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Adenosine deamination to inosine in isolated basolateral membrane from kidney proximal tubule: Implications for modulation of the membrane-associated protein kinase A

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ABSTRACT

In this work, the metabolism of adenosine by isolated BLM associated-enzymes and the implications of this process for the cAMP-signaling pathway are investigated. Inosine was identified as the major metabolic product, suggesting the presence of adenosine deaminase (ADA) activity in the BLM. This was confirmed by immunoblotting and ADA-specific enzyme assay. Implications for the enzymatic deamination of adenosine on the receptor-modulated cAMP-signaling pathway were also investigated. We observed that inosine induced a 2-fold increase in $[^{35}S]$ GTPγS binding to the BLM and it was inhibited by 10^{-6} M DPCPX, an A_1 receptor-selective antagonist. Inosine (10^{-7} M) inhibited protein kinase A activity in a DPCPX-sensitive manner. Molecular association between ADA and $G_{\alpha i\text{-}3}$ protein-coupled A_1 receptor was demonstrated by co-immunoprecipitation assay. These data show that adenosine is deaminated by A_1 receptor-associated ADA to inosine, which in turn modulates PKA in the BLM through A_1 receptor-mediated inhibition of adenylyl cyclase.

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Introduction

Extracellular adenosine (Ado)[2] has a key role in the physiological function of various organs, such as kidney, through autocrine and paracrine mechanisms [1,2]. The effect elicited by Ado depends on the local concentration and metabolism, as well as receptor subtype and the signaling pathway triggered by the receptor. The level of Ado is primarily defined by the action of five enzymes involved in its metabolism: 5′-nucleotidase (EC no. 3.1.3.5), adenosine kinase (EC no. 2.7.1.20), adenosine nucleosidase (EC no. 3.2.2.7), adenosine deaminase (EC no. 3.5.4.4) and *S*-adenosylhomocysteine hydrolase (EC no. 3.3.1.1).

It is well known that adenosine and adenine nucleotides such as ATP modulate hemodynamic and tubular functions [3–7] while little is known about other purine metabolites. For several years, Ado was regarded as the only endogenous purine nucleoside agonist for Ado receptors. Ado deamination to inosine catalyzed by adenosine deaminase (ADA) may be considered a mechanism to restrain the cellular effects induced by Ado and ATP [8,9]. However, inosine-induced biological effects mediated by Ado receptors in neuronal and immunologic system have also been demonstrated [10]. In renal tissue, the biological effects of inosine are not known and the role of plasma membrane-associated enzymes in the metabolism of adenosine is not fully understood.

Most Ado-induced effects are mediated by a family of G protein-coupled receptors [1]. In the last few years, our group has been studying the possible role of purines on the modulation of sodium transport in the renal cortex. We have demonstrated that Ado controls cAMP-modulated sodium transport in isolated basolateral membrane (BLM) of proximal tubules in a concentration-dependent biphasic manner through interaction with A_1 and A_2 receptors [11,12]. Recently, we have demonstrated

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[1] These authors contributed equally to the development of this work.
[2] *Abbreviations used:* ADA, adenosine deaminase; Ado, adenosine; ATP, adenosine triphosphate; CHA, N^6-cyclohexyladenosine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; db-cAMP, dibutyryl-adenosine 3′,5′-cyclic monophosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EDTA, ethylenediaminetetraacetic acid; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid; Ino, inosine; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(trishydroxymethyl)-aminomethane.

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that adenine, an Ado-derived nucleobase, binds to specific receptor in the kidney modulating the Na^+-ATPase activity [13]. These data suggest that other adenosine-derived metabolites could play an important role in the modulation of renal function.

In this work, the metabolism of Ado by isolated BLM associated-enzymes and the implications of this process for the cAMP-signaling pathway are investigated. Our results demonstrate that Ado is deaminated by A_1 receptor-associated ADA to inosine, which in turn modulates protein kinase A (PKA) activity in the BLM of proximal tubule through A_1 receptor-mediated inhibition of adenylyl cyclase activity.

Materials and methods

Reagents

[^{35}S]GTPγS (1065 Ci mmol^{-1}), [2-^{3}H]adenosine labeled in the 2-position of the adenine moiety (22 Ci mmol^{-1}) and [propyl-^{3}H]8-cyclopentyl-1,3-dipropylxanthine (DPCPX) labeled in the propyl group (118 Ci mmol^{-1}) were purchased from Amersham Biosciences, UK Limited. [^{32}P]Pi was obtained from the Institute of Energetic and Nuclear Research, São Paulo, SP, Brazil. ATP, ouabain, furosemide, inosine, adenosine, histone, dibutyryl-cAMP, GDPβS, GTPγS, ethylenediaminetetraacetic acid (EDTA), *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (HEPES), tris(trishydroxymethyl)-aminomethane (Tris), PKA inhibitor peptide from rabbit (Thr–Thr–Tyr–Ala–Asp–Phe–Ile–Ala–Ser–Gly–Arg–Thr–Gly–Arg–Arg–Asn–Ala–Ile–His–Asp) [14] were purchased from Sigma Chemical Co., St. Louis, MO, USA. DPCPX was purchased from Research Biochemicals International, Natick, MA, USA. Percoll was purchased from Pharmacia Biotech, Uppsala, Sweden. Goat polyclonal antibodies against adenosine deaminase, and A_2 and A_3 receptors were purchased from Santa Cruz Biotechnology Inc. (USA). Rabbit polyclonal antibodies against adenosine A_1 receptor and $G_{\alpha i\text{-}3}$ protein were purchased from Oncogene Research Products (Boston, MA). Anti-goat and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were purchased from Amersham Biosciences (*Piscataway*, NJ, USA). *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was purchased from Calbiochem, San Diego, USA. All other reagents were of the highest purity available. All solutions were prepared with deionized glass-distilled water. [γ-^{32}P]ATP was prepared as previously described [15].

Isolation of the BLM

The cortex homogenate and isolated basolateral membrane were obtained from adult pig kidney. The kidneys were removed immediately after the animal's death and maintained in cold solution (4 °C) containing: 250 mM sucrose, 10 mM Hepes–Tris (pH 7.6), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF). Thin slices of the cortex (cortex corticis) were removed with a scalpel. After dissection, slices were homogenized in the same cold solution with a Teflon and glass homogenizer. The homogenate was centrifuged for 10 min at 3000 rpm in an SCR20B centrifuge using an RP12-2 rotor (Hitachi) at 4 °C. The supernatant was collected and stored at −4 °C. The fraction containing the BLM was isolated using the Percoll gradient method [16]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 5–10 mg of protein ml^{-1} and stored at −4 °C. The specific activity of the BLM marker, (Na^+ + K^+)ATPase (220.6 ± 20.3 nmol Pi mg^{-1} min^{-1}) was enriched 8-fold when compared with that measured in kidney cortex homogenate.

Analysis of the metabolism of adenosine in the isolated BLM by thin layer chromatography (TLC)

Isolated BLM from proximal tubule (0.3 mg ml^{-1}) was incubated with 1.9 μM [^{3}H]adenosine (22 Ci mmol^{-1}) in HEPES–Tris 0.5 M (reaction volume 0.12 ml), for 10 min at 37 °C. The reaction was stopped by addition of 30 μl of 50% TCA. The samples were centrifuged for 15 min at 15,000 rpm in an Eppendorf centrifuge at 4 °C. An aliquot (60 μl) of the supernatant of each sample was spotted on the TLC plate (silica gel 60 F254, 16 × 4 cm, 0.25 mm layer thickness, Merck). The mobile phase consisted of methanol, ethyl acetate, butanol, ammonium hydroxide (3:4:7:4, v/v). An aliquot (30 μl) of a mix containing 5 mM of each purine standard (adenosine, inosine, hypoxanthine, adenine nucleotides and adenine) was also spotted over each sample. After running for 15 cm, the organic phase of the TLC was evaporated to dryness at room temperature. The areas corresponding to [^{3}H]adenosine-derived metabolites were visualized through reflection of UV light by co-migration with the respective purine standards, scraped from the plates and transferred to a scintillation vial containing 0.4% PPO and toluene, Triton X-100, water (8:4:1, v/v). The radioactivity contained in each area was quantified by liquid scintillation counting (Packard Tri-Carb 2100 TR).

ADA-specific enzyme assay

ADA activity in the BLM isolated from pig kidney proximal tubule was determined by spectrophotometric measurement of ammonia released in the deamination reaction of adenosine to inosine, according to the method described by O'Donovan [17]. Composition of the reaction medium was (0.15 ml): PBS (pH 7.0), 5 mM adenosine. The reaction was started by addition of the isolated BLM from proximal tubule (at a final protein concentration of 0.2 mg ml^{-1}). After 10 min incubation at 37 °C, the reaction was stopped by addition of 1 ml of solution I (50.4 mg ml^{-1} phenol, 0.4 mg ml^{-1} sodium nitroprussate), followed by addition of 1 ml of solution II (24 mg ml^{-1} NaOH, 2.1 mg ml^{-1} sodium hypochlorite). The ammonia released with inosine in the ADA-catalyzed reaction was determined stoichiometrically by absorbance at 635 nm in a spectrophotometer, using NH_4Cl as standard. ADA-specific activity was determined as the difference between ammonia released in the absence and in the presence of 5 × 10^{-8} M EHNA (an ADA-specific inhibitor).

[^{35}S]GTPS binding assay

The [^{35}S]GTPγS binding assay was carried out using isolated BLM as described by Lazareno [18] with some modifications. The composition of the reaction medium (0.05 ml) was: 100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0) and 5 nM [^{35}S]GTPγS (1065 Ci mmol^{-1}). The reaction was started by addition of isolated BLM (1 mg ml^{-1}). After 1 min at 37 °C, the reaction was stopped by addition of 5 ml of ice-cold buffer (100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0)). The content of each tube was immediately filtered and washed twice with 5 ml of ice-cold buffer through a glass fiber filter (MFS Advantec Inc. GB140, 25 mm diameter) under a vacuum. The radioactivity in the filters was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR). Specific binding was determined as the difference between the binding of [^{35}S]GTPγS in the absence and in the presence of 1 mM GTP.

[^{3}H]DPCPX binding assay

The [^{3}H]DPCPX binding assay was carried out using isolated BLM as previously described [13] with some modifications. Briefly, the composition of the reaction medium (0.05 ml) was: Tris–HCl

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