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Enzymological and pharmacological profile of T-0156, a potent and selective phosphodiesterase type 5 inhibitor

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Abstract

The enzymological and pharmacological properties of 2-(2-Methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156), a new phosphodiesterase type 5 inhibitor, were studied in vitro and in vivo. The inhibitory effects of T-0156 on six phosphodiesterase isozymes isolated from canine tissues were investigated. T-0156 specifically inhibited the hydrolysis of cyclic guanosine monophosphate (cGMP) by phosphodiesterase type 5, at low concentration (IC_{50} = 0.23 nM), in a competitive manner. T-0156 also inhibited phosphodiesterase type 6 with IC_{50} value of 56 nM, which was 240-fold higher than that for inhibition of phosphodiesterase type 5. T-0156 had low potencies against phosphodiesterase types 1, 2, 3, and 4 (IC_{50}>10 μM). In the isolated rabbit corpus cavernosum, T-0156 at 10 and 100 nM increased cGMP levels (100 nM T-0156-treated: 6.0 ± 1.5 pmol/mg protein, vehicle-treated: 1.1 ± 0.4 pmol/mg protein, $P < 0.05$), causing relaxation of the tissue. T-0156 at 1 to 100 nM potentiated the electrical field stimulation-induced relaxation in the isolated rabbit corpus cavernosum in a concentration-dependent manner (100 nM T-0156-treated: 76.9 ± 19.8%, vehicle-treated: 12.3 ± 10.1%, $P < 0.05$). Intraduodenal administration of T-0156 at 100 to 1000 μg/kg potentiated the pelvic nerve stimulation-induced tumescence in anesthetized dogs (1000 μg/kg T-0156-treated: 279.0 ± 38.4%, vehicle-treated: 9.8 ± 4.5%, $P < 0.05$). These results suggested that T-0156 enhanced the nitric oxide (NO)/cGMP pathway, probably through blockade of phosphodiesterase type 5 in vitro and in vivo experimental conditions. The present study clearly showed that T-0156 is a potent and highly selective phosphodiesterase type 5 inhibitor, which is a useful tool for pharmacological studies in vitro and in vivo.

Keywords: T-0156; Phosphodiesterase isozyme; Phosphodiesterase type 5 inhibitor; Cyclic guanosine monophosphate; Corpus cavernosum; Tumescence

1. Introduction

Cyclic nucleotide phosphodiesterases regulate physiological functions via the hydrolysis of cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP), which are intracellular second messengers. There are at least 11 distinct phosphodiesterase isozymes (types 1–11), differing in their substrate specificity, selective inhibition or stimulation by cofactors, selective inhibition by standard inhibitors and gene homology (Beavo, 1995; Soderling et al., 1998a,b, 1999; Fawcett et al., 2000). Phosphodiesterase type 5 is a specific enzyme for cGMP hydrolysis, and its activity has been found in the lung, vascular and tracheal smooth muscle, spleen, platelets, skeletal muscle, and corpus cavernosum (Lincoln et al., 1976; Coquil et al., 1985; Wallis et al., 1999). Therefore, it is possible to regulate the cGMP-related physiological functions by control of the phosphodiesterase type 5 activity in these tissues.

Zaprinast, an early phosphodiesterase type 5 inhibitor, has been used for pharmacological studies of phosphodiesterase type 5 functions (Beavo and Reifsnyder, 1990; Ahn et al., 1989; Souness et al., 1989; Hartell, 1996). However, phosphodiesterase type 5 inhibitory activity of zaprinast is weak (IC_{50}>100 nM) and approximately two-fold weaker than phosphodiesterase type 6 inhibition (Ballard et al., 1998), indicating nonpotent and nonselective phosphodies-

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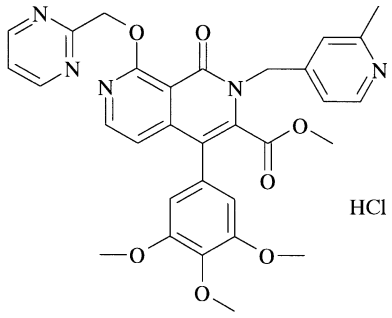

Fig. 1. Structure of T-0156.

terase type 5 inhibition. Recently, sildenafil, another phosphodiesterase type 5 inhibitor, was developed as an orally active agent for treatment of erectile dysfunction (Boolell et al., 1996; Goldstein et al., 1998). Sildenafil shows potent inhibition of phosphodiesterase type 5 with IC_{50} value of about 4 nM (Ballard et al., 1998; Boolell et al., 1996). Sildenafil increases intracellular cGMP levels in the corpus cavernosum, leading to enhanced relaxation of smooth muscle, and ultimately improves penile erection in men with erectile dysfunction. Sildenafil also inhibits phosphodiesterase types 1 and 6 (IC_{50} values of 281 and 33 nM, respectively, Ballard et al., 1998). These results indicate that sildenafil is not an ideal phosphodiesterase type 5 inhibitor for pharmacological studies.

Recently, we discovered a new compound, 2-(2-Methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156; Fig. 1), which potently and selectively inhibits phosphodiesterase type 5 as compared to sildenafil (Kikkawa et al., 2001). The purpose of this study is to examine the enzymological and pharmacological profile of T-0156 in vitro and in vivo.

2. Materials and methods

This study was approved by the Animal Research Committee of Tanabe Seiyaku.

2.1. Preparation of phosphodiesterase isozymes

Phosphodiesterase isozymes were isolated from canine tissues as described previously (Kotera et al., 2000). Male dogs at 48 weeks of age were anesthetized with sodium pentobarbital (50 mg/kg) before various tissues were excised. Phosphodiesterase types 1, 4, and 5 were partially purified from lung. Phosphodiesterase types 2, 3, and 6 were partially purified from adrenal gland, heart, and retina. The characteristics of phosphodiesterase isozymes, Ca^{2+}/calmodulin-stimulated phosphodiesterase (type 1), cGMP-stimulated phosphodiesterase (type 2), cGMP or milrinone-inhibited phosphodiesterase (type 3), cAMP-specific and rolipram-inhibited phosphodiesterase (type 4), cGMP-specific and sildenafil-inhibited phosphodiesterase (type 5), were verified by assessing the effectiveness of regulatory factors or the effect of selective inhibitors. We checked the purity of the isolated phosphodiesterase type 6 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Moreover, the identity of phosphodiesterase type 6 was also confirmed through its activation by trypsin.

2.2. Phosphodiesterase assay

The phosphodiesterase assay was performed by the radiolabeled nucleotide method (Thompson et al., 1979). Assay buffer contained 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.33 mg/ml of bovine serum albumin, 0.1 to 30 μl of enzyme solution, unlabeled cGMP or cAMP, and 12.5 nM [^{3}H]cGMP or 4.88 nM [^{3}H]cAMP. The reaction was started by mixing the substrate into 500 μl of the assay buffer, and tubes were incubated at 37 °C for 30 min. After boiling for 1.5 min, the mixtures were added to 100 μl of a 1 mg/ml solution of Crotalus atrox snake venom and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 500 μl methanol, and the resultant solutions were applied to a Dowex (1 × 8–400) column (volume 0.25 ml). Aqueous scintillation fluid was added to each eluate, and the radioactivity was measured. For studies of inhibition of phosphodiesterase activities, inhibitor was added to the assay buffer containing enzyme and preincubated for 5 min before reactions were initiated by the addition of substrate.

2.3. Rabbit corpus cavernosum preparations

Male New Zealand White rabbits weighing 2.5 to 3.6 kg were anesthetized with intravenously injected sodium pentobarbital (50 mg/kg) and killed by exsanguinations. The penis was rapidly removed and strips of corpus cavernosum were isolated. The tunica albuginea was carefully removed and the preparations of corpus cavernosum measuring approximately 3 × 3 × 5 mm were obtained. The preparation was suspended in a 10-ml tissue organ bath chamber containing a physiological salt solution (PSS), and connected to an isometric force transducer (transducer type UL, UL-10GR, UL-20GR; Minebea, Nagano, Japan). The bathing PSS was maintained at 37 ± 0.5 °C and was continuously aerated with 95% O_2 and 5% CO_2.

The initial resting isometric tension of each preparation was adjusted to about 1.5 g by gradual incremental stretching. After equilibration for 60 min, the high-KCl (120 mM) PSS was added to the preparation for characterization of the contractility. The high-KCl PSS was then replaced by standard PSS. The PSS contained 4.7 mM potassium chloride, 1.2 mM potassium dihydrogenphosphate, 1.2 mM magnesium sulfate heptahydrate, 23 μM EDTA, 1.5 mM calcium chloride dihydrate, 118 mM sodium chloride, 11 mM D-(+)-glucose, and 25 mM sodium hydrogen carbo-

nate. The high-KCl PSS contained 1.2 mM potassium dihydrogenphosphate, 1.2 mM magnesium sulfate heptahydrate, 23 μM EDTA, 1.5 mM calcium chloride dihydrate, 120 mM potassium chloride, 11 mM D-(+)-glucose, and 25 mM sodium hydrogen carbonate.

2.4. Relaxation and cyclic nucleotide levels in rabbit corpus cavernosum

The preparation of rabbit corpus cavernosum in the organ bath was contracted with phenylephrine (5 μM). T-0156 (10 or 100 nM) was then added to the preparation. After attainment of the maximal relaxant response to T-0156, the preparation was frozen immediately by liquid N_2. The frozen preparation was homogenized with in 1 ml of 6% trichloroacetic acid containing EDTA (1 mM). After centrifugation (5000 rpm, for 15 min, 4 °C), the supernatant was extracted with water-saturated diethyl ether, and aliquots of the aqueous phase were lyophilized to dryness and then reconstituted in 1 ml of 50 mM sodium acetate buffer (pH 6.2). The pellet was dissolved in 0.5 ml of 2 N NaOH, and used in the measurement of protein content. The cyclic nucleotide and protein contents in each solution were measured with commercially available cGMP and cAMP immunoassay kits (Amersham, Buckinghamshire, UK) and BCA protein assay kit (pierce, Rochford, IL, USA), respectively. The relaxation of the preparation induced by T-0156 was expressed as the percentage of the magnitude of phenylephrine-induced contraction. The cyclic nucleotide levels were expressed in pmol per mg protein.

2.5. Electrical field stimulation-induced relaxation

Electrical field stimulation-induced relaxation of the preparation of rabbit corpus cavernosum was performed according to the method of Takagi et al. (2001). Atropine and guanethidine were added to each organ bath chamber (1 and 5 μM, respectively) before contraction of the preparations by adding phenylephrine (5 μM). After the phenylephrine contractile response was stabilized, the preparation was subjected to electrical field stimulation (20 V, 0.2-ms pulse duration, for 40 s)-induced relaxation at intervals of 10 min. The stimulatory condition was selected by changing the frequency (1 to 16 Hz) in order to obtain approximately 10% relaxation of the phenylephrine-precontracted preparation. Electrical field stimulation was performed with the platinum electrode set up on both sides of the tissue strips and an electrical stimulator (SEN-3301 or SEN-7203; Nihon Kohden; Tokyo, Japan) and a power booster (PB 401; Physio-Tech; Tokyo, Japan). After the electrical field stimulation-induced relaxation at an interval of 10 min was stabilized, T-0156 (1 to 100 nM) or vehicle was added to the preparation at intervals of 30 min. Papaverine was added to each organ bath chamber (100 μM) to confirm the maximal relaxation of the preparation at the end of experiment. The potentiation was expressed as percentage of the amplitude of the relaxation response before treatment with T-0156 or vehicle.

2.6. Pelvic nerve stimulation-induced tumescence in anesthetized dogs

Pelvic nerve stimulation-induced tumescence in anesthetized dogs was performed according to the methods of Noto et al. (2000). Dogs weighting 11.4 to 18.7 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v. bolus injection, followed by 4.5 mg/kg/h i.v. infusion). An endotracheal tube was placed to ventilate (15 ml/kg/stroke, 20 strokes/min) with room air. The abdomen was opened through a midline abdominal incision. A polythene catheter was inserted into the duodenum and held in place by a ligature. The left pelvic nerve, located superior and lateral to the prostate was carefully isolated and placed on a bipolar electrode (IMT-1530; Inter Medical, Nagoya, Japan). A 21-gauge venous needle was placed in the corpus cavernosum on the left side and used for recording intracavernous pressure. The pelvic nerve was stimulated by electrical square pulse (10 V, 0.2-ms pulse duration, for 40 s) at frequencies from 2.5 to 20 Hz at intervals of 20 min. All experiments were started when the submaximal nerve stimulation evoked consistent responses. T-0156 or vehicle was administered intraduodenally (100, 300 or 1000 mg/kg) and the effect of T-0156 on the pelvic nerve stimulation-induced tumescence was assessed 10, 30, 50, 70 and 90 min after T-0156-treatment. For quantitative determination of the tumescence, we measured the area under the curve and expressed it as millimeters of mercury multiplied by minutes. The potentiation was expressed as percentage of the pelvic nerve stimulation-induced tumescence before treatment with T-0156 or vehicle.

2.7. Receptor binding and enzyme assays

The effects of T-0156 (10 or 100 μM) on radioligand bindings and on enzyme activities except for phosphodiesterase were assessed by MDS Pharma Services (Taipei, Taiwan).

2.8. Chemicals

T-0156, sildenafil, milrinone, and rolipram were synthesized by Discovery Research Laboratory, Tanabe Seiyaku, (Saitama, Japan). [^{3}H]cGMP (577 GBq/mmol) and [^{3}H]cAMP (1.55 TBq/mmol) were from Amersham. L-Phenylephrine hydrochloride, guanethidine sulfate, papaverine hydrochloride, cGMP, cAMP, and Dowex (1 × 8–400) were obtained from Sigma (St. Louis, MO, USA). Atropine sulphate monohydrate was purchased from Wako (Osaka, Japan). Crotalus atrox snake venom was purchased from Nakarai Tesque (Kyoto, Japan). All other chemicals were of analytical grade. T-0156 was dissolved in dimethylsulfoxide for the enzyme and receptor binding assays, and was

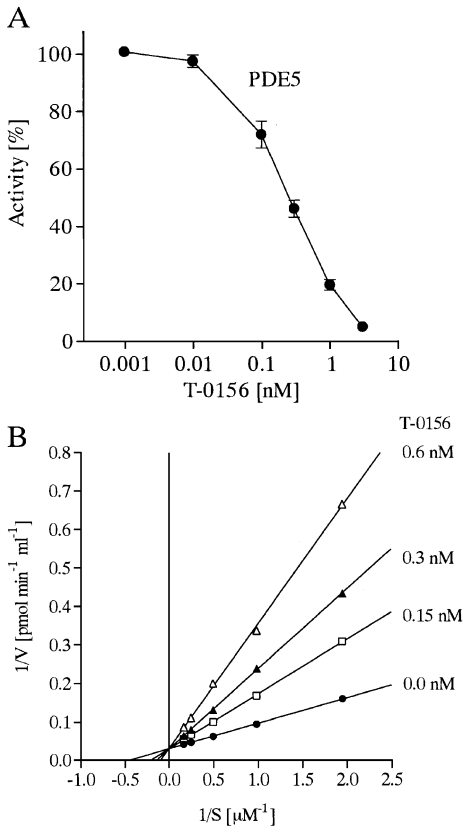

Fig. 2. (A) Inhibition of phosphodiesterase type 5 partially purified from canine lung by various concentrations of T-0156. Cyclic GMP hydrolytic activity (4.5 pmol/min/ml) in the absence of T-0156 was taken as 100%. Each point was shown as means ± S.E.M. for four assays. (B) Kinetic analysis of the inhibition of T-0156 on phosphodiesterase type 5 partially purified from canine lung. Lineweaver–Burk plots at concentrations of 0.51 to 6.01 μM cGMP are shown. Cyclic GMP hydrolytic activities were 32 to 35 pmol/min/ ml. All assays were performed in duplicate.

dissolved in hydrochloric acid (0.01 N) for other experiments to obtain the concentration of 10 mM following dilution with distilled water. All other chemicals were dissolved in distilled water.

2.9. Data analysis

Statistical analysis was performed with Dunnett's test (vs. vehicle in Fig. 3) and repeated measures two-way analysis of variance (ANOVA) followed by Student's *t*-test (unpaired values, T-0156 vs. vehicle in Fig. 4) and Dunnett's test (vs. vehicle in Fig. 5). *P*-values <0.05 were considered as statistically significant. Data are shown as means ± S.E.M.

3. Results

3.1. Inhibition of various phosphodiesterase isozymes by T-0156

T-0156 exhibited strong inhibition of the cGMP hydrolytic activity of phosphodiesterase type 5 (Fig. 2A), with an IC_{50} of 0.23 nM (Table 1). T-0156 also inhibited phosphodiesterase type 6 with IC_{50} value of 56 nM, which was 240-fold higher than that for inhibition of phosphodiesterase type 5. The cAMP hydrolytic activity of phosphodiesterase type 4 was inhibited weakly by T-0156 with IC_{50} value of 63 μM. T-0156 showed weak inhibitions of the cGMP hydrolytic activity of phosphodiesterase types 1 and 2 as well as the cAMP hydrolytic activity of phosphodiesterase type 3 (IC_{50}>100 μM).

3.2. Kinetic analysis of the effect of T-0156 on phosphodiesterase type 5

To elucidate the mechanism underlying the inhibition by T-0156, Lineweaver–Burk plots were constructed at concentrations of 0.51 to 6.01 μM cGMP substrate (Fig. 2B). The phosphodiesterase type 5 partially purified from canine lung had a cGMP K_m value of 6.2 μM, and T-0156 inhibited the phosphodiesterase type 5 activity in a competitive manner with respect to cGMP hydrolysis. The inhibition constant (K_i value) of T-0156 for phosphodiesterase type 5, calculated by drug concentration vs. slope replots, was 0.22 ± 0.08 nM.

3.3. Effects of T-0156 on isometric tension and cyclic nucleotide levels in phenylephrine-precontracted rabbit corpus cavernosum

T-0156 at 10 or 100 nM caused an increment in cGMP levels. The isometric tension of corpus cavernosum decreased with cGMP elevation (Fig. 3). The increase in

Table 1
Inhibitory effects of T-0156 and sildenafil on phosphodiesterase isozymes

Compounds	IC_{50} (nM)					
	PDE1	PDE2	PDE3	PDE4	PDE5	PDE6
T-0156	>100000	>100000	>100000	63000 ± 31000	0.23 ± 0.027	56 ± 1.9
Sildenafil	270 ± 38	43000 ± 1500	>100000	11000 ± 3100	3.6 ± 0.26	29 ± 0.93

Phosphodiesterase types 1, 4, and 5 were isolated from canine lung, type 3 was isolated from canine heart. Phosphodiesterase types 2 and 6 were prepared from canine adrenal gland and canine retinas, respectively. Phosphodiesterase types 1, 2 and 5 activities were assayed with 1 μM cGMP as a substrate, and types 3 and 4 activities were assayed with 1 μM cAMP. After phosphodiesterase type 6 was activated by trypsin, the activity was measured with 10 μM cGMP. In each experiment, phosphodiesterase activity was 2–35 pmol/min/ml. The IC_{50} values were determined by nonlinear regression (sigmoidal curve fit). Data are shown as mean ± S.E.M. for three to four experiments. The data of sildenafil were cited from the previous studies (Kotera et al., 2000).

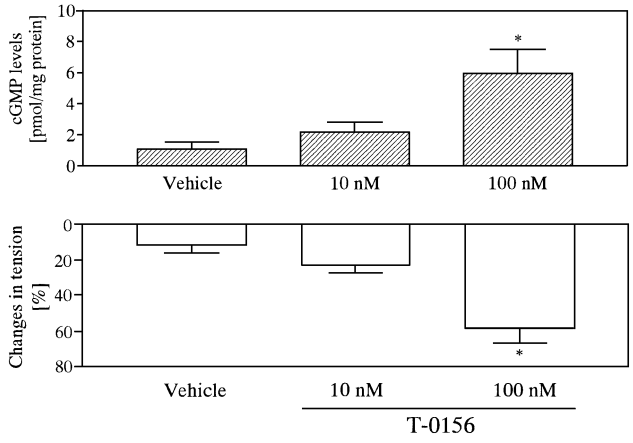

Fig. 3. Changes in cGMP levels and isometric tension by treatment with T-0156 in the isolated rabbit corpus cavernosum. T-0156 (10 or 100 nM) were added to preparations precontracted with phenylephrine. After the maximal change in tension was obtained, the preparation was immediately frozen in liquid N_2. Cyclic GMP levels were measured with a commercially available kit. Data are shown as means ± S.E.M. for five preparations. * $P<0.05$ vs. vehicle-treated.

cGMP levels by T-0156 at a concentration of 100 nM was approximately five-fold higher than that of vehicle (T-0156-treated: 6.0 ± 1.5 pmol/mg protein, vehicle-treated: 1.1 ± 0.4 pmol/mg protein, $P<0.05$). In contrast, cAMP levels were not altered by T-0156 (100 nM T-0156-treated: 25.0 ± 8.7 pmol/mg protein, vehicle-treated: 13.3 ± 3.7 pmol/mg protein, $P=0.48$).

3.4. The potentiation of electrical field stimulation-induced relaxation by T-0156 in phenylephrine-precontracted rabbit corpus cavernosum

Fig. 4 shows typical tracings of the influence of T-0156 on electrical field stimulation-induced relaxation in the rabbit corpus cavernosum. T-0156 at 1 to 100 nM produced a concentration-dependent potentiation of electrical field stimulation-induced relaxation (Fig. 5). The potentiation by T-0156 at a concentration of 100 nM was statistically

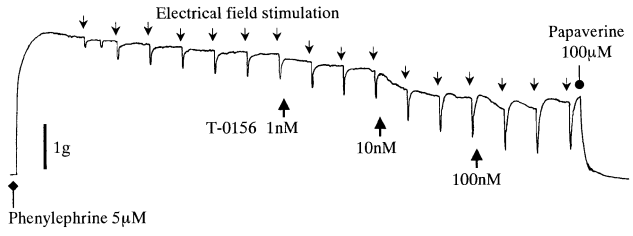

Fig. 4. Typical tracing showing the effect of T-0156 on electrical field stimulation-induced relaxation in the isolated rabbit corpus cavernosum precontracted with phenylephrine.

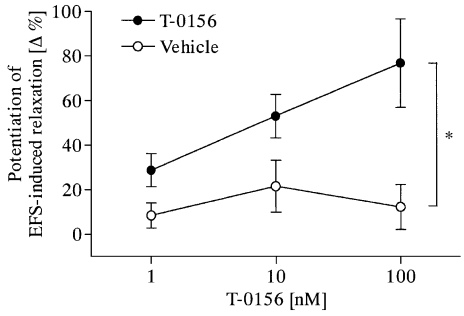

Fig. 5. Effect of T-0156 on electrical field stimulation-induced relaxation in the isolated rabbit corpus cavernosum precontracted with phenylephrine. The electrical field stimulation-induced relaxation in the absence of 100 nM T-0156 was expressed as 100%. Data are shown as means ± S.E.M. for five or six preparations. * Significant difference between 1000 nM T-0156-treated and vehicle-treated preparations at $P<0.05$.

significant (T-0156-treated: 76.9 ± 19.8%, vehicle-treated: 12.3 ± 10.1%, $P<0.05$).

3.5. The potentiation of pelvic nerve stimulation-induced tumescence by T-0156 in anesthetized dogs

Intraduodenal administration of T-0156 at 100 to 1000 μg/kg potentiated a pelvic nerve stimulation-induced tumescence in a dose-dependent manner and the potentiations at 300 and 1000 μg/kg were statistically significant (Fig. 6). The maximal potentiating effect of T-0156 was observed 10 to 30 min after the intraduodenal administration, and the potentiation was sustained at least for 90 min.

3.6. Effects of T-0156 on radioligand binding and on enzymes activities

T-0156 at a high concentration of 10 μM inhibited [^{3}H]CGP-39653 binding to the glutamate NMDA recep-

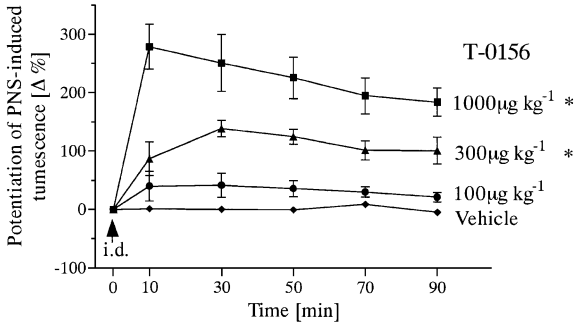

Fig. 6. Effect of intraduodenal treatment with T-0156 on pelvic nerve stimulation-induced tumescence in anesthetized dogs. The pelvic nerve stimulation-induced tumescence in the absence of T-0156 was expressed as 100%. Data are shown as means ± S.E.M. for four or five dogs. * $P<0.05$ vs. vehicle-treated dogs.

tor and the activity of calpain, a Ca^{2+}-activated neutral protease, (percentage of inhibition: 32% and 24%, respectively). T-0156 at 10 or 100 μM did not inhibit either binding of several radioligands bindings or activities of several enzymes as summarized in Table 2.

Table 2
Lack of effect of 10 μM T-0156 on radioligand bindings and on enzyme activities

Receptors	Ligand	% Inhibition
Nicotinic acetylcoline, central	[^{3}H]Cytisine	− 4
Purinergic P_{2x}	[^{3}H]-α, β-methylene-ATP	16
Purinergic P_{2y}	[^{35}S]ATP-α S	− 3
Serotonin 5-HT_{1A}	[^{3}H]8-OH-DPAT	12
Sigma σ_1	[^{3}H]Haloperidol	12
Sodium channel, site 2	[^{3}H]Batrachotoxinin	2
Tachykinin NK_1	[^{3}H]SR-140333	8
Thromboxane A_2 (TXA_2)	[^{3}H]SQ-29548	1
Vascular endothelial growth factor (VEGF)	[^{125}I]$VEGF_{165}$	− 15
Vasoactive intestinal peptide VIP_1	[^{125}I]VIP	− 14
Adenosine A_{2A}	[^{3}H]CGS-21680	− 9
Angiotensin AT_1	[^{125}I][Sar1, Ile^8]-Angiotensin II	6
Bradykinin B_2	[^{3}H]Bradykinin	18
Dopamine $D_{4.2}$	[^{3}H]Spiperone	− 2
Endothelin ET_A	[^{125}I]Endothelin-1	11
Endothelin ET_B	[^{125}I]Endothelin-1	3
Galanin	[^{125}I]Galanin	17
Glutamate, kainate	[^{3}H]Kainate	− 14
Glutamate, NMDA, agonist	[^{3}H]CGP-39653	32
Glutamate, NMDA, Phencyclidine	[^{3}H]TCP	− 3
Histamine H_3	[^{3}H]*N*-α-Methylhistamine	13
Leukoteriene B_4	[^{3}H]Leukotriene B4	− 17
Muscarinic M_1	[^{3}H]*N*-Methylscopolamine	16
Muscarinic M_2	[^{3}H]*N*-Methylscopolamine	14
Muscarinic M_4	[^{3}H]*N*-Methylscopolamine	− 2
Neuropeptide Y_2	[^{3}H]PYY	3
Enzymes	**Source**	**% Inhibition**
Protease, calpain	Human erythrocytes	24
Protein kinase PKCα[a]	Human recombinant (insect Sf9 cells)	5
Protein kinase PKCβ (I & II)[a]	Rat brain	8
Protein kinase, EGF receptor tyrosine kinase	Human recombinant (insect Sf9 cells)	13
Protein kinase, ERK1 serine/threonine kinase[a]	Human recombinant (bacterial)	9
Protein phosphatase, calcineurin PP2B phosphatase	Rat brain	− 1

Values are means for two experiments.
[a] Concentration of T-0156 was 100 μM.

4. Discussion

T-0156 was recently discovered as a new structural class of phosphodiesterase type 5 inhibitor in our laboratory (Kikkawa et al., 2001). The present work was designed to investigate the enzymological and pharmacological profile of T-0156 in vitro and in vivo.

In enzyme assays using six phosphodiesterase isozymes obtained from canine tissues, T-0156 only showed potent inhibition of phosphodiesterase type 5 with IC_{50} value of 0.23 nM. The potency of phosphodiesterase type 5 inhibition was 240-fold higher than that of phosphodiesterase type 6 inhibition and 290 000-fold higher than that of phosphodiesterase type 4. T-0156 had low potency against phosphodiesterase types 1, 2 and 3 (IC_{50}>100 μM). Potency and selectivity of phosphodiesterase type 5 inhibition by T-0156 were higher than those of sildenafil, which is an agent for treatment of erectile dysfunction (Goldstein et al., 1998). Thus, T-0156 is a potent and highly selective phosphodiesterase type 5 inhibitor.

The structure of T-0156 is quite different from that of cGMP, whereas other phosphodiesterase type 5 inhibitors such as zaprinast and sildenafil are structurally related to cGMP. Nevertheless, the kinetic analysis revealed that T-0156 is a competitive inhibitor as has been shown for zaprinast (Turko et al., 1998) and sildenafil (Ballard et al., 1998). It has been reported that residues such as Tyr^{602}, His^{607}, His^{643} and Asp^{754}, which are located in the catalytic domain of phosphodiesterase type 5, might form important interactions with sildenafil (Turko et al., 1999). The elucidation of the phosphodiesterase type 5 binding site of T-0156 will be of interest.

Phosphodiesterase type 5 is a predominant cGMP-hydrolyzing enzyme in corpus cavernosum (Boolell et al., 1996), and its inhibition causes elevation of cGMP levels in this tissue (Jeremy et al., 1997). In the present study, T-0156 increased cGMP levels but not cAMP levels in rabbit corpus cavernosum, showing that T-0156 has a pharmacological profile as a specific phosphodiesterase type 5 inhibitor. T-0156 also caused relaxation of this preparation. It has been reported that nitric oxide (NO) released from NO synthase (NOS)-containing neurons or arteriolar and sinusoidal endothelial cells causes elevation of intracellular cGMP through activation of guanylate cyclase, which in turn leads to the relaxation of smooth muscle of corpus cavernosum (Trigo-Rocha et al., 1993a). In the present study, T-0156 caused an accumulation of cGMP and relaxation of the preparation without NO from NOS-containing nerves, suggesting that the effects of T-0156 were dependent on activation of guanylate cyclase by preexisting NO likely derived from endothelial cells of corpus cavernosum.

We also examined the effect of T-0156 on electrical field stimulation-induced relaxation in the isolated rabbit corpus cavernosum, which was an alternative bioassay to evaluate the influence of the NO/cGMP pathway. In our previous study, electrical field stimulation-induced relaxation was

attenuated by pretreatment with tetrodotoxin or N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, and the L-NAME-inhibited relaxation was restored by excess L-arginine, suggesting that electrical field stimulation-induced relaxation was associated with NO from NOS-containing nerves (Takagi et al., 2001). T-0156 potentiated the electrical field stimulation-induced relaxation in a concentration-dependent manner. This result indicated that T-0156 is efficient for phosphodiesterase type 5 inhibition in vitro, and produces its pharmacological effort by enhancing the NO/cGMP pathway. In addition, it was also observed that the enhancing effects of T-0156 on the NO/cGMP pathway in the isolated rabbit corpus cavernosum were not saturated at 10 nM in spite of T-0156 at this concentration produced 100% inhibition of phosphodiesterase type 5 activity. One possible explanation for this observation is that the cell membrane permeability of T-0156 may be low and higher concentrations of T-0156 may be needed for its inhibitory effect on phosphodiesterase type 5 in isolated tissues. Additional research is needed to clarify the precise mechanism of these phenomena.

Direct electrical stimulation of the pelvic nerves induces penile tumescence mediated by the NO/cGMP pathway in dogs (Trigo-Rocha et al., 1993b). This pelvic nerve stimulation-induced tumescence has been used as a model for the pharmacological evaluation of phosphodiesterase type 5 inhibitors. It has been reported that intraduodenal treatment with sildenafil induces a dose-dependent potentiation of tumescence in this model, implying that sildenafil is an orally active inhibitor of phosphodiesterase type 5 (Noto et al., 2000). When T-0156 was administered intraduodenally, the dose-dependent potentiation of tumescence was observed. This result indicates that T-0156 is an orally effective phosphodiesterase type 5 inhibitor in dogs. The efficacy of T-0156 at a dose of 300 μg/kg was almost the same as that of sildenafil (Noto et al., 2000) in spite of T-0156 was 16 times more potent as an inhibitor of PDE 5 than sildenafil. The efficacy of the drug administered orally or intraduodenally is generally regulated by its bioavailability, serum protein bindings, cell membrane permeability, and so on. The same efficacy of T-0156 with sildenafil in vivo may be due to the low bioavailability of T-0156 (about 9%; our preliminary data). Since the time to reach the maximal potentiation of tumescence by T-0156 (10 to 30 min) was shorter than that by sildenafil (30 min), the absorption of T-0156 is likely to be more rapid than that of sildenafil (Noto et al., 2000).

T-0156 at 10 μM did not have significant effects on binding of radioligands to several receptors and did not affect several enzyme activities as summarized in Table 2, indicating more than 40000-fold selectivity for phosphodiesterase type 5 inhibition. The results support the utility of T-0156 as a selective phosphodiesterase type 5 inhibitor.

In summary, T-0156 is considered to be a useful tool as a potent and selective phosphodiesterase type 5 inhibitor in enzymological and pharmacological studies.

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