 8.0. For determining the effects of pH, the buffers were 100 mM Tris–HCl (pH 7–8.5) and 100 mM Tris–glycine (pH 8.5–10). Oligomycin (0.1 mg/ml) and adenylyl (3′-5′)-adenosine pentaphosphate ($Ap_5A$) (0.5 mM) were added to inhibit mitochondrial ATPase and adenylate kinase, respectively, when the activities were measured in homogenates. Only oligomycin was added during kinetic characterization and $M_r$ determination when using microvillar membranes. The activity assays after isoelectrofocusing where done in the absence of any inhibitor.

Glutamate and lactate dehydrogenase were assayed as in Leighton *et al.* (1968). γ-Glutamyltranspeptidase activity (γ-GT) was determined according to Tate and Meister (1981). The $(Na^+,K^+)$-ATPase activity was calculated from the difference in Pi liberation from ATP in the presence and absence of ouabain 2 mM as described by Kinne-Saffran and Kinne (1974). 5′-Nucleotidase was measured according to Beaufay *et al.* (1974).

### *Protein determination*

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

### *Solubilization of membrane proteins*

Membrane fractions (with a protein concentration between 1 and 5 mg/ml) were incubated for 10 min at 30°C with *n*-octylglucoside

(10 mg/mg protein). Insoluble material was removed by centrifugation at 100,000 *g* for 1 hr.

*Gel isoelectrofocusing*

The gel mixture was as described in Sánchez *et al.* (1980), in the presence of 0.2% *n*-octylglucoside. Ampholines ranged from pH 3 to pH 10. The sample was added before gel polymerization to distribute it uniformly along the gel. Voltage was constant at 300 V. The current decreased to a constant value in approx. 100 min. Apyrase activity was measured in samples eluted from gel slices (0.2 mm) with assay medium plus 0.2% *n*-octylglucoside or with the gel slices directly. After isoelectrofocusing, gel slices with ATPase–ADPase activities were electroeluted in $NaHCO_3$–SDS as described by Dumbar (1987). After elution, fractions were freeze-dried and solids were resuspended in SDS–PAGE buffer. The purity of this fraction with apyrase-like activity was checked by SDS–PAGE according to Laemmli (1970). Protein bands were stained with Coomassie brilliant blue.

*Immunowestern blotting*

Proteins separated on SDS–PAGE according to Laemmli (1970) were transferred to nitrocellulose paper as described by Towbin *et al.* (1979). After blocking with TBS–buffer containing 5% non-fat dry milk, the nitrocellulose blots were incubated overnight with a 1/2000 dilution of polyclonal placental apyrase antisera (prepared in the laboratory) in 1% milk buffer. After washings and incubation for 1 hr with the secondary antibody (anti-rabbit Ig conjugated to peroxidase at a dilution 1/3000), the binding was detected by the appearance of a coloured product produced by peroxidase using 0.05% (mass/vol) 4-chloro-1-naphthol dissolved in pure cold methanol and 0.05% (by vol) $H_2O_2$ (at 30%) as substrates. The protein used for raising antibodies in rabbits was obtained as follows: a microsomal placental fraction was partially purified by isoelectrofocusing, proteins focused at pI 4.5 contained both ATPase–ADPase activities (Kettlun *et al*., 1994), these proteins were further separated by SDS–PAGE and electrotransfered to nitrocellulose membranes (as described above). The main protein band which has the same $M_r$ (62 kDa) of the native enzyme (Kettlun *et al*., 1994), considered to be a highly purified placental apyrase, was used to inoculate rabbits. These antibodies recognized a single protein band of $M_r$ 62 kDa in placental microvillar membranes.

*Chemical modification of amino acid residues*

Modification reactions were as specified by Means and Feeney (1964). The modification time, temperature and reagent concentration employed are listed in Table 1. Reactions were stopped by dilution of portions of incubations in assay medium, which were subsequently assayed for ATPase–ADPase activities. Controls contained solvent only. Either ATP or ADP (10 mM) was added as protecting agent. The buffer conditions were: 0.1 M Tris–HCl, pH 8.0, for *bis*-dithionitrobenzoic acid (DTNB), PMSF, tetranitromethane (TNM) and methylmethanethiosulfonate (MMTS); 0.1 M sodium bicarbonate, pH 8.0, for the phenylglyoxal reaction; 0.05 M MES, pH 6.0, for reaction with Woodward K; 0.08 M sodium acetate, pH 5.0, for modifications with diethylpyrocarbonate (DEP) and Koshland reagent; 0.05 M Tricine, pH 9.0, for the modification with maleic anhydride.

Table 1. Chemical modification of microvillar membrane ATPase–ADPase activities. Details are given in Materials and Methods. All experiments were run at least in duplicate

| Reagent | Inactivation (%) ATPase | ADPase |
|---|---|---|
| Phenylglyoxal (50 mM, 30 min, 30°C) | 84 | 79 |
| +ATP (5 mM) | 12 | 14 |
| +ADP (5 mM) | 26 | 38 |
| Woodward K (5 mM, 5 min, 0°C) | 71 | 71 |
| +ATP (5 mM) | 27 | 26 |
| +ADP (5 mM) | 32 | 32 |
| Koshland reagent (50 mM, 5 min, 30°C) | 63 | 66 |
| +ATP (5 mM) | 72 | 74 |
| +ADP (5 mM) | 51 | 50 |
| Tetranitromethane (50 mM, 30 min, 30°C) | 72 | 74 |
| Maleic anhydride (50 mM, 25 min, 30°C) | 51 | 50 |
| Diethylpyrocarbonate (50 mM, 10 min, 0°C) | 96 | 87 |

*Determination of* $M_r$

The $M_r$ of ATPase and ADPase activities was determined by the irradiation–inactivation method using $^{60}Co$ (Kempner and Schlegel, 1979) at the Comisión Chilena de Energia Nuclear in a $^{60}Co$ irradiator Naratom 3500. Control samples were kept at the same temperature as those irradiated. The 37% dose ($D_{37}$) was used for empirical calculation of the $M_r$.

*Perfusion studies*

Isolated rat kidneys were perfused as described by Roblero *et al.* (1976). The perfusion medium contained 3% BSA in 0.15 M NaCl. Nucleotides (2.5 mM, lower concentrations were completely hydrolysed) with $CaCl_2$ (5 or 25 mM) in perfusion medium were infused directly into the perfusate inflow line near the mesenteric cannula (in a volume of 200 µl). The nucleotide concentration used in the perfusion medium was selected after testing different concentration ranges; with lower amounts of nucleotides all the substrate was hydrolysed up to adenosine. The infusion rate was approx. 1.5 ml/min. The absence of tissue damage was checked by measuring lactate dehydrogenase activity (Leighton *et al.*, 1968) in the perfusate at the end of each experiment. Controls were performed with perfusion medium with or without $CaCl_2$; no Pi liberation was observed. In experiments with apyrase antibodies, a volume of 100 µl of the immune serum (diluted 1/3 in the perfusion medium) was infused immediately before the infusion of 200 µl of ATP or ADP solution in the presence of the same dilution of serum. Pre-immune serum served as control (100% of Pi liberated from nucleotide hydrolysis). The initial Pi contamination from serum (pre-immune and immune) was substracted from the total amount of Pi determined in the perfused fractions.

*Chromatographic separation of nucleotides*

Polyethylene imine plates (PEI-$F_{254}$) from Merck were developed in ascending chromatography with 1 M LiCl according to Randerath and Randerath (1967). The adenine ring was detected by fluorescence quenching at 254 nm. Portions of 100 µl of the two perfused fractions containing the maximum of Pi were previously concentrated by liophilization and resuspended in 20 µl of distilled water. Aliquots of 5 µl of these samples were applied to the plates.

## RESULTS

*Localization of ATPase–ADPase activities*

ATPase and ADPase activities were concentrated at the same sucrose interface as microvillar membranes, as assessed by the enrichment of the microvillar marker, γ-GT (Fig. 1). The purity of this fraction was checked by the absence of glutamate and lactate dehydrogenase activities (mitochondrial and cytosolic markers, respectively). This preparation was contaminated with basolateral membranes, as judged by the presence of $(Na^+,K^+)$-ATPase. Basolateral membranes do not, however, exhibit a $Ca^{2+}/Mg^{2+}$-ATPase activity. Any possible ATPase activity due to $(Na^+,K^+)$-ATPase was discounted because of the insensitivity to ouabain (5 mM); the ATPase activity was also unaffected by verapamil (0.1 mM), $Ca^{2+}$ channel blocker or by an alkaline phosphatase inhibitor, levamisole (10 mM). Oligomycin (0.1 mg/ml) inhibited ATPase activity by approx. 30%, probably because of contamination with mitochondrial ATPase. Oligomycin was therefore routinely included in the ATPase assay medium.

The ADPase activity can be mimicked by adenylate kinase acting together with an ATPase. ADPase activity however, was, unchanged when either $Ap_5A$ (adenylate kinase inhibitor) or glucose and hexokinase were added.

The 'right side out' orientation of microvillar vesicles was confirmed by assaying two characteristic ecto-enzymes, γ-GT and 5′-nucleotidase (Tate and Meister, 1981; Stanley *et al.*, 1982) in the presence and absence of Triton X-100. The activities of these enzymes in the absence of

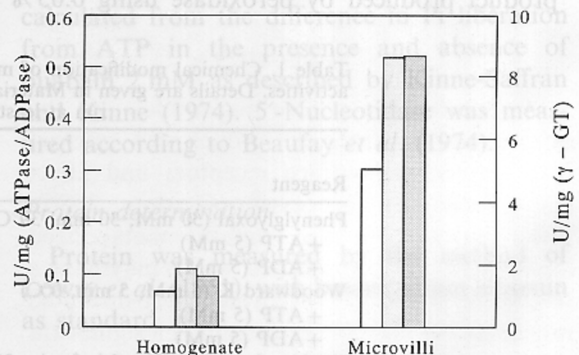


Fig. 1. Microvillar membrane preparation. Renal cortex homogenate was centrifuged on a discontinuous sucrose gradient as described in Materials and Methods. Assays for (⬚) ATPase, (□) ADPase and (■) γ-GT were perfomed in the homogenates and in the sucrose layer interfaces.

detergent were 86 and 96%, respectively, of the activities determined in the presence of 1% Triton X-100. Because Triton X-100 denatured ATPase–ADPase activities, saponin (0.2 mg/ml) or digitonin (2.0 mg/ml) were used in this case. The ecto-localization was confirmed by the lack of effect of these detergents.

*Kinetic charaterization of microvillar ATPase and ADPase*

The pH dependence curves, using either ATP, ADP, UTP or UDP as substrates, showed the highest activity at pH 8.0, with 50% activity around 7.5 and 9.5.

Both activities were stimulated by bivalent cations, $Ca^{2+}$ being the most effective, followed by $Mg^{2+}$ and $Mn^{2+}$. ATPase activity in the presence of 5 mM $Mg^{2+}$ was unaffected by addition of $Ca^{2+}$ (0.5 mM), a behavior characteristic of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase. No inhibition by bivalent cations (up to 5 mM) was observed. Addition of EGTA or CDTA (1.0 mM) decreased ATPase activity to below 10%, but ADPase activity remained at approx. 40%. Higher concentrations (10 mM) of chelators decreased this figure to 23%. But the decrease in ADPase activity observed at this high chelator concentration could be due to direct inhibitory action of EGTA or CDTA on the enzyme.

*Kinetic parameter determination and inhibition studies*

The *Km* values for ATPase and ADPase activities calculated from the Pi liberation at pH 8.0 were 15 and 21 μM, respectively, similar to the values reported for apyrases from other rat tissues like salivary gland, mammary gland and uterus (Valenzuela *et al*., 1989). Both AMP-PcP and AMPcP were competitive inhibitors with either ATP or ADP as substrates. These results support the view that both activities share a common active site.

*Modification of amino acid residues*

Participation of aliphatic hydroxyl and sulfhydryl groups in kidney ATPase and ADPase activities were excluded because 0.1 mM PMSF (seryl protein modifier) and 1 mM DTNB or 0.1 mM MMTS (-SH modifiers) did not affect either of these activities.

Inactivation by several reagents is summarized in Table 1. The loss of activity observed after treatment with phenylglyoxal (Arg modifier) and Woodward K (carboxylic group modifier) were largely prevented when the enzyme was preincubated with ATP or ADP. But on the contrary, inactivations produced by Koshland reagent (Trp group modifier), TNM (Tyr modifier), maleic anhydride (amino group modifier) and DEP (His modifier) were not prevented by the substrates.

Similar effects of the different chemical reagents used on both ATPase and ADPase activities are also in accordance with the existence of a single active site.

*Determination of the $M_r$ in native conditions*

ATPase and ADPase activities present in the microvillar membrane fraction decayed as a simple exponential function of the radiation dose. Similar $M_r$ values of 65.6 and 67.4 kDa were calculated for ATPase and ADPase activities, respectively, from the activity decay curves ($r > 0.98$). These data support the proposal that both activities are expresed by the same protein.

*Partial purification of ATPase–ADPase activities*

*Solubilization and isoelectrofocusing of plasma membrane proteins*. Up to 50% of ATPase and ADPase activities were solubilized, with < 10% of the activity remaining in the 100,000 *g* pellet. The solubilized fraction was submitted to isoelectrofocusing. Addition of urea and *n*-octylglucoside was essential for detection of the single phosphohydrolase peak, suggesting that these activities were associated with highly

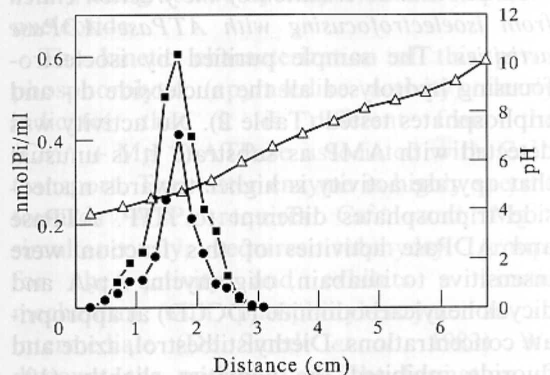


Fig. 2. Gel electrofocusing of the solubilized microvillar preparation. Ampholines ranged from pH 3.5 to pH 10; the sample was added before gel polymerization. (△) pH profile; (●) ATPase and (■) ADPase activities expressed in mU/ml. The upper electrolyte was 0.2% $H_2SO_4$ (anode) and the lower electrolyte was 0.2 N NaOH (cathode).

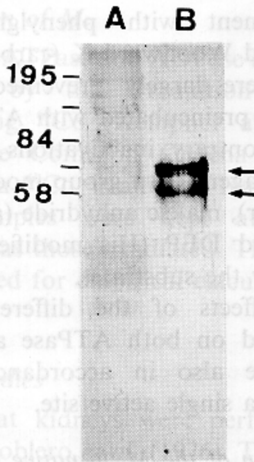


Fig. 3. Immunowestern blot of microvillar vesicles. Lane A: molecular weight markers ($\alpha_2$-macroglubulin 195 kDa, β-galactosidase 116 kDa, fructose-6-phosphate kinase 84 kDa, pyruvate kinase 66 kDa, fumarase 58 kDa, lactic dehydrogenase 36.5 kDa, triosephosphate isomerase 26.6 kDa). Lane B: 20 μg of microvillar sample.

aggregable protein(s). Both activities were found to focus at pH 5.0 (Fig. 2). (This value was obtained in three independent experiments.) SDS–PAGE of the protein fraction eluted at this pI showed the presence of three bands of 128, 67 and 53 kDa, respectively. Immunowestern blot showed that the 67 and 53 kDa bands crossreacted with the polyclonal antibodies directed against human placental apyrase (not shown). Immunowestern blot of the crude microvillar membrane fraction also revealed a similar pattern with the same two bands (Fig. 3). The band with smaller mass probably represents a degradation product of the native protein of $M_r$ 67 kDa.

*Kinetic characterization of the fraction eluted from isoelectrofocusing with ATPase–ADPase activities*. The sample purified by isoelectrofocusing hydrolysed all the nucleoside di- and triphosphates tested (Table 2). No activity was detected with AMP as substrate It is unusual that apyrase activity is higher towards nucleoside triphosphates different to ATP. ATPase and ADPase activities of this fraction were insensitive to ouabain, oligomycin, $Ap_5A$ and dicyclohexylcarbodiimide (DCCD) at appropriate concentrations. Diethylstilbestrol, azide and fluoride inhibited the activities slightly (10–20%), while orthovanadate (boiled 5 min at 100°C just before use to avoid polymerization, personal communication Dr S. Verjovski-Almeida) produced a significant effect on both ATPase and ADPase activities (Table 2).

*Perfusion studies*

In isolated, perfused kidneys, nucleotides were hydrolysed to give substantial amounts of Pi. Endothelial membranes remained undamaged during the experiments as judged by the lack of lactate dehydrogenase activity in the perfusate. Figure 4(A) and (B) shows Pi liberation when either ATP or ADP was added. Hydrolysis was inhibited by AMP–PcP and AMPcP. The presence of oligomycin, ouabain, verapamil or levamisole had no effect. These results rule out other ATPases as possible sources of activity or alkaline phosphatase. Other nucleotides, like GTP, CTP, UTP, GDP, CDP and UDP, were also hydrolysed, which indicates the non-specificity of the hydrolysing activity of the endothelium. 5′-Nucleotidase activity was also observed when AMP was added to the perfusion medium, but was lower than ATPase or ADPase activity. Upon perfusion with the same amount of nucleotide (1 μmol), ATP produced 1.5 μmol, ADP 0.96 μmol and AMP 0.34 μmol of Pi. Consistent with this, chromatographic analysis of the perfusate showed almost complete disappearance of the nucleoside di- or triphosphate, and substantial accumulation of AMP.

The presence of apyrase antibodies had a considerable inhibitory effect on ATPase (40%)

Table 2. Substrate specificity of the partially purified ATPase–ADPase activities and effect of inhibitor on these activities. The enzyme was a solubilized preparation submitted to isoelectrofocusing (see text). The Pi released was determined according to Ernster *et al.* (1950). All nucleotides were 2 mM (final concentration). Assay conditions are described in Materials and Methods. Data correspond to two independent electrofocusing experiments

**Nucleotide specificity**

| Nucleotide | Activity (%) | Nucleotide | Activity (%) |
|---|---|---|---|
| ATP | 100 | ADP | 181 |
| GTP | 233 | GDP | 74 |
| UTP | 175 | UDP | 80 |
| CTP | 174 | CDP | 114 |
| dTTP | 176 | dTDP | 114 |

**Effect of inhibitors**

| Inhibitor | Conc. | Inhibition (%) ATPase | Inhibition (%) ADPase |
|---|---|---|---|
| $Ap_5A$ | 0.5 mM | 0 | 0 |
| Ouabain | 5.0 mM | 0 | 0 |
| DCCD | 1.0 mM | 0 | 0 |
| Oligomycin | 0.1 mg/ml | 3 | 9 |
| Fluoride | 10.0 mM | 21 | 9 |
| DES | 1.0 mM | 30 | 11 |
| Azide | 1.0 mM | 27 | 25 |
| Orthovanadate | 10 μM | 59 | 53 |
| | 100 μM | 73 | 77 |
| Orthovanadate | 0.1 mg/ml | ATPase | ADPase |

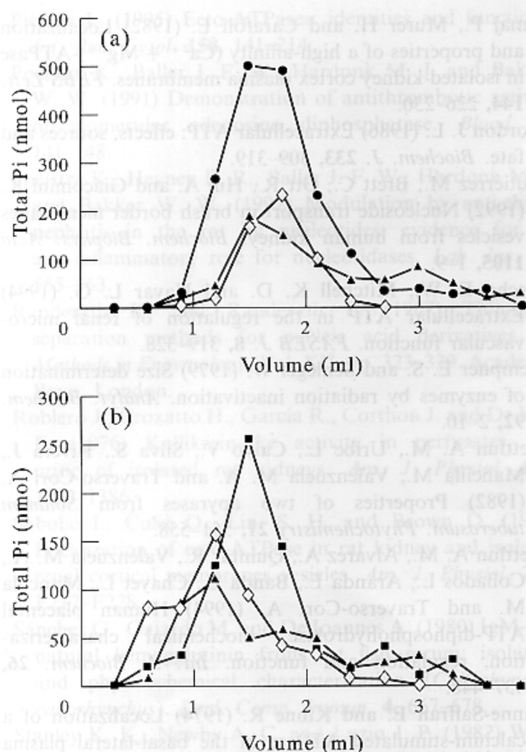


Fig. 4. Perfusion of isolated rat kidney with nucleotides. Perfusion was as described in Materials and Methods. The Pi produced in the perfusate after addition of 1 μmol of ATP (a) or ADP (b) was determined by the Fiske and SubbaRow method (1925) after precipitation of proteins with 6% TCA. When added, nucleotide analogues were at the same concentration as the respective substrate. (▲) Plus AMP–PcP; (◇) plus AMPcP.

and ADPase (57%) activities despite the absence of any pre-incubation period. This strongly supports the proposal that the hydrolytic activity observed in vascular endothelium corresponds to apyrase.

## DISCUSSION

Our experiments indicate that ATPase and ADPase activities in rat kidney microvillar plasma membrane preparations are associated with a single protein. The kinetic properties of the fraction with ATPase and ADPase activities are similar to those reported for ATP–diphosphohydrolases from different tissues (LeBel *et al*., 1980; Valenzuela *et al*., 1989; Battastini *et al*, 1991; Vasconcelos *et al*., 1993; Kettlun *et al*., 1994). These characteristics include broad specificity towards the nucleoside di- and triphosphates, absence of monophosphoesterase activity, stimulation by $Ca^{2+}$ or $Mg^{2+}$, insensitivity towards ouabain, oligomycin and $Ap_5A$, an alkaline pH optimum, the lack of essential —SH and aliphatic —OH amino acid residues and the possible participation of carboxyl groups and arginine in the catalytic activity (as evidenced by the degree of substrate protection from chemical modification). The similar response of the ATPase and ADPase activities to group-specific reagents suggests that both substrates interact with the same active site, as does the competitive inhibitory effect of analogues of ATP and ADP on both ATPase and ADPase activities. It is interesting to note that 10 μM, the concentration which has been decribed to produce a complete loss of $(Ca^{2+} + Mg^{2+})$-ATPase activity from kidney cortex plasma membranes orthovanadate did not completely inhibit both activities (Gmaj *et al*., 1982).

The similar $M_r$ values of both activities determined by the irradiation–inactivation technique, which reveals the size of the smallest unit required to carry out the enzyme function (Kempner and Schlegel, 1979), also indicate involvement of a single protein. The values reported here agree with those for other mammalian apyrases (LeBel *et al*. 1980; Valenzuela *et al*., 1989; Pieber *et al*., 1991; Picher *et al*., 1993; Kettlun *et al*., 1994).

Although most of the studied apyrases have an absolute requirement for bivalent metals (Valenzuela *et al*, 1989; Pieber *et al*, 1991), the renal cortex ADPase activity retains considerable activity in the presence of chelating agents. This difference in ATPase and ADPase activities has also been observed with apyrases from *Solanum tuberosum* (Kettlun *et al*., 1982). The placental enzyme required lower levels of $Ca^{2+}$ for maximum stimulation (Kettlun *et al*., 1994), being able to hydrolyse the uncomplexed nucleotide.

The kinetic characterization of this pyrophosphorolytic (apyrase-like activity) clearly indicates that it is different from the $(Ca^{2+} + Mg^{2+})$-ATPase associated with $Ca^{2+}$ transport. This latter enzyme is highly specific towards ATP, requires $Ca^{2+}$ and $Mg^{2+}$ simultaneously, requires sulphydryl groups for the activity and exhibits a kinetic mechanism with phosphoryl-enzyme as an intermediate (De Smedt *et al*., 1983). We discount the possibility of the formation of such intermediate during apyrase catalysis because AMPcP, a product analogue, is a competitive inhibitor. Kinetic studies with pancreas apyrase (Laliberté and Beaudoin, 1983) also support our conclusions.

Gradient centrifugation studies indicate that the ($Ca^{2+}/Mg^{2+}$)-ATPase activity reported previously in the renal cortex microvillar membrane (Busse *et al.*, 1980; Mörtl *et al.*, 1984; Van Erum *et al.*, 1988,1995) is the ATP-diphosphohydrolase. This is further supported by the crossreactivity of this enzyme with antibodies directed against placental ATP-diphosphohydrolase.

Apyrase appears to be localized as an ecto-enzyme in both the microvillar membranes and the vascular endothelium. The latter is evidenced by the inhibition of nucleotide hydrolysis by apyrase antibodies. This ecto-enzyme localization supports our thesis that this enzyme may have an important role in the extracellular metabolism of nucleotides.

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