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A novel non-xanthine adenosine A_1 receptor antagonist

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Abstract

FK453, (+)-(*R*)-[(*E*)-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl) acryloyl]-2-piperidine ethanol, was examined for adenosine receptor antagonistic activity using isolated guinea-pig atria and aorta and for affinity for adenosine receptors in the rat cerebral cortex and striatum in comparison with FR113452 (*S* enantiomer of FK453), PD116948 (1,3-dipropyl-8-cyclopentylxanthine), theophylline (1,3-dimethylxanthine) and CGS15943 ([1,2,4]triazolo[1,5-*c*]quinazolone). FK453 showed potent inhibition of the negative inotropic activity elicited by 10 μM adenosine with an IC_{50} of 560 pM in guinea-pig atria. However, FK453 was less potent in inhibiting the relaxation induced by 3.2 μM adenosine and had an IC_{50} of 1.18 μM in guinea-pig aorta. The IC_{50} values for FR113452, PD116948, theophylline and CGS15943 were 1.18 μM, 1.31 nM, 20.2 μM and 74.2 nM in atria and > 100 μM, 656 nM, 239 μM, 127 nM in aorta respectively. In the binding study, FK453 antagonized [^{3}H]N^6-cyclohexyladenosine binding to the rat cortical adenosine A_1 receptor with an IC_{50} of 17.2 nM. The IC_{50} values for FR113452, PD116948, theophylline and CGS15943 were 10.1 μM, 4.7 nM, 67.7 μM and 241 nM respectively. FK453 inhibited [^{3}H]5′-*N*-ethylcarboxamideadenosine binding to rat striatum adenosine A_2 receptor with an IC_{50} of 11.3 μM. FK453 had no adenosine A_1 receptor agonistic activity, since it had no negative inotropic activity up to 100 μM in isolated guinea-pig atria. These results demonstrate that FK453 is a novel non-xanthine adenosine receptor antagonist and is potent and selective for the adenosine A_1 receptor subtype.

Keywords: Adenosine receptor; FK453; Atrium; Aorta; Binding assay

1. Introduction

Adenosine receptors located on cell membranes are classified into two major subtypes, adenosine A_1 and A_2 receptors. The classification was originally based on the effects of a series of adenosine analogs on adenylate cyclase activity; activation of the adenosine A_1 receptor inhibits adenylate cyclase and conversely activation of the adenosine A_2 receptor stimulates adenylate cyclase (Van Calker et al., 1979; Londos et al., 1980). It has been proposed that the relative pharmacological potencies of a series of reference adenosine analogs with different adenosine A_1 and A_2 receptor binding activities, such as *R*-N^6-phenylisopropyladenosine, N^6-cyclohexyladenosine and 5′-*N*-ethylcarboxamideadenosine, should be used to characterize adenosine receptors (Williams, 1989; Olsson and Pearson, 1990).

Based on the relationship between the receptor binding and pharmacological activity of several adenosine analogs, there is indirect evidence that the cardiovascular effects of adenosine are mediated by two different types of receptors. Many adenosine analogs which display preferential affinity for adenosine A_1 receptors produce depressions of heart rate, myocardial contractility and impulse conduction velocity (Evans et al., 1982; Collis, 1983; Belardinelli et al., 1982; Edvinsson and Fredholm, 1983), whereas those analogs exhibiting greater affinity for adenosine A_2 receptors produce vasodilation (Collis and Brown, 1983; Kusachi et al., 1983; Mustafa and Askar, 1985; Haleen and Evans, 1985). However a number of workers have found that in many tissues containing receptors classi-

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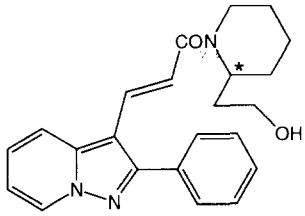

Fig. 1. Chemical structures of FK453 and FR113452. FK453: (+)-(*R*)-1-[(*E*)-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl) acryloyl]-2-piperidine ethanol. FR113452: (−)-(*S*)-enantiomer of FK453. Asterisk denotes the asymmetric carbon atom.

fied as adenosine A_1 receptors, the potency of *R*-N^6-phenylisopropyladenosine is different from that of 5′-*N*-ethylcarboxamideadenosine. It was suggested that the adenosine A_1 receptors should be sub-divided into adenosine A_1 and A_3 receptor subtypes. As knowledge of the different types of adenosine receptors increases, these receptors may prove to be possible targets for novel pharmacologically active drugs.

At present, the xanthines are the major class of adenosine receptor antagonists, such as theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine). Several more potent alkylxanthines, including PD116948 (1,3-dipropyl-8-cyclopentylxanthine) and 8-[4-(2-aminoethyl) amino) carbonyl methyl oxyphenyl]1,3-dipropylxanthine, have been described (Williams, 1989). FK453 is a novel pyrazolopyridine derivative (Fig. 1) which was synthetized by Fujisawa Pharmaceutical Co. (Shiokawa et al., 1992). FK453 was found to possess effective diuretic activity similar to that of hydrochlorothiazide, a typical diuretic, and theophylline. Although the chemical structure of FK453 did not resemble the structure of either of them, the preliminary pharmacological profile of FK453 was similar to that of theophylline, such as its diuretic activity and renal vasodilatory activity (Terai et al., 1990). The present studies were performed to examine the effects of FK453 on the adenosine receptors, by using functional assay and radioligand binding assay techniques, in comparison to the effects of FR113452 (*S* enantiomer of FK453, Fig. 1), PD116948 (adenosine A_1 receptor antagonist), theophylline (non-selective adenosine receptor antagonist) and CGS15943 ([1,2,4]triazolo[1,5-*C*]quinazolone; adenosine A_2 receptor antagonist) (Williams et al., 1987).

2. Materials and methods

All experimental procedures were approved by the Animal Care and Use Committee in our laboratories and were in accordance with National Institutes of Health Guideline for the care and use of animals.

2.1. Materials

FK453, FR113452, PD116948, CGS15943 and dipyridamole were synthetized by Fujisawa Pharmaceutical Co. [^{3}H]N^6-cyclohexyladenosine and [^{3}H]5′-*N*-ethylcarboxamideadenosine were obtained from New England Nuclear and adenosine deaminase (type VI: calf intestinal mucosa) was purchased from Sigma Chemical Co. Other drugs used were as follows: adenosine (Kohjin, Japan), theophylline and hydrochlorothiazide (Sigma, USA), erythro-9-(2-hydroxyl-3-nonyl) adenine (Burroughs Wellcome, USA) N^6-cyclohexyladenosine and 5′-*N*-ethyl-carboxamide-adenosine (Sigma, USA).

All test solutions were prepared fresh before use. As light can cause photochemical *trans-cis* isomerization of FK453 and FR113452 in solution, all experiments with these compounds were conducted in the dark.

2.2. Effect on excretion of urine and urinary electrolytes in conscious rats

Male Sprague-Dawley strain rats aged 6 weeks, weighing 170–210 g, were used after food deprivation for 18 h. The test drugs suspended in 0.5% methylcellulose solution were given to the rats (5 ml/kg p.o.) and simultaneously physiological saline was given (20 ml/kg p.o.). The rats were housed in groups of three in metabolism cages and the urine was collected. Urine volume was measured with a volumetric cylinder and urinary electrolytes (Na^+ and K^+) were measured with a Stat/Ion System (Technicon). The tests were conducted with three groups of three animals for each dose.

2.3. Adenosine-mediated functional assay with isolated tissues from guinea-pigs

Adenosine A_1 receptor antagonist and agonist activity in guinea-pig atria

Male Hartley strain guinea-pigs, weighing 500–650 g, were killed by bleeding and the hearts were removed. Atrial strips were obtained and suspended in organ baths containing 50 ml of Tyrode's solution with 10 μM dipyridamole and 10 μM erythro-9-(2-hydroxy-3-noynyl) adenine maintained at 30° C and aerated with a gas mixture of 95% O_2-5% CO_2. The atria were connected to a strain gauge under an initial tension of 0.4–0.6 g. After constant contraction had been obtained, the test compound was added to the organ bath 15 min prior to 10 μM adenosine treatment. Adenosine receptor antagonistic activity was evaluated by measuring the inhibition of adenosine-induced negative inotropic activities of the test compound. Adeno-

sine receptor antagonistic activity is expressed as IC_{50} value.

Adenosine receptor agonist activities were evaluated by measuring the inotropic effect of the test compounds on the isolated guinea-pig atria without adenosine addition.

Adenosine A_2 receptor antagonistic activity in guinea-pig aorta

Male Hartley guinea-pigs, weighing 250–500 g, were killed by bleeding and the thoracic aorta was removed. Spiral strips of thoracic aorta were mounted in organ baths containing oxygenated Krebs solution. The nucleoside transport inhibitor dipyridamole (10 μM) and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (10 μM) were added to the Krebs solution. The tissues were placed under a resting tension of 0.9–1.0 g and allowed to equilibrate for 1 h. Submaximal contractions of the aortic strips were evoked by addition of 3 μM phenylephrine. Then 3.2 μM adenosine was added to the bath contents and the evoked relaxation was measured in the absence and/or presence of the test compounds. Adenosine receptor antagonistic activity is expressed as IC_{50} value.

2.4. Radioligand binding assay

Adenosine A_1 receptor binding

Cerebral cortices obtained from male Sprague-Dawley rats, weighing 200–300 g, were homogenized in 20 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4) using a Polytron homogenizer. The homogenate was centrifuged at $41\,000 \times g$ for 30 min (at 4° C) and resulting pellets were resuspended in 20 volumes of the buffer (50 mM, pH 7.4). Adenosine deaminase was added (2 units/ml) and then incubated at 37° C for 30 min to remove endogenous adenosine. The treated homogenate was then recentrifuged and the resulting pellets were frozen at −70° C until the assay.

The adenosine A_1 receptor antagonistic activity of each compound was evaluated in three separate experiments. All assays were run in duplicate in a final volume of 1 ml containing 1 nM [^{3}H]N^6-cyclohexyladenosine with a specific activity of 925 GBq/mmol (25 Ci/mmol). Non-specific binding was determined in the presence of 10 μM cyclohexyladenosine. Reactions were initiated by the addition of the adenosine deaminase-treated membrane homogenates at a final protein concentration of 200–300 μg/ml, and this mixture was incubated at 25° C for 3 h. After incubation the bound radioactivity was isolated by filtration under vacuum over Whatman GF/B glass fiber filter strips, and unbound radioactivity was removed with two washes of 5 ml of ice-cold buffer. Filter strips were placed in glass vials to which 10 ml of Aquasol was added. After equilibration for at least 12 h, radioactivity was determined in a conventional liquid scintillation counter (Oei et al., 1988).

Adenosine A_2 receptor binding

Striata obtained from male Sprague-Dawley rats were homogenized in 20 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, using a Polytron homogenizer. The homogenate was centrifuged at $41\,000 \times g$ for 30 min (at 4° C) and the resulting pellets were resuspended in 20 volumes of the buffer (50 mM, pH 7.4). Adenosine deaminase was added (2 units/ml) and then the suspension was incubated at 37° C for 30 min to remove endogenous adenosine. The treated homogenate was then recentrifuged and the resulting pellets were frozen at −70° C until the assay.

The adenosine A_2 receptor antagonistic activity of each compound was assessed in at least three separate experiments. All assays were run in duplicate in a final volume of 1 ml containing 5 nM [^{3}H]5′-*N*-ethylcarboxamideadenosine with a specific activity of 770 GBq/mmol (20.8 Ci/mmol). 50 nM of N^6-cyclopentyladenosine was added to eliminate the adenosine A_1 component. Non-specific binding was determined in the presence of 20 μM 5′-*N*-ethylcarboxamideadenosine. Reactions were initiated by the addition of the adenosine deaminase-treated membrane homogenate at a final protein concentration of 200–300 μg/ml, and this mixture was incubated at 25° C for 120 min. Then, bound radioactivity was isolated and measured by the same procedure as described for [^{3}H]N^6-cyclohexyladenosine binding (Bruns et al., 1986; Oei et al., 1988).

2.5. Other pharmacological and biochemical profiles

Norepinephrine-induced contraction

Vas deferens strips, isolated from male Wistar strain rats (270–330 g), were suspended in an organ bath containing oxygenated Tyrode's solution under an initial tension of 0.5 g at 37° C. Contraction was induced by 5.9 μM norepinephrine (Kura et al., 1991).

Acetylcholine- or histamine-induced contraction

Ileal strips, isolated from male Hartley strain guinea-pigs (350–500 g), were suspended in an organ bath containing oxygenated Tyrode's solution under an initial tension of 0.5 g at 37° C. Contraction was induced by 5.5 μM acetylcholine or 5.4 μM histamine (Yamada et al., 1991).

Serotonin-induced contraction

Stomach strips, isolated from male Wistar strain rats (260–320 g), were suspended in an organ bath containing oxygenated Tyrode's solution under an initial tension of 1.0 g at 37° C. Contraction of the stomach was induced by 0.32 μM serotonin (Kojima et al., 1991).

Table 1
Effects of FK453 and hydrochlorothiazide on the urine volume and the excretion of urinary electrolytes in conscious rats

Compounds	Dose (mg/kg)	UV (ml/kg)	Na^+ (μEq/kg)	K^+ (μEq/kg)
Vehicle		16.3 ± 1.4	2076 ± 189	615 ± 85
FK453	0.32	18.0 ± 1.0 [b]	2345 ± 163	727 ± 58
	1.0	24.6 ± 0.5 [b]	3289 ± 187 [a]	833 ± 10
	3.2	31.9 ± 1.4 [b]	4109 ± 257 [b]	831 ± 40
	10	44.4 ± 4.0 [b]	5819 ± 200 [b]	1070 ± 101 [a]
	32	44.7 ± 1.5 [b]	6103 ± 132 [b]	1219 ± 54 [b]
Vehicle		12.6 ± 0.6	2002 ± 350	539 ± 89
Hydrochlorothiazide	0.32	23.5 ± 1.2 [b]	3711 ± 355 [a]	799 ± 69
	1.0	25.9 ± 1.6 [b]	3992 ± 298 [a]	926 ± 38 [a]
	3.2	28.9 ± 0.5 [b]	4591 ± 93 [b]	948 ± 83 [a]
	10	33.3 ± 2.4 [b]	5234 ± 397 [b]	1038 ± 104 [a]

All values are expressed as means ± S.E. (three groups of three rats for each dose). Values that are significantly different from the vehicle (0.5% methylcellulose) are indicated by [a] $P < 0.05$ and [b] $P < 0.01$. Urine was collected for 6 h. UV: urine volume, Na^+: sodium excretion, K^+: potassium excretion.

cAMP phosphodiesterase inhibitory activity

A crude phosphodiesterase enzyme preparation was obtained as a supernatant fraction after centrifugation (100000 × *g* for 40 min) of the homogenates of rat aorta. The phosphodiesterase assay was performed by the method of Thompson and Appleman (1971).

2.6. Data analysis

The dose-response curve was fitted to a straight line by performing a logit-log transformation of the data. The responses for each concentration of antagonist were obtained by interpolation from the linear regression analysis of the logit-log plot. These values were then averaged to form the dose-response curves and then the IC_{50} values were determined.

Statistical evaluation was performed with Dunnett's multiple comparison test and *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Effect on excretion of urine and urinary electrolytes in conscious rats

FK453, given to rats in single oral doses from 0.32 to 32 mg/kg, significantly increased urine volume and urinary electrolyte (Na^+ and K^+) excretion when compared with those of vehicle-treated animals. The increase in urine volume and sodium excretion reached a plateau at 10 mg/kg (2.7-fold and 2.8-fold respectively).

Hydrochlorothiazide significantly increased urine volume and urinary electrolytes at doses from 0.32 to 10 mg/kg (Table 1).

3.2. Adenosine receptor antagonistic activities in guinea-pig atria and aorta

Adenosine induced a negative inotropic response in guinea-pig atria and induced a dose-dependent relaxation response in guinea-pig aorta (Figs. 2 and 3).

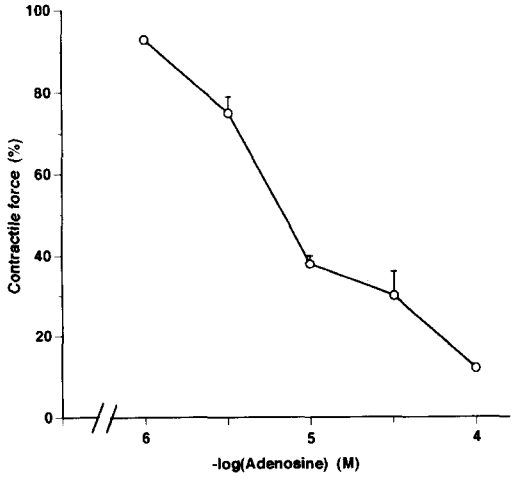

Fig. 2. The effect of adenosine on the contractile force of guinea-pig atria. Pre-dosing contractions of guinea-pig atria were 1–1.5 g tension. Data are expressed as means ± S.E. ($n = 5$).

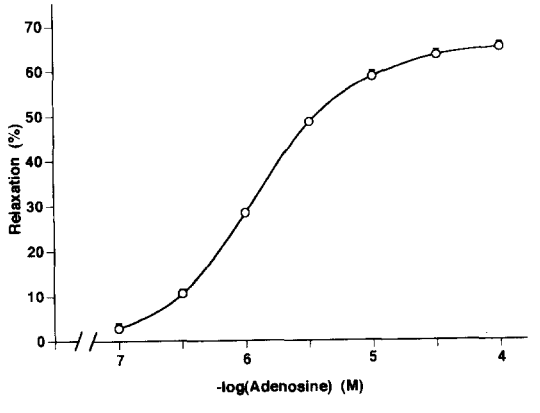

Fig. 3. The effect of adenosine on the tension elicited by phenylephrine. Mean contractions in response to 3 μM phenylephrine are 1.86 ± 0.13 g tension. Data are expressed as means ± S.E. ($n = 5$).

Table 2
Adenosine antagonistic activities of FK453, FR113452 and other adenosine antagonists in guinea-pig atria and aorta

Compounds	A_1 (atria) IC_{50} (nM) [Conc. range]	A_2 (aorta) IC_{50} (nM) [Conc. range]	A_2/A_1 ratio
FK453	0.56±0.08 [0.01–10 nM]	1180±128 [0.1–10 μM]	2107
FR113452	1180±215 [0.1–10 μM]	>100000 [1–100 μM]	(–)
PD116948	1.31±0.20 [0.1–100 nM]	656±91 [0.1–10 μM]	501
Theophylline	20200±1170 [1–100 μM]	239000±37600 [10–320 μM]	11.8
CGS15943	74.2±17.0 [0.01–1 μM]	127±36 [0.032–10 μM]	1.7

Four or five different concentrations were used for each assay. IC_{50} values are expressed as means±S.E. (n = 4–5 for each test). Conc. range: concentration range.

Table 3
Binding activities of FK453, FR113452 and other adenosine antagonists in rat brain membranes

Compounds	A_1([^{3}H]CHA) IC_{50} (nM) [Conc. range]	A_2([^{3}H]NECA) IC_{50} (nM) [Conc. range]	A_2/A_1 ratio
FK453	17.2±4.5 [0.001–1 μM]	11300±2100 [0.32–100 μM]	657
FR113452	10100±600 [0.1–100 μM]	130000±9000 [1–320 μM]	12.9
PD116948	4.7±0.6 [0.32–320 nM]	1130±30 [0.0032–10 μM]	240
Theophylline	67700±4800 [0.0032–1 mM]	64500±8500 [0.01–1 mM]	0.95
CGS15943	241±83 [0.01–1 μM]	51±9 [0.0032–1 μM]	0.21

Binding activities were evaluated in three separate assays. Each assay was run in duplicate. Six or seven different concentrations were used for each assay. IC_{50} values are expressed as means±S.E. [^{3}H]CHA: [^{3}H]N^6-cyclohexyladenosine, [^{3}H]NECA: [^{3}H]5′-N-ethylcarboxamideadenosine, Conc. range: concentration range.

FK453 and FR113452 inhibited the negative inotropic activity of 10 μM adenosine in isolated guinea-pig atria and their IC_{50} values were 560 pM and 1.18 μM respectively. FK453 and FR113452 were less potent in the inhibition of the relaxation induced by 3.2 μM adenosine in the guinea-pig aorta strips. The IC_{50} values for FK453 and FR113452 were 1.18 μM and >100 μM. The A_2/A_1 ratio of FK453 was 2107 (Table 2).

PD116948, theophylline and CGS15943 had similar inhibitory effects on adenosine-induced negative inotropic activity. Their IC_{50} values were 1.31 nM, 20.2 μM and 74.2 nM respectively (Table 2).

The inhibitory effects of reference adenosine receptor antagonists on the relaxation response of adenosine were compared in isolated guinea-pig aorta. The IC_{50} values of PD116948, theophylline and CGS15943 were 656 nM, 239 μM and 127 nM respectively. The A_2/A_1 selectivity of reference compounds is shown in Table 2.

3.3. Adenosine A_1 and A_2 receptor binding assay with rat brain membranes

FK453 and FR113452 inhibited the specific binding of [^{3}H]N^6-cyclohexyladenosine to rat cortical membranes with IC_{50} values of 17.2 nM and 10.1 μM respectively (Fig. 4 and Table 3).

FK453 and FR113452 inhibited the specific binding of [^{3}H]5′-N-ethyl-carboxamideadenosine to rat striatal membranes with IC_{50} values of 11.3 μM and 130 μM. The A_2/A_1 ratios of FK453 and FR113452 were 657 and 12.9 respectively (Fig. 4 and Table 3).

We compared the effects of PD116948, theophylline and CGS15943 on radioligand binding to adenosine receptors. The IC_{50} values of these compounds at adenosine A_1 receptors were 4.7 nM for PD116948, 67.7 μM for theophylline and 241 nM for CGS15943.

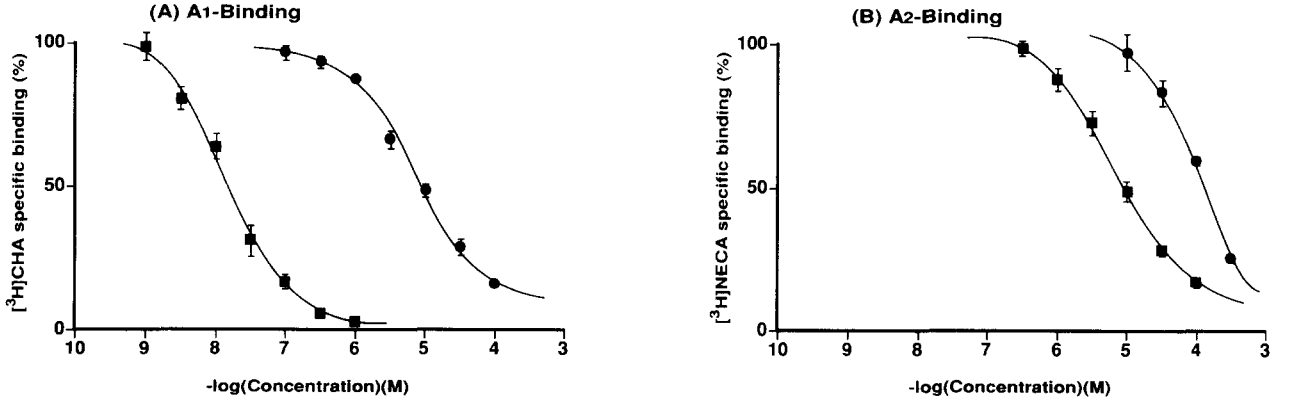

Fig. 4. Displacement curves for FK453 and FR113452. (A) [^{3}H]CHA ([^{3}H]N^6-cyclohexyladenosine) in rat cortical membrane. (B) [^{3}H]NECA ([^{3}H] 5′-N-ethylcarboxamideadenosine) in striatal membrane. FK453: (■). FR113452: (●).

PD116948 had the most potent adenosine A_1 receptor binding activity. The IC_{50} values at adenosine A_2 receptor were 1.13 μM for PD116948, 64.5 μM for theophylline and 51 nM for CGS15943 (Table 3).

Fig. 5 shows the relationship between adenosine A_1 receptor antagonistic activities in guinea-pig atria and the affinity for adenosine A_1 receptors in the rat cortical membrane. The relationship for FK453 was to the left of that for the other compounds.

3.4. Effects of FK453, FR113452, reference adenosine antagonists and adenosine agonists on the isolated guinea-pig atria

FK453 (1–100 μM) caused concentration-dependent increases in the spontaneous force of contraction of guinea-pig atria. These increases at 1 μM, 10 μM and 100 μM were 8.1%, 22.7% and 81.2% respectively. FR113452 caused 21.0% increase at 100 μM and the positive inotropic effect of FR113452 was smaller than that of FK453 (Fig. 6).

PD116948 at 1 μM and 10 μM caused 6.3% and 16.1% increases in the spontaneous force of contraction of guinea-pig atria and these increases were slightly smaller than those of the same concentration of FK453. CGS15943 and theophylline had little effect on the isolated guinea-pig atria.

N^6-Cyclohexyladenosine (adenosine A_1 receptor-selective agonist) and 5′-*N*-ethylcarboxamideadenosine (non-selective adenosine receptor agonist) decreased the contractile force of atria at concentrations of 10–100 nM (Fig. 6).

3.5. Other pharmacological and biochemical profiles of FK453

The results are shown in Table 4. FK453 had little effect on norepinephrine-, acetylcholine-, histamine- and serotonin-induced contractions in isolated organs. FK453 inhibited phosphodiesterase activities in rat aorta extract with IC_{50} values of 8.4 μM and 14.0 μM with cAMP and cGMP as substrate respectively.

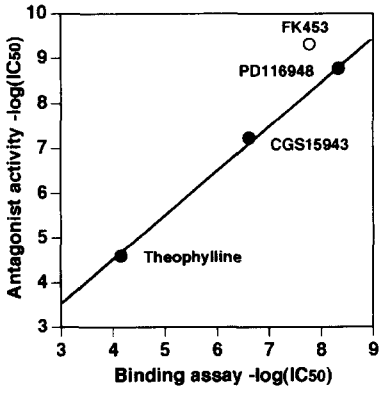

Fig. 5. Relationship between adenosine A_1 antagonistic activities in guinea-pig atria and the affinity for adenosine A_1 receptors in rat cortical membranes. Solid line is the linear regression ($y = 1.0048 \pm 0.46585$; correlation coefficient = 0.9989).

Table 4
Effect of FK453 on norepinephrine-, acetylcholine-, histamine- and serotonin-induced contractions in isolated organs and phosphodiesterase activities in rat aorta extract

Contractions and enzyme	IC_{50} (μM)
Norepinephrine-induced contraction (rat vas deference)	> 20
Acetylcholine-induced contraction (guinea-pig ileum)	> 20
Histamine-induced contraction (guinea-pig ileum)	> 20
Serotonin-induced contraction (rat stomach)	> 20
Phosphodiesterase (rat aorta)	8.4 ± 0.1 [a]
	14.0 ± 0.3 [b]

[a] 1.5×10^{-7} M cAMP was used as enzyme substrate ($n = 3$). [b] 1.5×10^{-7} M cGMP was used as enzyme substrate ($n = 3$). $n = 4$ for other tests.

4. Discussion

Most adenosine receptor antagonists reported to date are xanthine derivatives. Theophylline is a prototypic adenosine receptor antagonist but it is relatively weak and has other pharmacological activities (Williams, 1989). Recently many potent and selective xanthine type adenosine receptor antagonists have been developed such as 8-phenyltheophylline, BW-A844U (1-propyl-3-(3-aminophenethyl)-8-cyclopentylxanthine) and PD116948 (Jacobson et al., 1992). PD116948 is one of the most potent adenosine A_1 receptor-selective antagonists, with activity in the nanomolar range. The availability of rapid binding assays for adenosine A_1 and A_2 receptor subtypes has led to the discovery of novel reagents that interact with adenosine receptors. A number of non-xanthine type adenosine antagonists have been synthesized by many pharmaceutical companies. These are reviewed by Jacobson et al. (1991,, 1992 and Williams (1989). The present study showed that FK453 possessed potent adenosine receptor antagonistic activity with an IC_{50} of 560 pM in guinea-pig atria and antagonized [^{3}H]N^6-cyclohexyladenosine binding with an IC_{50} of 17.2 nM. A_2/A_1 ratios in the functional assay and the binding assay were 2107 and 657 respectively. As FK453 had little effect on adrenergic, muscarinic, histaminergic and serotonergic receptors up to 20 μM (Table 4), it can be considered to be a potent and selective adenosine A_1 receptor antagonist.

In guinea-pig atria, FK453 had a clear positive inotropic activity at higher concentrations, similar to that of the selective adenosine A_1 receptor antagonist, PD116948 (Fig. 6). N^6-Cyclohexyladenosine (adenosine A_1 receptor agonist) and 5′-*N*-ethylcarboxami-

deadenosine (non-selective adenosine receptor agonist) had negative inotropic activities. These effects induced by N^6-cyclohexyladenosine and 5′-*N*-ethylcarboxamideadenosine are considered to be due to the stimulation of adenosine A_1 receptor in the atria (Lerman and Belardinelli, 1991). Therefore we can conclude that FK453 does not possess adenosine A_1 receptor agonist activity. The positive inotropic activity in guinea-pig

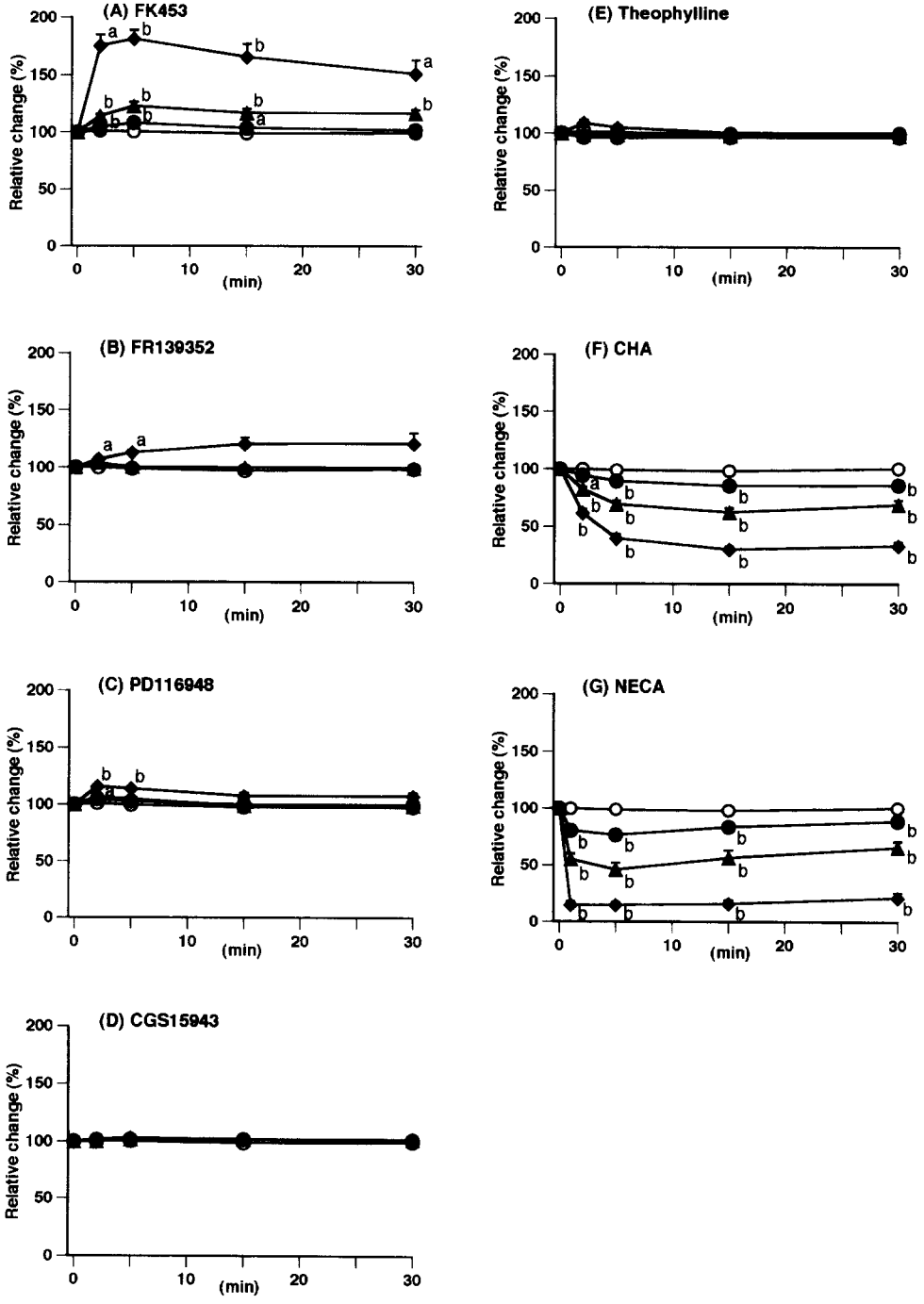

Fig. 6. Lack of adenosine receptor agonistic effect of FK453 in comparison with FR113452, other adenosine antagonists, N^6-cyclohexyladenosine and 5′-*N*-ethylcarboxamideadenosine. The effects of the drugs on the contractile force are plotted as a percentage of the control values (before the drug addition) at various times. Each value is the means ± S.E. ($n = 5$). (A) FK453: vehicle (○), 10^{-6} M (●), 10^{-5} M (▲), 10^{-4} M (◆). (B) FR113452: vehicle (○), 10^{-5} M (●), 10^{-4} M (◆). (C) PD116948: vehicle (○), 10^{-7} M (●), 10^{-6} M (▲), 10^{-5} M (◆). (D) CGS15943: vehicle (○), 10^{-7} M (●), 10^{-6} M (◆). (E) Theophylline: vehicle (○), 10^{-6} M (●), 10^{-5} M (▲), 10^{-4} M (◆). (F) CHA (N^6-cyclohexyladenosine): vehicle (○), 10^{-8} M (●), 3.2×10^{-8} M (▲) and 10^{-7} M (◆). (G) NECA (5′-*N*-ethylcarboxamideadenosine): vehicle (○), 10^{-8} M (●), 3.2×10^{-8} M (▲) and 10^{-7} M (◆). Data are expressed as means ± S.E., $^aP < 0.05$, $^bP < 0.01$, significantly different from vehicle.

atria at the high concentrations of FK453 is possibly due to the phosphodiesterase inhibitory activity (Table 4). However, this phosphodiesterase inhibitory activity in a crude enzyme preparation of rat aortas was about 500–20 000 times weaker than the adenosine A_1 receptor antagonistic activity.

FK453 is a non-xanthine type adenosine receptor antagonist and contains one asymmetric carbon atom. The affinity for the adenosine A_1 receptor subtype of the *R*-(+) enantiomer was 587-fold higher in the binding assay and 2107-fold higher in the atrial functional assay than that of the *S*-(−) enantiomer. However, the affinity of both enantiomers for the adenosine A_2 receptor was markedly weak in both the atrial functional assay and the brain binding assay. These results demonstrate that the 2-position of piperazine ring of FK453 is important for adenosine A_1 receptor binding.

FK453 possessed diuretic, saliuretic and kaliuretic properties similar to those of a thiazide diuretic. However, hydrochlorothiazide had no adenosine receptor antagonistic activity (data not shown). Furthermore the diuretic pattern (relationship between potency and efficacy) of FK453 was obviously different from that of hydrochlorothiazide (Table 1). These results suggest that the diuretic mechanism of FK453 was different from that of hydrochlorothiazide. Adenosine A_1 receptor antagonists, such as 8-phenyltheophylline and PD116948, are reported to have diuretic activity in rats (Collis et al., 1986; Knight et al., 1993). As the diuretic effect of FK453 seems to be related to the adenosine receptor antagonistic activity, we intend to describe the diuretic mechanism of FK453 in another paper.

We used PD116948 (adenosine A_1 receptor antagonist), theophylline (non-selective adenosine receptor antagonist) and CGS15943 (adenosine A_2 receptor antagonist) for comparison of adenosine receptor antagonist potency. The order of adenosine A_1 receptor antagonist potency in guinea-pig atria was FK453 > PD116948 > CGS15943 > theophylline. However, the order of adenosine A_1 receptor antagonist potency in the radioligand binding assay with rat brain membranes was PD116948 > FK453 > CGS15943 > theophylline. Fig. 5 shows the relationship between the adenosine A_1 receptor antagonistic effect in guinea-pig atria and in rat brain membrane binding assay and we observed that the relationship for FK453 was to the left of that of the other three reference compounds. This result suggests that the affinity of FK453 for adenosine A_1 receptors might be greater in atria than in brain.

Comparative studies of the affinity of xanthine derivatives for adenosine A_1 receptor binding in the rat and the guinea-pig brain membranes have reported that the affinity is often significantly higher for rat brain membranes, i.e. there is a species difference (Ukena et al., 1986). However, Collis et al. demonstrated that there were no marked species differences in the activity of alkylxanthines in functional assays of atria from rats and guinea-pigs (Collis et al., 1988).

Furthermore, Collis et al. compared the antagonist potency of some xanthine derivatives (1,3-dipropyl-8-phenylxanthine and 8-[4-(2-aminoethyl amino) carbonyl methyl oxyphenyl]1,3-dipropylxanthine) in a radioligand binding assay with rat brain tissue and a functional assay with guinea-pig atria (Collis et al., 1987). They showed that the pK_i values of alkylxanthines for binding to rat brain are greater than their pA_2 values in guinea-pig atria. In another paper, Collis and coworkers showed that the pA_2 value for PD116948 in the guinea-pig atrium (7.9) is markedly less than the pK_i value reported for binding studies with rat brain membrane (Collis et al., 1989). These reports strongly suggested that the affinity of alkylxanthines is higher for rat brain membranes than for guinea-pig atria.

Although we showed that PD116948, CGS15943 and theophylline had similar potency in both assays in the present studies, it was obvious that FK453 was more potent in the atria functional assay than in the brain membrane binding assay (Tables 2 and 3, and Fig. 5). These results suggest that FK453 may be a new ligand which recognizes preferentially the atria adenosine A_1 receptor subtype, which may be different from the brain adenosine A_1 receptor.

The adenosine A_1 receptor subtype has been classified on the basis of the higher affinity or potency of N^6-substituted adenosine analogs compared with the 5′-substituted adenosine compound 5′-*N*-ethyl carboxamideadenosine, and by an inhibitory effect on adenylate cyclase. Ribeiro and Sebastiao (1986) have observed that in atrial tissue, and at pre-junctional sites on neuronal tissue, the N^6- and 5′-substituted analogs of adenosine are equipotent. There is also little evidence for an effect of adenosine receptors on adenylate cyclase in these tissues. This prompted the idea that receptors with these characteristics represent a third subclass: the adenosine A_3 receptor.

Recently, the cDNAs that encode adenosine A_1, A_{2A}, A_{2B} and A_3 receptors have been cloned and expressed (Tucker and Linden, 1993; Collis and Hourani, 1993). We intend to study the effect of FK453 on these receptors. This is now under investigation in our laboratories.

In conclusion, FK453 is a novel non-xanthine adenosine receptor antagonist which is very potent and selective for the adenosine A_1 receptor subtype.

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