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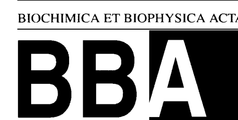

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Inhibitory effect of KW-3902, an adenosine A_1 receptor antagonist, on *p*-aminohippurate transport in OK cells

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Abstract

KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine) is a novel potent and selective adenosine A_1 receptor antagonist. We examined the effect of KW-3902 on *p*-aminohippurate (PAH) transport in opossum kidney (OK) epithelial cells. Pretreatment for 3 h with KW-3902 inhibited the transcellular transport of PAH across OK cell monolayers from the basal to the apical side. The uptake of PAH across the basolateral membrane of OK cells was inhibited by KW-3902 pretreatment in a time- and concentration-dependent manner. A kinetic analysis revealed that the inhibitory effect of KW-3902 on the basolateral PAH uptake was due to an increase in the Michaelis constant (K_m) as well as a decrease in the maximum uptake rate (V_{max}), showing that the inhibition was a mixed type. Pretreatment with adenosine deaminase or 8-cyclopentyl-1,3-dipropylxanthine, another selective adenosine A_1 receptor antagonist, also decreased the basolateral PAH uptake. KW-3902 pretreatment had no effect on the concentration of intracellular α-ketoglutarate which exchanges for PAH across the basolateral membrane of OK cells. These results suggest that KW-3902 has an inhibitory effect on PAH transport in OK epithelial cells. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Organic anion transport; Renal secretion; Adenosine A_1 receptor antagonist; Opossum kidney cell; *p*-Aminohippurate/dicarboxylate exchanger

1. Introduction

The organic anion transport system in the renal proximal tubule is the major system responsible for the elimination of a wide variety of anionic compounds, including endogenous metabolites, drugs and xenobiotics or their metabolites [1,2]. This function is important physiologically because many organic anions are toxic and need to be removed as promptly as possible. Several studies have indicated that renal organic anion secretion could be modulated by various exogenous and endogenous agents. Gemba et al. [3] reported that trifluoperazine, a calmodulin antagonist, stimulated *p*-aminohippurate (PAH), a typical organic anion, accumulation in rat kidney cortical slices. Metabolic substrates such as acetate, pyruvate, lactate and intermediates of the citric acid cycle have been shown to stimulate organic anion transport in vitro and in vivo [4–7]. Furthermore, Halpin and Renfro [8] showed dopaminergic inhibition and α-adrenergic stimulation of organic

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anion secretion by flounder proximal tubule primary cultures.

Cultured epithelial cells derived from the kidney are useful for study on a variety of renal cellular functions, including transcellular transport of solutes such as organic cations [9–12] and regulation of transport by hormones and drugs [13]. A series of reports have demonstrated that OK cells, which were established from the American opossum kidney [14], are useful as an in vitro model system to study the organic anion secretion in the renal proximal tubules [15–17]. Recently, we showed that the PAH transport in OK cells is under the regulatory control of protein kinase C [18]. A subsequent work indicated that parathyroid hormone (PTH) inhibits PAH transport in OK cells, and that protein kinase C activation is involved in the inhibitory effect of PTH on PAH transport [19].

Adenosine is an autacoid that regulates a number of renal functions, including renal hemodynamics, renin secretion and erythropoietin production, via interactions with A_1 and A_2 adenosine receptors, differentiated by affinity for various adenosine analogues. The A_1 receptor is coupled to inhibition of adenylate cyclase and/or activation of phospholipase C, whereas the A_2 receptor is coupled to adenylate cyclase activation. It has been reported that adenosine stimulates several transporters in the proximal tubules, including Na^+–phosphate cotransporter [20], Na^+–glucose cotransporter [20] and Na^+–$3HCO_3^-$ cotransporter [21]. It was suggested that the increase in Na^+–phosphate cotransport and Na^+–glucose cotransport might contribute to the antidiuretic action of adenosine and that the stimulation of the basolateral Na^+–$3HCO_3^-$ cotransport might reduce the metabolic work of $(Na^+ + K^+)$-ATPase, leading to the conservation of ATP.

KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine) is a newly synthesized selective adenosine A_1 receptor antagonist [22]. KW-3902 has been shown to induce significant diuretic and natriuretic actions, resulting from an inhibition of water and sodium reabsorption in tubular sites [23]. In addition, KW-3902 possesses a renal protective effect in rats with acute renal failure induced by cisplatin [24] and cephaloridine [25]. It is reported that the nephrotoxicity of these drugs can also be reduced by coadministration of probenecid, a typical organic anion transport inhibitor, and other organic anions [26,27]. Therefore, the renal protective effect of KW-3902 against cisplatin- and cephaloridine-induced acute renal failure may be due to a decrease in the intracellular concentration of these drugs in the renal proximal tubular cells, following a change in renal organic anion transport activity.

The purpose of this study is to examine whether KW-3902 modulates the renal organic anion transport, using OK epithelial cells. The results show that KW-3902 inhibits PAH transport in OK cell monolayers.

2. Materials and methods

2.1. Cell culture

OK cells were cultured in medium 199 (Flow Laboratories, Rockville, MD, USA) containing 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD, USA) without antibiotics, in an atmosphere of 5% CO_2–95% air at 37°C, and subcultured every 7 days using 0.02% EDTA and 0.05% trypsin [15]. OK cells were used between passages 77 and 106.

2.2. Transport measurements

PAH transport was measured in OK cell monolayers cultured in Transwell chambers (Costar, Cambridge, MA, USA). To prepare cell monolayers, cells were seeded at a density of 4×10^5 cells/cm^2 on polycarbonate membranes (3 μm pore size) in Transwell cell chambers (4.71 cm^2 surface area), which were placed in 6-well cluster plates. The volume of medium inside and outside the chambers was 1.5 and 2.6 ml, respectively. Fresh medium was replaced every 2 or 3 days, and the cells were used on the 5th or 6th day after seeding. Transport was measured at 37°C in Dulbecco's phosphate-buffered saline (PBS buffer containing in mM, 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, and 0.5 $MgCl_2$) supplemented with 5 mM D-glucose.

The transcellular transport of [^{14}C]PAH across OK cell monolayers and cellular [^{14}C]PAH uptake were measured as described previously [15,17]. D-[^{3}H]Mannitol was used to correct for paracellular

flux or extracellular trapping and non-specific uptake of PAH in each transport experiment.

2.3. Cell treatment

KW-3902, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), phorbol 12-myristate 13-acetate (PMA) and staurosporine were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO during exposure was 0.25–0.5%. The compounds were applied to both the basolateral and apical sides for a specified period unless stated. The control cells were incubated with the same concentration of DMSO in each experiment. Adenosine deaminase (10 U/ml) was added to both the basolateral and apical sides and incubated for 2 h. Finally, the cell monolayers were washed three times with the PBS containing 5 mM D-glucose before measuring the PAH transport.

2.4. Analytical methods

The radioactivity was determined in 5 ml of ACSII (Amersham International, Buckinghamshire, UK) by liquid scintillation counting using an external standard to correct for quenching. The appropriate crossover correction was given to separate the radioactivities of ^{3}H and ^{14}C. Protein was determined by the method of Bradford [28] with bovine γ-globulin as the standard. Intracellular α-ketoglutarate concentration was determined as described before [29].

Statistical analysis was performed by Student's *t*-test, or by the one-way analysis of variance with the Scheffé test for post hoc analysis ($P < 0.05$ for significance).

2.5. Materials

p-[glycyl-1-^{14}C]Aminohippurate (1.53–2.0 GBq/mmol) and D-[^{3}H]mannitol (729 GBq/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA, USA). KW-3902 was a gift from Kyowa Hakko Kogyo (Tokyo, Japan). PMA, DPCPX and adenosine deaminase were purchased from Sigma (St. Louis, MO, USA). Staurosporine was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of the highest purity available.

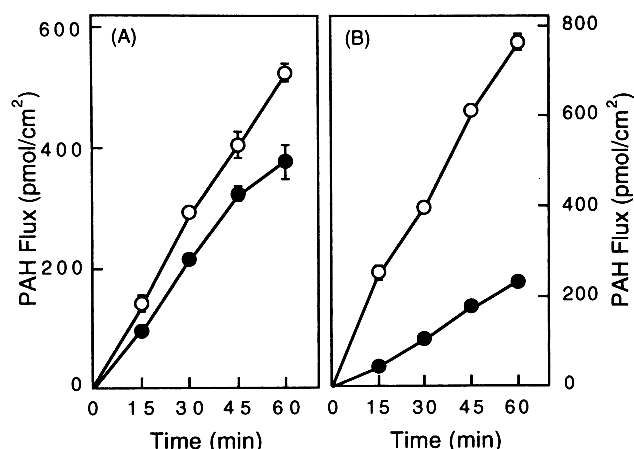

Fig. 1. Effect of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902) on basal-to-apical transport of PAH by OK cell monolayers. Confluent monolayers were incubated for 3 h without (open circles) or with (closed circles) KW-3902 of 3 μM (A) and 30 μM (B). After washing the cells, [^{14}C]PAH (15 μM) and D-[^{3}H]mannitol (15 μM) were added to the basal side of monolayers. After 15, 30, 45, and 60 min, the medium on the apical side was collected (100 μl), and radioactivity levels were counted to determine transcellular transport of [^{14}C]PAH. D-[^{3}H]Mannitol was used to correct for paracellular flux. Each point represents the mean ± S.E. of three monolayers of a typical experiment.

3. Results

3.1. Effect of KW-3902 on the basal-to-apical PAH transport

Fig. 1 shows the transcellular transport of [^{14}C]PAH across OK cell monolayers incubated in the absence (control) or presence of KW-3902 for 3 h before transport measurements. Pretreatment with 3 μM KW-3902 decreased the transcellular transport of PAH from the basal side to the apical side (71.8 ± 5.5% of control at 60 min flux) (Fig. 1A), and 30 μM KW-3902 more strongly inhibited the transcellular PAH transport (30.6 ± 0.5% of control at 60 min flux) (Fig. 1B). To analyze further the effect of KW-3902 on PAH transport in OK cells, we examined the effects of KW-3902 on PAH uptake across the basolateral membrane of OK cells.

3.2. Effect of KW-3902 pretreatment on basolateral PAH transport

We examined the effect of various concentrations

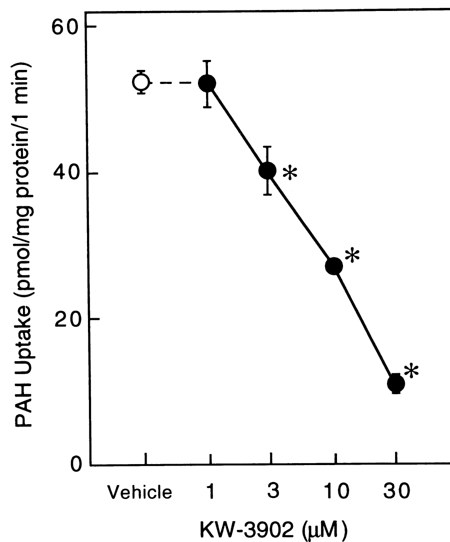

Fig. 2. Concentration-dependent effect of KW-3902 on PAH uptake from the basal side of OK cell monolayers. Confluent monolayers were incubated for 3 h with various concentrations of KW-3902 (1–30 μM) or its vehicle. After washing the cells, [^{14}C]PAH (15 μM) and D-[^{3}H]mannitol (15 μM) were added to the basal side of monolayers, and [^{14}C]PAH uptake for 1 min at 37°C was measured. Each point represents the mean ± S.E. of four monolayers of two separate experiments. *$P<0.05$, significant difference from vehicle.

of KW-3902 on PAH uptake from the basal side of OK cells. OK cells were treated with various concentrations (1–30 μM) of KW-3902 for 3 h, then the basolateral PAH uptake for 1 min was measured. As shown in Fig. 2, KW-3902 inhibited the basolateral PAH uptake in a concentration-dependent manner with an apparent half-maximal inhibitory concentration (IC_{50}) of 10.7 μM.

Fig. 3 shows the effect of KW-3902 (30 μM) pretreatment period (2 min–3 h) on PAH uptake from the basal side of OK cells. Exposure to KW-3902 caused a time-dependent decrease in PAH uptake, and it was significant in all the periods examined.

Then, the effect of KW-3902 pretreatment side on the basolateral PAH uptake was examined. Pretreatment of 30 μM KW-3902 on either the apical or basolateral side decreased the basolateral PAH uptake, and showed a weaker inhibitory effect than the pretreatment of both sides (Fig. 4).

3.3. Influence of KW-3902 on kinetic parameters of basolateral PAH uptake

To determine the effect of KW-3902 on the kinetic parameters of PAH uptake from the basal side, OK cells were incubated for 1 h in the absence or presence of KW-3902 (10 μM). The kinetic constants of the basolateral PAH uptake in OK cells were analyzed by measuring the 1 min uptake of PAH over a concentration range of 15–400 μM. As shown in Fig. 5, the basolateral PAH uptake was saturable and inhibited by KW-3902 at all concentrations of PAH examined. Eadie-Hofstee plots were linear in control and KW-3902-treated cells (Fig. 5, inset). The Michaelis constants (K_m) in the cells incubated without and with KW-3902 were 53.0 ± 0.3 and 75.6 ± 4.0 μM, respectively (means ± S.E. of three experiments), and the maximum uptake rates (V_{max}) in the cells incubated without and with KW-3902 were 222.0 ± 27.0 and 166.0 ± 25.1 pmol (mg protein)$^{-1}$ min^{-1}, respectively. Thus, the inhibition of the basolateral PAH transport by KW-3902 was apparently a mixed type.

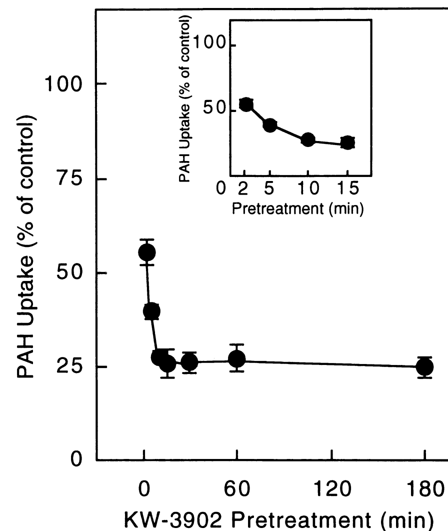

Fig. 3. Effect of pretreatment time on KW-3902-induced inhibition of PAH uptake from the basal side of OK cell monolayers. Confluent monolayers were incubated for various periods (2, 5, 10, 15, 30, 60, and 180 min) with KW-3902 (30 μM) or its vehicle (control), and [^{14}C]PAH uptake from the basal side of monolayers was measured as described in Fig. 2. The inset shows PAH uptake in OK cells pretreated within 15 min. Each point represents the mean ± S.E. of four monolayers of two separate experiments.

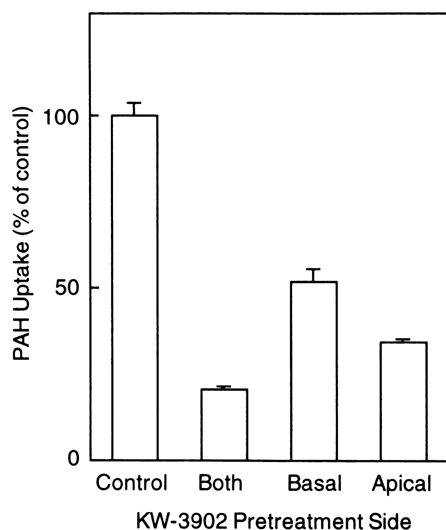

Fig. 4. Effect of KW-3902 pretreatment side on PAH uptake from the basal side of OK cell monolayers. KW-3902 (final 30 μM) was applied to the basolateral and/or apical side of monolayers for 15 min. After washing the cells, [^{14}C]PAH uptake for 1 min from the basal side of monolayers was measured as described in Fig. 2. Each column represents the mean ± S.E. of three monolayers of a typical experiment.

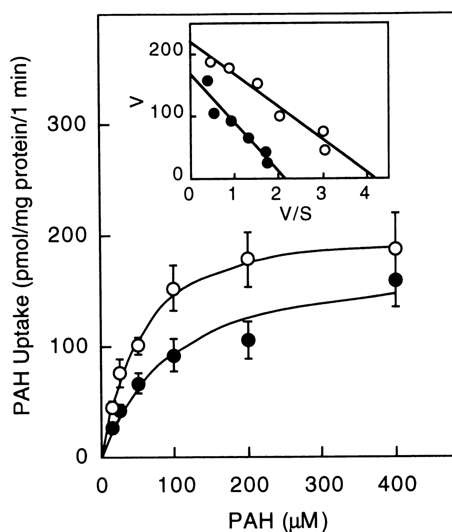

Fig. 5. Influence of KW-3902 on kinetic parameters of PAH uptake from the basal side of OK cell monolayers. Confluent monolayers were incubated for 1 h in the absence (open circles) or presence (closed circles) of KW-3902 (10 μM), and [^{14}C]PAH uptake from the basal side of monolayers was measured as described in Fig. 2. Inset: Eadie-Hofstee plots of the data. Each point represents the mean ± S.E. of three separate experiments.

3.4. *Effect of staurosporine on KW-3902-induced inhibition of basolateral PAH uptake in OK cells*

Previous results showed that PAH transport in OK cells was inhibited by activation of protein kinase C [18]. Therefore, we examined whether the KW-3902-induced inhibitory effect of PAH transport in OK cells is due to activation of protein kinase C. As a result, staurosporine, a protein kinase C inhibitor, had no effect on the inhibition of PAH transport by KW-3902, though it blocked the inhibition by PMA, a protein kinase C activator (Fig. 6).

3.5. *Effect of KW-3902 on intracellular α-ketoglutarate concentration*

Recent reports suggest that α-ketoglutarate is a major physiological dicarboxylate participating in organic anion secretion via PAH/dicarboxylate exchange [1,6,30], and PAH/dicarboxylate exchange is modulated by changes in the intracellular concentration of α-ketoglutarate [6,29]. Therefore, we examined whether KW-3902 affects intracellular α-keto-

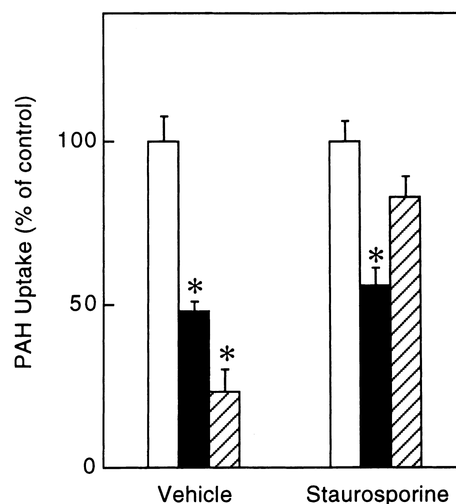

Fig. 6. Effect of staurosporine on KW-3902-induced inhibition of PAH uptake from the basal side of OK cell monolayers. Confluent monolayers were exposed for 15 min to 1 μM staurosporine or its vehicle, followed by an additional 3 h incubation in the absence (control; open columns) or the presence of 10 μM KW-3902 (solid columns) or 100 nM PMA (hatched columns). After washing the cells, [^{14}C]PAH (15 μM) uptake from the basal side of monolayers was measured as described in Fig. 2. Each column represents the mean ± S.E. of four monolayers of two separate experiments. $*P < 0.05$, significant difference from each control.

glutarate concentration. However, the intracellular α-ketoglutarate concentration was not significantly changed by KW-3902 pretreatment (0.3–30 μM concentration) (Fig. 7).

3.6. *Effect of adenosine deaminase and DPCPX on basolateral PAH uptake*

To determine whether endogenous adenosine is involved in PAH transport in OK cells, OK cells were pretreated for 2 h with adenosine deaminase (10 U/ml) to remove endogenous adenosine. As shown in Fig. 8, adenosine deaminase pretreatment significantly decreased the basolateral PAH uptake. Moreover, we studied the effect of DPCPX, another selective adenosine A_1 receptor antagonist, on the basolateral PAH uptake in OK cells. The basolateral PAH uptake was significantly inhibited by pretreatment for 3 h with 30 μM DPCPX (Fig. 8). Thus, the effect of KW-3902 on PAH uptake was at least in part mediated by the adenosine receptor blockage.

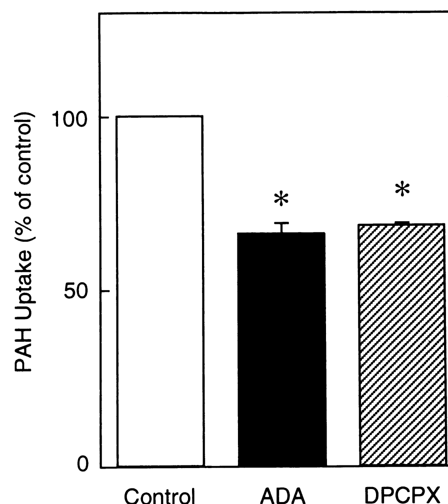

Fig. 8. Effect of adenosine deaminase (ADA) and DPCPX on PAH uptake from the basal side of OK cell monolayers. Confluent monolayers were incubated for 2 h with ADA (10 U/ml) or for 3 h with DPCPX (30 μM). The control cells were incubated with its vehicle. After washing the cells, [^{14}C]PAH (15 μM) uptake from the basal side of monolayers was measured as described in Fig. 2. Each column represents the mean ± S.E. of three monolayers of a typical experiment. *$P < 0.05$, significant difference from each control.

4. Discussion

It has been reported that KW-3902 showed significant diuretic and natriuretic actions, being due to inhibition of water and sodium reabsorption mainly in the proximal tubule [22,23]. Furthermore, Cai et al. [31,32] observed that KW-3902 inhibited sodium-dependent phosphate transport in rat cultured proximal tubular cells. In addition, Nagashima et al. [25] reported that KW-3902 possesses a renal protective effect in rats with cephaloridine-induced acute renal failure, which might be due to the inhibition of renal organic anion transport by KW-3902. Thus, KW-3902 might modulate the activity of the organic anion transporter in renal proximal tubular cells. Therefore, we examined the effect of KW-3902 on PAH transport in OK cells, which are a useful in vitro model system to study the organic anion transport across intact epithelial cells.

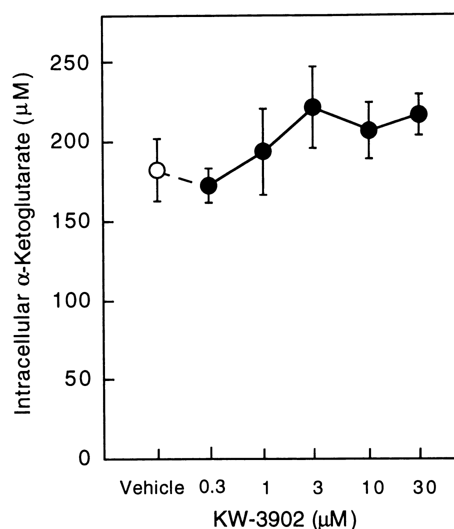

Fig. 7. Effect of KW-3902 on the intracellular α-ketoglutarate concentration in OK cell monolayers. Confluent monolayers were exposed to various concentrations of KW-3902 (0.3–30 μM) or its vehicle for 3 h. After washing the cells, intracellular α-ketoglutarate concentration was determined. Each point represents the mean ± S.E. of four monolayers.

In the present study, KW-3902 inhibited the transcellular transport of PAH from the basal to the apical side in OK cells. Furthermore, the basolateral PAH uptake in OK cells was inhibited by KW-3902 in a pretreatment time- and concentration-dependent manner. The kinetic analysis in the present study revealed an increase in K_m and a decrease in V_{max} by KW-3902 pretreatment, showing that the

inhibition by KW-3902 was a mixed type. On the one hand, our previous report showed that the inhibition by benzylpenicillin, an organic anion, was a competitive type because the analysis showed an increase in K_m without changing V_{max} [16]. In addition, the application of KW-3902 to the apical side of OK cells showed an inhibitory effect on the PAH uptake from the basal side to which KW-3902 was not applied. Thus, the inhibitory effect of KW-3902 on PAH transport in OK cells seems to be due to not the competitive interaction with the PAH transport but to the change in the PAH transport activity.

The concentration-response relationship for the KW-3902-induced inhibition of basolateral PAH uptake in OK cells ($IC_{50} = 10.7$ μM) was not so different from that of phosphate transport in the rat cultured renal proximal tubular cells ($IC_{50} = 2.0$ μM), reported by Cai et al. [31]. However, there are several differences of KW-3902-induced inhibitory characteristics between PAH transport and phosphate transport. The pretreatment of KW-3902 for 15 min showed the maximal inhibition of the basolateral PAH transport, whereas it did not significantly inhibit the phosphate transport [31]. Furthermore, a kinetic analysis showed that KW-3902 elicited a decrease in both the maximal velocity and the affinity of basolateral PAH uptake, while it has been reported that the affinity of phosphate transport was not changed by KW-3902 [31]. Thus, it is likely that KW-3902 affects various transporters in the kidney, and that the mechanisms of regulation vary with transporters.

We observed that removal of endogenous adenosine by adenosine deaminase significantly decreased the basolateral PAH uptake in OK cells. Furthermore, DPCPX, another selective adenosine A_1 receptor antagonist, inhibited the basolateral PAH uptake in OK cells. Therefore, it is likely that the effect of KW-3902 on PAH uptake was, at least in part, mediated by the adenosine receptor blockage. On one hand, the concentration (3 μM) at which KW-3902 affected the basolateral PAH transport was much higher than the dissociation constant value (1.3 nM) of KW-3902 for adenosine A_1 receptor. Therefore, the possibility that other mechanisms rather than the adenosine receptor-mediated regulation are involved in the action of KW-3902 could not be excluded. However, the inhibitory effect of the basolateral PAH transport by the apical pretreatment of KW-3902 suggests the involvement of a receptor-mediated regulation, though the specific receptor remains to be clarified in the present work.

Adenosine A receptors are supposed to be coupled to phospholipase C and/or adenylate cyclase through the inhibitory guanosine 5′-triphosphate-binding protein G_i [33]. Thus, the adenosine receptor appears to be coupled to intracellular regulatory pathways, including activation and/or inhibition of protein kinase C and protein kinase A. Our previous results showed that PAH transport in OK cells was inhibited by the activation of protein kinase C [18], while it was not affected by protein kinase A activators [19]. Therefore, we examined whether the KW-3902-induced inhibition of PAH transport in OK cells was due to the activation of protein kinase C. As a result, staurosporine, a protein kinase C inhibitor, did not recover the decrease in the PAH transport activity by KW-3902. Thus, protein kinase C activation does not seem to be involved in the inhibitory effect of KW-3902 on PAH transport in OK cells.

Studies with basolateral membrane vesicles, renal cortical slices, and isolated renal tubules have shown that PAH is transported via PAH/dicarboxylate exchange in renal basolateral membrane, and that α-ketoglutarate could be a physiological substrate for PAH/dicarboxylate exchange [6,30,34,35]. In addition, PAH transport in rat renal cortical slices was modulated by changes in intracellular α-ketoglutarate concentration or the outwardly oriented α-ketoglutarate gradient [6]. In OK cells, we found that the basolateral PAH uptake was inhibited in accordance with the decrease in the intracellular α-ketoglutarate concentration [29]. Therefore, we examined whether the KW-3902-induced inhibitory effect on PAH transport in OK cells is due to changes of intracellular α-ketoglutarate concentration. The intracellular α-ketoglutarate concentration in OK cells, however, was not significantly affected by KW-3902. Thus, it is likely that the inhibitory effect of KW-3902 on the PAH transport is mediated by a mechanism independent of decrease in intracellular α-ketoglutarate concentration. Further studies are needed to clarify the mechanism underlying the inhibition of PAH transport by KW-3902.

In the present study, we showed that KW-3902

inhibits both the transcellular transport of PAH from the basal to the apical side and the basolateral PAH transport in OK cells. It is possible that the inhibition by KW-3902 of the basolateral organic anion transport reduces the accumulation of anionic drugs in the renal proximal tubular cells. Therefore, the present result might explain the protective effect of KW-3902 against the nephrotoxicity induced by cisplatin and cephaloridine, which was reported previously in in vivo experiments [24,25].

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