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Identification and characterization of adenosine A_1 receptor-cAMP system in human glomeruli

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Identification and characterization of adenosine A_1 receptor-cAMP system in human glomeruli. Although adenosine is known to affect renal function through stimulating adenosine receptors, little is known about A_1 receptors in human glomeruli. Thus, we attempted to identify the adenosine A_1 receptor-cyclic AMP (cAMP) system in human glomeruli. Normal renal cortical tissues were obtained at nephrectomy of patients with renal cell carcinoma. Glomeruli were isolated using a graded sieving method or dissected manually under a stereomicroscope. Radioligand binding assay using 2-chloro-N-[^{3}H] cyclopentyl adenosine ([^{3}H]CCPA, an A_1 agonist ligand) was performed at 30°C for 90 minutes. Cyclic AMP (cAMP) produced in glomeruli was measured after incubation with different concentrations of N^6-cyclohexyladenosine (CHA; A_1 agonist) and a phosphodiesterase inhibitor. The specific binding was saturated within 60 minutes and reversible by adding 1 mM of theophylline. Scatchard plot analysis revealed a single class of binding site (K_d = 1.78 ± 0.21 nM, B_{max} = 271.7 ± 35.8 fmol/mg protein). The specific binding was inhibited dose-dependently by various agents in an order suggesting A_1 receptor specificity. CHA inhibited the production of cAMP in microdissected human glomeruli. This inhibitory effect was antagonized by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 antagonist). This is the first study revealing the presence of the A_1 receptor-cAMP system in human glomeruli using a radioligand binding assay method and by measuring the cAMP production.

Adenosine has been shown to be involved in the regulation of a variety of renal functions such as renal blood flow, glomerular filtration rate [1] and renin secretion [2, 3] through adenosine receptors in the kidney, affecting urinary flow and electrolyte excretion [4, 5]. According to current concepts [6–9], the action of adenosine is mediated by extracellular receptors designated A_1 and A_2, which are coupled to adenylate cyclase through the inhibitory guanine nucleotide binding protein Gi and stimulatory protein Gs. The stimulation of A_1 receptors was reported to inhibit cAMP production in heart muscle cells [10, 11], adipocytes [8], the central nervous system [12], and renal tissues including glomeruli [13], mesangial cell [14], thick ascending limbs [15] and collecting tubules [4, 16].

Recent animal studies have suggested that the stimulation of the A_1 receptors causes the glomerular contraction [17, 18], the decreases in glomerular filtration rate, renal blood flow [19–21] and sodium chloride transport [22, 23], and the inhibition of renin secretion [2, 3], erythropoietin secretion [24] and neurotransmitter release [25, 26]. However, the characterization of A_1 receptors by using a direct radioligand binding assay has not been performed extensively in the kidney [27, 28], and little is known about human glomeruli. Thus, we attempted to identify and characterize the A_1 receptor-cAMP system in human glomeruli by radioligand binding assay using 2-chloro-N-[^{3}H] cyclopentyladenosine ([^{3}H]CCPA, a highly selective A_1 agonist ligand [29]) and cAMP assay.

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Methods

Isolation of glomeruli for binding assay

Human kidneys were obtained at nephrectomy from patients with renal cell carcinoma. Techniques for isolation of glomeruli were according to those reported previously [30], with some modification. Normal cortical tissues were immediately separated from medulla, dissected and minced to a paste-like consistency. The paste was successively pushed through 120 μm stainless sieve which excluded the tubules, and through a 89 μm sieve which retained glomeruli. The suspension of glomeruli obtained was centrifuged at 120 × g for three minutes and the supernatant was discarded. This operation was repeated three times. The purity of the glomeruli was more than 97%. The glomeruli were rapidly frozen and kept at −80°C until study of radioligand binding assay. All frozen samples were used within two months.

Radioligand binding assay

For the radioligand binding assay, the glomeruli were homogenized in a Dounce homogenizer in a buffer solution containing 5 mM Tris-HCl and 5 mM EDTA, pH 7.4, at 4°C. Homogenates were centrifuged at 30,000 × g for 30 minutes at 4°C. The glomerular membrane pellets were resuspended to a final concentration of 0.3 to 0.5 mg of protein/ml in a buffer containing 50 mM Tris-HCl, 120 mM NaCl and 3 U/ml of adenosine deaminase, pH 7.4, and preincubated at 30°C for 30 minutes in order to remove endogenous adenosine. Protein concentration of the membrane suspension was determined by the Lowry method [31]. Fifty microliters of the same buffer containing various concentrations of [^{3}H]CCPA were added to 100 μl of the membrane suspension. Nonspecific binding was determined by adding 1 mM of theophylline, an antagonist of adenosine receptors. The incubation was carried out at 30°C for 90 minutes, unless otherwise specified. The reaction was terminated by

filtration through polyethylenimine-treated Whatman GF/C filters with ice-cold incubation buffer containing 50 mM Tris-HCl, 120 mM NaCl and 0.1% BSA. The filters were then dried and their radioactivities were counted with a scintillation counter after adding scintillator. Binding capacity (B_{max}), dissociation constant (K_d) and results from competition studies were analyzed with the LIGAND computer program [32].

Preparation of glomeruli for cAMP assay

A method for preparing isolated glomeruli has been reported previously [33]. Briefly, normal renal cortical tissues were obtained at nephrectomy from patients with renal cell carcinoma. The tissues were sliced and incubated at 30°C for 45 minutes in a Krebs-Ringer bicarbonate buffer solution (KRB, pH 7.4), which contained 0.1% BSA and 0.1% collagenase and was exposed to 95% O_2/5% CO_2. Glomeruli were dissected under a stereomicroscope in an ice-cold modified Hanks' HEPES solution (pH 7.4) containing 3 U/ml of adenosine deaminase and 2.5×10^{-4} M of Ro 20-1724, a phosphodiesterase inhibitor. Isolated glomeruli were without capsules and arterioles and the structure was well preserved. Two glomeruli were transferred to 20 μl of a modified Hanks' HEPES solution in a siliconized glass culture tube for enzyme assay.

Assay of cyclic AMP

Techniques for incubation and assay of cellular cyclic AMP in glomeruli were similar to those reported previously [33]. After five minutes of preincubation at 37°C, 20 μl of modified Hanks' HEPES solution containing various concentration of N^6-cyclohexyladenosine (CHA; a selective adenosine A_1 agonist), 7.3×10^{-7} M of parathyroid hormone (PTH, 1-34; human) and/or 5×10^{-5} M of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; a selective adenosine A_1 antagonist) were added to the incubation medium. The incubation was continued for an additional two minutes at 37°C. The reaction was terminated by adding 50 μl of ice-cold 10% trichloroacetic acid and the solution was extracted three times with 0.8 ml of water-saturated ether. Cyclic AMP was determined by radioimmunoassay after acetylation (New England Nuclear kit). Data were expressed as means ± SE. Statistical analysis were performed by Student's *t*-test after ANOVA.

Materials

[^{3}H]CCPA was obtained from New England Nuclear (Boston, Massachusetts, USA). The following compounds were obtained from Sigma Chemical Company (St. Louis, Missouri, USA): collagenase (type I), adenosine deaminase, N-2-hydroxyethylpiperazine (HEPES), PTH, CHA, DPCPX and 5'-N-ethylcarboxamidoadenosine (NECA, an A_1 and A_2 agonist). YT-146, a selective adenosine A_2 agonist [34], was supplied by Toa Eiyo Ltd. (Fukushima, Japan). Ro 20-1724 was supplied by Hoffmann LaRoche Ltd. (Basel, Switzerland).

Results

Radioligand binding assay

The binding of [^{3}H]CCPA for human glomerular membranes was saturable (Fig. 1) and reversible (Fig. 2). Maximum binding was obtained within 60 minutes after the start of incubation. The apparent rate constant of this pseudo first-order reaction has been calculated to be 0.103 min^{-1} (r = 0.98). The dissociation studies were performed by adding 1 mM of theophylline, a nonspecific adenosine receptor antagonist, to the reaction mixtures after saturation (Fig. 2). Dissociation was also rapid. The K_d value (1.62 nM) calculated from the association and dissociation studies correlated reasonably well with the value obtained from the Scatchard plot analysis. Scatchard analysis revealed the presence of single high-affinity binding site with a K_d of 1.78 ± 0.21 nM and a B_{max} of 271.7 ± 35.8 fmol/mg of protein for the glomerular membranes (N = 10). A representative saturation curve and its Scatchard plot analysis are shown in Figure 3. Binding to the low affinity state was not reliably detected under these conditions. Nonspecific binding was about 30% of the total binding at K_d. Competition studies with various adenosine analogues showed that the binding of [^{3}H]CCPA to human glomerular membranes was inhibited in a dose-dependent manner (Fig. 4). Ki values (means of 3 experiments) against the binding of [^{3}H]CCPA were calculated using the LIGAND and the results were as follows; 1.6 nM for DPCPX (A_1 antagonist), 2.1 nM for CHA (A_1 agonist), 7.2 nM for NECA (A_1 and A_2 agonist), 66 nM for YT-146 (A_2 agonist), 6000 nM for theophylline (nonselective antagonist) (Table 1). The results suggested an adenosine A_1 receptor specificity.

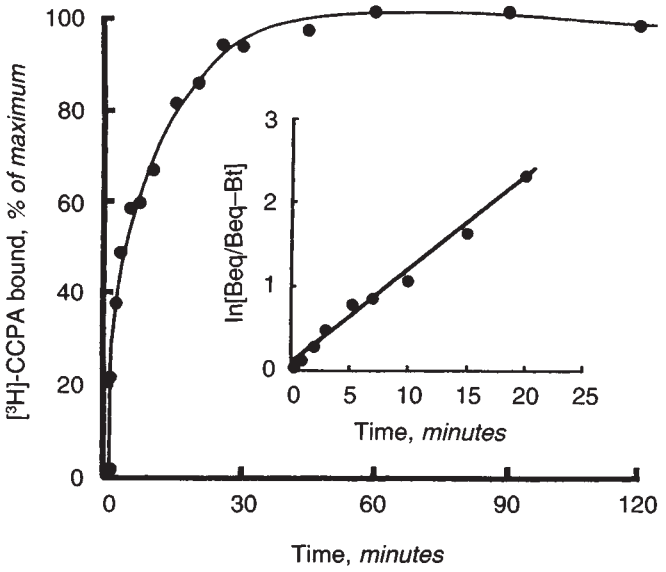

Fig. 1. *Kinetic analysis of [^{3}H] CCPA binding to the human glomerular membranes as a function of time.* The specific binding of the radioligand was rapid and saturable. Maximal binding was obtained within 60 minutes. Insert: Pseudo-first order kinetic plot of [^{3}H] CCPA binding. Data were used to determine Bt (amount of [^{3}H] CCPA bound at time "t") and Beq (amount of [^{3}H] CCPA bound equilibrium). This line (r = 0.98) has a slope, Kap, equal to the observed rate constant of the pseudo-first order reaction.

Glomerular cAMP system

Our previous study [35] showed that cAMP production increased as a function of time, and reached virtually a maximum within two minutes after the start of the incubation. Thus, glomeruli were incubated for two minutes in this study. A linear relationship was observed between the number of glomeruli and cAMP formed in the presence of 7.3×10^{-7} M of PTH during two minutes of incubation. Therefore, two glomeruli were incubated in one tube. The dose-response relationship between

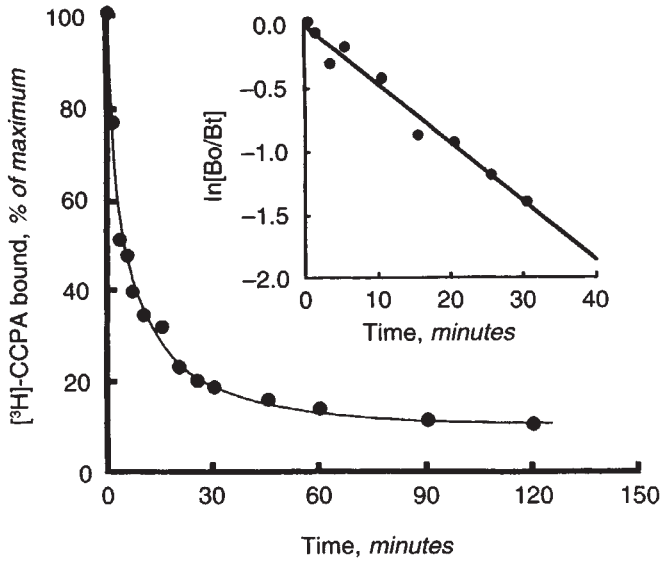

Fig. 2. *Reversibility of [^{3}H] CCPA binding to the human glomerular membranes.* Dissociation curve was determined by adding theophylline to a final concentration of 1 mM after radioligand was first incubated with the membranes for 60 minutes. Insert; First order rate plot of the dissociation of the receptor-ligand complex. Data were used to determine Bt (amount of [^{3}H] CCPA binding at time "t") and Bo (amount of [^{3}H] CCPA binding at time zero). The line, determined by linear regression analysis (r = 0.99) has a slope, K_2, equal to the first order rate constant.

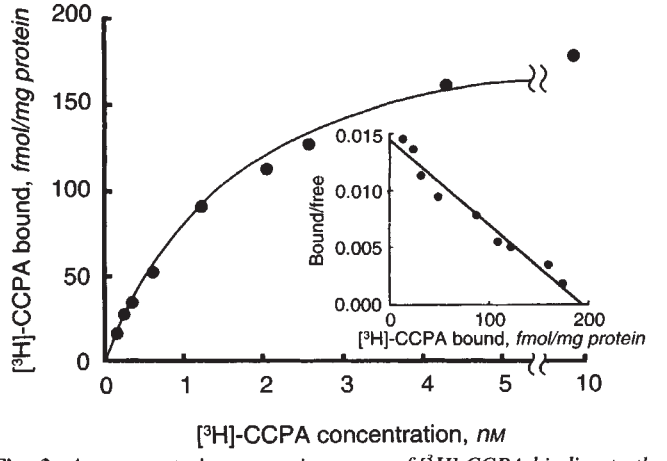

Fig. 3. *A representative saturation curve of [^{3}H] CCPA binding to the human glomerular membranes.* Membrane solution prepared from human isolated glomeruli was incubated with [^{3}H] CCPA solution (0.06 to 9.8 nM). Nonspecific binding was determined by adding 1 mM of theophylline. Incubation was done at 30°C for 90 minutes. Insert; Scatchard analysis shows a straight line, indicating a single binding site.

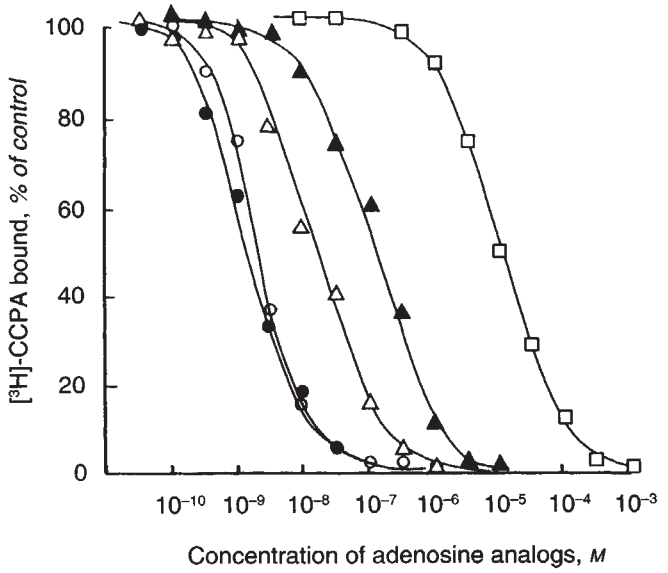

Fig. 4. *Representative competition curves of various adenosine analogs with [^{3}H] CCPA binding to the human glomerular membranes.* Ki of each analogue is as follows: 8-cyclopentyl-dipropylxanthine (DPCPX, an adenosine A_1 antagonist, ●——●), 1.4 nM; N^6-cyclohexyladenosine (CHA, an adenosine A agonist, ○——○), 1.9 nM; 5′-N-ethyl- carboxamidoadenosine (NECA, an adenosine A_1, A_2 agonist △——△, 6.8 nM; 2-octinyl-adenosine (YT-146, an adenosine A_2 agonist, ▲——▲, 63 nM; and theophylline (□——□), 5400 nM.

Table 1. Ki values of adenosine analogs for the binding of [^{3}H]CCPA to the human glomerular membranes

Adenosine analogs	Ki nM
DPCPX; A_1 antagonist	1.6
CHA; A_1 agonist	2.1
NECA; A_1 and A_2 agonist	7.2
YT-146; A_2 agonist	66
Theophylline	6000

Data are means of 3 experiments.

PTH and cAMP production in the glomeruli showed that half-maximum stimulation occurred at 1×10^{-7} M PTH [35]. Thus, PTH at a concentration of 7.3×10^{-7} M was chosen to stimulate adenylate cyclase. Under these assay conditions, CHA, an adenosine A_1 agonist, significantly inhibited cAMP production at concentrations of 5×10^{-8} M and 5×10^{-7} M compared to basal value (from 28.4 ± 3.4 to 23.6 ± 2.2 and 22.1 ± 2.0 fmol/glomerulus, respectively: $P < 0.05$). However, this inhibitory effect was no longer apparent at 5×10^{-5} M (Fig. 5). In the presence of 5×10^{-5} M of DPCPX, an adenosine A_1 antagonist, the inhibitor effect of CHA was disappeared. Moreover, high concentrations of CHA (5×10^{-5} M) stimulated cAMP production (from 26.4 ± 3.4 to 36.1 ± 3.2 fmol/glomerulus; $P < 0.05$) possibly through the A_2 receptor. PTH (7.3×10^{-7} M) stimulated cAMP production in human glomeruli from 28.4 ± 3.4 to 123.4 ± 17.8 fmol/glomerulus. CHA at concentrations ranging from 5×10^{-8} to 5×10^{-5} M significantly inhibited PTH-stimulated cAMP production (Fig. 6). This inhibitory effect was antagonized by DPCPX (Fig. 6).

Discussion

The present study first demonstrated in human glomeruli the presence of a single class of binding sites for [^{3}H]CCPA, an A_1 agonist, by using the radioligand binding assay and the inhibitory effect on cAMP production through stimulation of the A_1 receptor.

Rabbit glomeruli were previously reported to contain A_1 receptors with a B_{max} of 7.7 fmol/mg protein using binding experiments with [^{125}I]HPIA, an A_1 agonist ligand [27]. The present study showed that human glomeruli contain a much higher density of A_1 receptors (B_{max} = 271.7 ± 35.8 fmol/mg

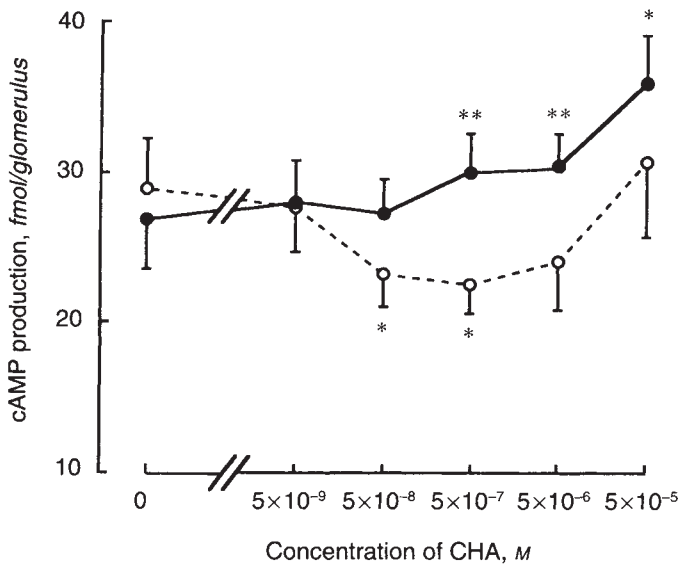

Fig. 5. *Effect of CHA (an A_1 agonist) on basal cAMP production in human glomeruli in the absence (○----○) and presence (●——●) of 5 × 10^{-5} M of DPCPX (an A_1 antagonist).* For incubation conditions see **Methods**. Each point is the mean ± SE of 8 separate experiments, each in triplicate. * $P < 0.05$ compared to the basal value (no CHA added). ** $P < 0.05$ compared to the values at same concentration of CHA (no DPCPX added).

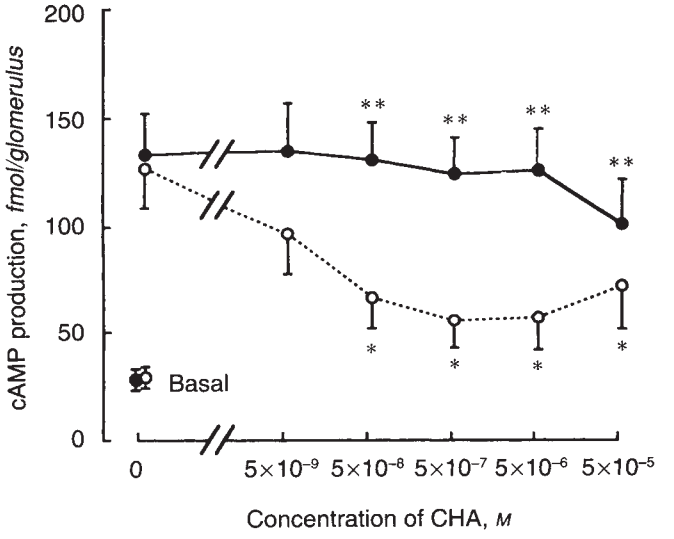

Fig. 6. *Effect of CHA on PTH-stimulated cAMP production in isolated human glomeruli in the absence (○----○) and presence (●——●) of 5 × 10^{-5} M of DPCPX.* For incubation conditions see ***Methods***. Each data point is the mean ± SE of 8 separate experiments, each in triplicate. Value without PTH is 39.2 ± 2.1 fmol/glomerulus (no DPCPX added) or 42.3 ± 4.7 fmol/glomerulus (DPCPX added). * $P < 0.05$ compared to the value without CHA. ** $P < 0.05$ compared to the each value at the same concentration of CHA (no DPCPX added).

protein) compared to those of the rabbit, even if the difference in methods is considered. The high concentration of [^{3}H]CHA binding sites in human glomeruli and the low concentration in the guinea pig glomeruli have been demonstrated previously by autoradiography [36], while the biochemical characterization as well as intracellular signal transduction systems of the A_1 receptors has not been evaluated. The Ki values of adenosine analogs for the binding of [^{3}H]CCPA to the human glomerular membranes were slightly higher than the previous data in the rat brain [29]. For example in the rat brain using [^{3}H]CCPA as a ligand, Ki values of DPCPX, NECA, and theophylline were 0.3, 2.8, and 5750 nM respectively. The actual reason for these differences are not clear. However, differences of species and organs as well as assay conditions may be the possible reasons for these differences of B_{max} and Ki.

In this study, we measured cAMP levels in isolated intact glomeruli. Because direct measurement of adenylate cyclase activity required excess ATP as a substrate, we had to use the broken cell. However, in the broken cell, the inhibitory effect of adenylate cyclase could not be always demonstrated [33, 37], possibly due to damage of the linkage between receptor and adenylate cyclase. Therefore, we used intact glomeruli and found inhibition of adenylate cyclase by stimulating the adenosine A_1 receptor.

In this study, we demonstrated that CHA inhibited cAMP production in isolated human glomeruli through stimulation of the A_1 receptor. Furthermore, we found that in the absence of DPCPX, a high concentration (5×10^{-5} M) of CHA had no effect or even a stimulatory effect on basal cAMP production in the presence of DPCPX. These results suggest that human glomeruli might contain not only A_1 receptors but also A_2 receptors, and therefore A_2 receptor-induced stimulation of cAMP production might be added to A_1 receptor-induced inhibition of cAMP. In the presence of PTH we could not find A_2 receptor-stimulated cAMP production. This may be explained by the fact that the adenylate cyclase system was already stimulated maximally by PTH. These results are consistent with the fact that adenosine has a high affinity for A_1 receptors and low affinity for A_2 receptors. The inhibitory effect on cAMP production of the A_1 receptor stimulation in the human glomeruli was greater than that in rat glomeruli [13]. Because we used the same method for both studies, this difference may be due to the difference of A_1 receptor density between the two species.

There are several types of cells in the glomerulus. Because in this study an interaction of A_1 receptor and PTH on cAMP formation was observed in human glomeruli, one of the possible sites of localization of the adenosine A_1 receptor is in the cell type that responds to PTH. A previous immunohistochemical study showed that cAMP fluorescence increased in visceral epithelium (podocyte) after perfusion with PTH [38]. Therefore, the A_1 receptor might exist in this cell type. However, a recent report showed that A_1 receptor stimulation inhibits cAMP production in the rat mesangium [14]. Thus, further studies will be required to identify the cell types where the A_1 receptor exists in the human glomerulus.

Recent studies suggest the existence of an additional cellular pathway for the action of adenosine, the phosphoinositol pathway, in which calcium is the intracellular messenger. In renal tissues it has been reported that adenosine stimulates the turnover of inositol phosphates and the elevation of cytosolic free calcium through stimulation of the A_1 receptor [4, 14, 39, 40].

In summary, this is the first study to characterize the adenosine A_1 receptor by direct radioligand binding assay and show the inhibitory effect on cAMP production through this A_1 receptor in human glomeruli. More studies will be required to

investigate the physiological role of this A_1 receptor-cAMP system in human glomeruli.

Acknowledgments

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