Comparison of the biochemical properties, regulation and function of ATP-diphosphohydrolase from human placenta and rat kidney

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Presented at the X Annual Meeting of the Federação de Sociedades de Biologia Experimental, Serra Negra, SP, Brasil, August 23-26, 1995.

Research supported by Fondecyt (No. 193-1003) and DTI (No. B-3345-9433).

Abstract

ATP-diphosphohydrolase (apyrase, EC 3.6.1.5) has both ATPase and ADPase activity that are stimulated by bivalent metals, with Ca^{2+} being the most effective. The possible physiological function of this enzyme, associated with placental and renal microvilli, is related to the extracellular metabolism of nucleotides. A comparison of the biochemical properties of human placenta and rat kidney apyrase is presented, showing similarities in Mr, bivalent metal stimulation, nucleotide nonspecificity, insensitivity towards specific ATPase inhibitors, and lack of essential sulfhydryl and aliphatic hydroxyl groups. We describe the treatment of membrane preparations from both tissues with different detergents and the isoelectric focusing of the solubilized proteins to partially purify apyrase. An ectoenzyme localization is assigned both in microvillus membranes and in the vasculature on the basis of organ perfusion experiments with nucleotides in the presence of antibodies. Placental and kidney microvillus membranes inhibited ADP-induced platelet aggregation, in agreement with an extracellular role. Initial studies on enzyme regulation suggested the existence of at least two types of modulatory proteins: an activating protein in the cytosol of both tissues, and an inhibitory protein associated with placental microsomes. Possible hormonal regulation was investigated in kidneys using *in vivo* estradiol treatment, but only slight changes in total apyrase activity were observed.

Key words
- ATP-diphosphohydrolase
- Apyrase
- Modulatory proteins

at the ratio of 1 g/6 ml using a Potter Elvejhem (glass pestle) to break cells and the homogenate was centrifuged at 12,000 *g* for 20 min to eliminate the mitochondrial fraction and further centrifuged at 100,000 *g* for 1 h to separate the microsomal fraction from the soluble fraction. Proteins in the 100,000-*g* supernatant and proteins solubilized from the microsomal fraction with 2 M NaCl were precipitated separately with 50% ammonium sulfate (17). The pellet was dissolved in 50 mM Tris-HCl, pH 7.5, and heated at 100°C for 5 min to inactivate apyrase activity and denature other proteins. The supernatant was freeze-dried, dissolved in distilled water and exhaustively dialyzed against 0.1 M NaCl. The fraction was called Fr-50 (17). The presence of regulatory proteins of apyrase activity was tested on the placental and kidney microvillus preparations previously washed with 2 M NaCl (to remove modulatory proteins). Bovine serum albumin (BSA) at the same protein concentration as Fr-50 was used as control and enzyme activity demonstrable in the presence of BSA was assumed to represent 100% activity.

Results and Discussion

Kinetic and physicochemical characterization of ATPase and ADPase activity from placenta and kidney microvilli

The conservation of the original "right side of orientation" is a characteristic of microvillus vesicles. This was confirmed by the similar amount of 5'-nucleotidase activity measured in the presence or absence of Triton X-100. Other detergents (see below) did not release more activity than Triton X-100. However, the maximum ATPase and ADPase activity of these membrane preparations was observed in the absence of detergent. This led us to conclude that ATPase-ADPase activity measured in the presence of Ca^{2+} has an ectoenzyme localization. Both activities were stimulated by bivalent metal ions, Ca^{2+} being the most efficient, followed by Mg^{2+}>Mn^{2+}. The measured Ca^{2+}-ATPase activity was not due to the calcium transport enzyme, known as (Ca^{2+} + Mg^{2+})-ATPase, because activity determined in the presence of 5 mM Mg^{2+} was not further stimulated by the addition of micromolar concentrations of Ca^{2+}. The activating effect of millimolar concentrations of Ca^{2+} is a common characteristic of all apyrases studied (6,7,9-14).

The ATPase and ADPase activity of microvillus vesicles from placenta and kidney was not reduced by the addition of a sulfhydryl reagent such as *bis*-dithionitrobenzoic acid (DTNB) or the aliphatic hydroxyl-modifying reagent phenylmethylsulfonyl fluoride (PMSF). These results agree with previous reports that the functional groups of these amino acids are not necessary for apyrase activity (6,11,27).

The molecular mass of the enzyme present in these two tissues was determined by ^{60}Co radiation-inactivation. This technique indicates the size of the smallest unit required to carry out the enzyme function and has been widely used to determine the Mr of enzymes in the native membranes (23). The inactivation of ATPase and ADPase activity was an exponential function of irradiation dose as shown for placenta (Figure 1A) and kidney (Figure 1B) microvilli. Similar values for Mr were obtained for both the ATPase and ADPase activities present in membrane fractions from both tissues (see Table 1). These values, 60 to 68 kDa, are consistent with those determined by gel filtration for mammalian apyrases from pig pancreas (7) and rat placenta and uterus (6,8).

Solubilization and partial purification

Table 2 summarizes the results obtained when different detergents were used to solubilize apyrase activity from membrane fractions. Data are reported for solubilized ADPase activity present in the 100,000-*g* supernatant and activity remaining in the

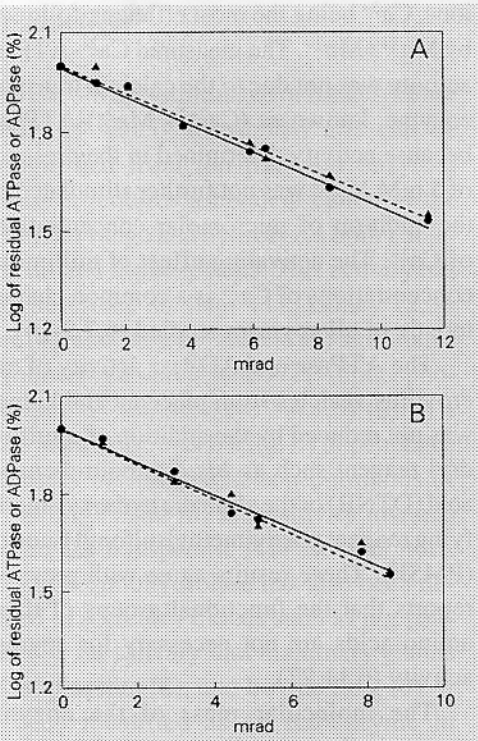

Figure 1 - ^{60}Co inactivation of the ATPase and ADPase activity of membrane-bound apyrase. The microvillus membranes from placenta or kidney were freeze-dried in vacuum-sealed ampoules and irradiated at a dose rate of 1 mrad/day. The activity of membranes maintained at the same temperature as those irradiated was considered to be 100%. Each point is the average of three independent samples (r>0.98). *A*, Placental membranes; *B*, kidney membranes. ●, ATPase activity; ▲, ADPase activity.

Table 1 - Characterization of ATPase and ADPase activity prepared from placental and renal microvillus membranes.

Apyrase activity was solubilized from membranes with n-octylglucoside (10 mg/mg protein). Material was separated by isoelectrofocusing (IEF) and ATPase-ADPase activity was detected after gel elution with the assay medium (without the substrate). Fractions eluted from IEF gels having most of the observed activity were submitted to SDS-PAGE. Protein associated with each band was determined by densitometry after staining and the distribution is reported as % within parentheses. The pI reported for ATPase-ADPase activity was the average of three independent determinations.

	Placenta		Kidney	
	ATPase	ADPase	ATPase	ADPase
Mr (kDa)	62.7	60.5	65.6	67.4
pI	4.5	4.5	5.0	5.0
Protein bands at the pI determined by SDS-PAGE				
	210 kDa (10%)		128 kDa (30%)	
	98 kDa (10%)		67 kDa (40%)	
	64 kDa (80%)		53 kDa (30%)	

pellet. ATPase activity behaved similarly (data not shown). The experiments were carried out at a protein concentration of 1 mg/ml and at the detergent concentrations indicated because they provided the maximum solubilization and recovery of enzyme activity and reproducibility (data not shown). The behavior of ATPase activity was similar to that reported in the table for ADPase (data not shown). N-Octylglucoside (alone or combined with other non-ionic detergents such as digitonin and laurylsarcosine) solubilized 50% of the ADPase activity from placenta and kidney membrane preparations. ADPase activity from placenta was more stable in the presence of detergents compared to enzyme activity prepared from kidney cortex microvilli. Triton X-100, Tween 20, Nonidet P-40 and lithium diiodosalicylate inactivated 100% of the ATPase-ADPase activity and others such as Brij-35 were unable to release apyrase from the membrane. The solubilization of apyrase from different animal tissues has been difficult due to the poor recovery of activity. Solubilization is tissue dependent as indicated by the fact that the enzyme is removed from bovine aorta membranes with Triton X-100 in close to 40% yield (28), whereas pig pancreas apyrase was solubilized with Triton X-100 with only a 6% recovery (7).

Preliminary purification of the enzyme solubilized with n-octylglucoside (10 mg/mg protein) by polyacrylamide gel by electrofocusing in the pH range of 3.5 to 10 showed that both hydrolytic activities (ATPase-ADPase) from each source focused at the same pH: 4.5 for the placental and 5.0 for the kidney microvillus preparations (Table 1). The solution eluted from the isoelectrofocusing gel with enzyme activity contained at least three different proteins with the same pI (4.5 or 5.5) on SDS-PAGE (Table 1). The main protein band of 64 kDa detected on SDS-PAGE from the placental tissue after electrofocusing corresponded to 80% based on protein staining and densito-

metry. The three protein bands obtained from kidney after separation by SDS-PAGE were recovered in similar proportions and all had similar pI = 5.0. We assume that the protein bands of molecular mass 64 and 67 kDa obtained from placental and kidney tissues, respectively, correspond to apyrase because the Mr values are similar to those determined by radiation-inactivation.

The effect of specific inhibitors has been used to distinguish transport and mitochondrial ATPase activity from ATP-dihydrophosphohydrolase activity. Ouabain (5 mM) and oligomycin (0.1 mg/ml) which are specific inhibitors of (Na^+,K^+)-ATPase, respectively, did not modify the ATPase or ADPase activity of the soluble partially purified fractions prepared from placenta and kidney microvilli. Similarly, the adenylate kinase inhibitor Ap_5A (29) at 0.5 mM did not affect the hydrolytic activity of the membrane fractions from either source. These observations indicate that the ADPase activity is a true ADPase activity and is not the result of the coupled action of adenylate kinase and an ATPase. Specificity studies of enzyme prepared by isoelectric focusing showed that all nucleoside di- and triphosphates tested were hydrolyzed (Table 3), but the preparation did not have monophosphoesterase activity. Taken as a whole, these results confirm and extend the demonstration of ecto-apyrase activity in human placenta and rat renal cortex microvilli, which present similar characteristics reported by us (6,8) and by others (7,9,10-12) who studied the enzyme from other tissues.

Table 2 - Solubilization of ADPase activity from placental and renal microvillus membranes.

Membranes (1 mg protein/ml) were preincubated at 30°C for 30 min with detergent and later separated into supernatant and pellet fractions by centrifugation at 100,000 *g* for 1 h at 4°C. Activity is reported as percent of activity present in microvillus membranes before detergent treatment. ND, Not determined.

	ADPase recovery (%)			
	Placenta		Kidney	
	Supernatant	Pellet	Supernatant	Pellet
n-Octylglucoside (5 or 10 mg/mg protein)	25	36	51	4
Digitonin (1 or 2%)	36	39	10	17
N-Laurylsarcosine (1%)	51	16	20	0
Digitonin (1%) + n-octylglucoside (5 mg/mg protein)	54	17	21	0
N-Laurylsarcosine (1%) + n-octylglucoside (5 mg/mg protein)	54	5.5	ND	ND
N-Laurylsarcosine (1%) + digitonin (1%)	43	26	ND	ND
CHAPS (0.5 %) + lysophosphatidyl choline (0.5%) + 0.6 M KI + 0.1 M EDTA	39	23	27	21

Table 3 - Substrate specificity of placental and renal fractions eluted from the isoelectrofocusing gel.

Nucleotide concentration was 2 mM in the presence of 5 mM Ca^{2+} in 100 mM Tris-HCl buffer, pH 8.0.

Nucleotide	Placenta	Kidney
	Relative activity (%)	
ADP	100	100
GDP	112	74
UDP	67	80
CDP	71	114
dTDP	62	114
ATP	83	87
GTP	92	203
UTP	95	152
CTP	71	153
dTTP	56	153
AMP	0	0

Perfusion experiments

In perfused isolated placenta and kidney, the Ca^{2+} complex of 1 mM ATP or ADP was hydrolyzed to Pi (data not shown). There was no damage to endothelial membranes, as indicated by the absence of the cytosol marker lactate dehydrogenase activity in the perfusate. Perfusion of nucleotides in the

presence of specific ATPase inhibitors such as oligomycin (0.1 mg/ml), ouabain (5 mM) and verapamil (0.05 mM) (a Ca^{2+} channel blocker) and levamisole (1 mM) (a phosphatase inhibitor) did not modify the total amount of Pi released into the perfusate from either substrate.

Antibodies to the 64-kDa apyrase prepared by SDS-PAGE from placental microvilli after solubilization with n-octylglucoside (10 mg/mg protein) significantly inhibited both ATP and ADP hydrolysis during perfusion through isolated placenta and kidney preparations, as shown in Table 4. The amount of Pi released in the presence of rabbit pre-immune serum was considered to be 100% hydrolysis. These data indicate that the vasculature of both the placenta and kidney contains apyrase activity with an ectoenzyme localization.

Regulation of apyrase activity

Modulator proteins

The data in Table 5 show that the Fr-50 fraction prepared from the cytosol fraction of placenta and kidney activated the apyrase activity of washed microvilli. Placental Fr-50 activated placental microvilli activity 20-24% and kidney Fr-50 activated kidney microvilli apyrase activity 33-35%. In contrast, the Fr-50 fraction prepared from placental microsomes inhibited placental membrane apyrase activity by 16-17%, whereas microsomal Fr-50 had no effect on kidney membrane apyrase activity. The placental microsome protein present in Fr-50 respon-

Table 4 - Perfusion of isolated placenta and kidney with calcium-nucleotide complexes.

Ectonucleoside di- and triphosphatase activity was monitored by measuring the appearance of Pi in the perfusates. $CaCl_2$ concentration was twice that of the nucleotide concentration. Pi released in preparations perfused with pre-immune serum instead of the antibodies (immune serum) was considered to be 100% ectonucleotidase activity. The antibody and pre-immune serum were present in the perfusate at a dilution of 1/3.

Addition	Ecto-ATPase activity (%)	Ecto-ADPase activity (%)
Placenta		
10 µmol ATP or ADP + pre-immune serum	100	100
10 µmol ATP or ADP + immune serum	50	66
Kidney		
1 µmol ATP or ADP + pre-immune serum	100	100
1 µmol ATP or ADP + immune serum	60	43

Table 5 - Effect of placental and renal heat-stable proteins on their corresponding ATPase and ADPase membrane activity.

The amounts of protein in the Fr-50 fractions tested were: cytosol Fr-50, 1 mg/ml from placenta and 2 mg/ml from kidney. For microsomal Fr-50 they were 0.17 mg/ml from placenta and 0.5 mg/ml from kidney.

	Placenta membrane		Kidney membrane	
	ATPase (%)	ADPase (%)	ATPase (%)	ADPase (%)
Calmodulin (potato or bovine)	100	100	100	100
Fr-50 isolated from cytosol	124	120	135	133
Fr-50 isolated from microsomes	84	83	100	100

sible for inhibition is not an integral part of the membrane, as indicated by the fact that it can be removed by washing with 2 M NaCl. In view of the procedure used to prepare the Fr-50 fractions (see Methods), we conclude that these modulating proteins are heat stable, dialyzable and soluble in 50% ammonium sulfate.

The effect seems to be specific and not due to a "nonspecific protein effect" because BSA was used at equal concentrations as control to determine the 100% activity reference value. Furthermore, heat-stable calmodulin can be ruled out because potato tuber (17) or bovine brain calmodulin had no effect on activity, as indicated in Table 5. The data in Figure 2 show that a protein with pI 8.4 present in kidney cytosol Fr-50 can activate the ADPase activity of kidney microvilli.

Other proteins in addition to calmodulin (30) can modulate enzyme activity. We have described an apyrase-activating protein and an apyrase-inhibiting soluble protein in *S. tuberosum* tubers (17). The presence of proteins that activate (31) or inhibit (32) a high-affinity Ca^{2+}-stimulated Mg^{2+}-dependent ATPase has been reported in rat liver. The presence of proteins that modulate apyrase activity in animal tissues suggests that this enzyme may participate in the regulation of other physiological processes as an ecto-nucleotidase which, acting together with 5'-nucleotidase, may regulate ATP and ADP levels and also facilitate the reincorporation of adenosine.

Effect of estradiol on ATPase and ADPase activity in rat kidney

The possible regulatory effect of estradiol on kidney apyrase activity was evaluated in ovariectomized rats implanted with silastic capsules (5 cm x 1 mm I.D.) containing 200 µg/ml of estradiol, which maintained the hormone concentration at levels equivalent to those of proestrus for 8 days.

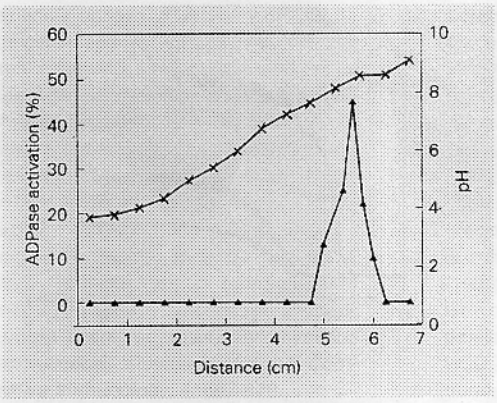

Figure 2 - Isoelectrofocusing of the cytosolic Fr-50 prepared from rat kidney. After measuring the pH gradient, the gel was cut into 2-mm slices and placed in the assay medium overnight. The activating protein was detected by the addition of 1 µl of the microvillus membranes (containing apyrase activity) and 2 mM ADP to each slice. An isoelectrofocused gel without Fr-50 was used as control. ▲, ADPase activation; X, pH.

The apyrase activity of our ovariectomized rats treated with estradiol and ovariectomized rats did not differ from that of intact rats, which was 0.0678 ± 0.0009 (ATPase) and 0.0600 ± 0.0083 (ADPase) µmol Pi min^{-1} mg $protein^{-1}$.

Receptors for estradiol have been described in renal tissue (33). Suzuki et al. (34,35) have reported an increase in Mg^{2+}-HCO_3-ATPase activity after peritoneal injection of estradiol. Although this increase was not very large it was statistically significant. The lack of response of apyrase to estradiol treatment in our experiment implies that apyrase is not regulated by this hormone in renal tissue. However, these results do not rule out a possible regulation of apyrase activity by other hormones. Neither lactic dehydrogenase nor gamma-glutamyl transpeptidase activity was affected by the hormonal treatment. Gamma-glutamyl transpeptidase is a characteristic ectoenzyme of renal cortex microvilli (36).

Functional studies

Both membrane fractions inhibited the platelet aggregation induced by ADP in a concentration-dependent way (Figure 3A,B) because of their ADPase activity. We suggest that apyrase activity in the placental microvilli could be important in regulating the ADP levels available for platelet aggregation. Some investigators have proposed that the elevated ADPase activity in human

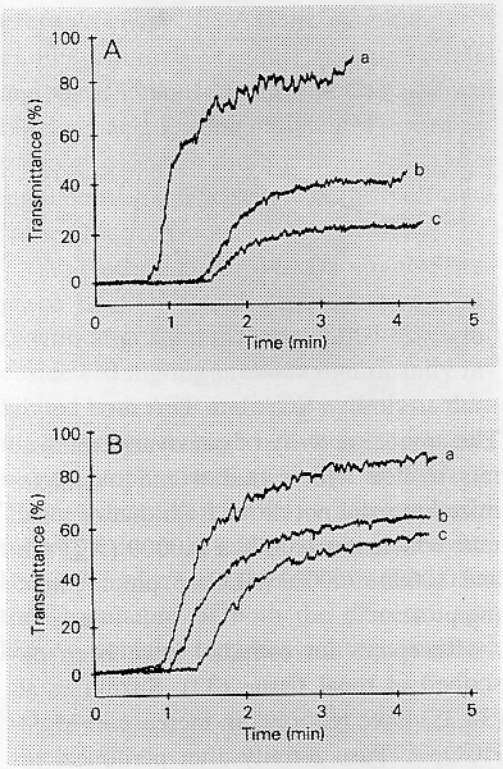

Figure 3 - Inhibition of ADP-induced platelet aggregation of microvillus membranes. The aggregation control in response to 4 µM ADP is indicated by tracing *a*. *Panel A*, Addition of placental membranes containing 0.023 U/ml ADPase (b) and 0.046 U/ml ADPase (c). *Panel B*, Addition of kidney membranes containing 0.022 U/ml ADPase (b) and 0.029 U/ml ADPase (c)

placenta may compensate for the limited amounts of prostacyclin, the major vascular prostaglandin, a vasodilator and a very potent inhibitor of platelet aggregation (37). The main function of apyrase localized in the renal microvilli may be the salvage of purine nucleobases present in primary urine, whereas the function of apyrase in both placental and kidney vasculature may be related to the regulation of the levels of ATP and ADP, which, together with adenosine, exert important physiological functions acting on their respective purinoceptors (15,16,38-40). The regulation of extracellular ATP levels may also modulate the ectoprotein kinase activity already demonstrated in endothelial cells (41).

Conclusions

The kinetic characterization of ATPase and ADPase activity of microvillus membranes prepared from human placenta and rat kidney cortex indicates that they correspond to the single enzyme ATP-diphosphohydrolase or apyrase. Similarities in pI and Mr of both pyrophosphohydrolytic activities are consistent with the existence of a single protein with ecto-ATPase and ecto-ADPase activity. The significant inhibition by anti-apyrase antibodies of both ecto-ATPase and ecto-ADPase activities present in placental and renal vasculature indicates the existence of apyrase in the endothelium with an ectoenzyme localization. ATP-diphosphohydrolase seems to be regulated by one or two modulatory proteins. The inhibitory effect of the membranes observed on the ADP-dependent platelet aggregation illustrates one of the functions proposed for this enzyme, at least in the placenta, which is the inhibition of platelet aggregation.

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