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Sildenafil and T-1032, phosphodiesterase type 5 inhibitors, showed a different vasorelaxant property in the isolated rat aorta

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Abstract

The vasorelaxant effects of sildenafil and T-1032 [methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate], two phosphodiesterase type 5 inhibitors, were examined in the isolated rat aorta. Sildenafil and T-1032, both of which have almost the same potency and selectivity regarding phosphodiesterase type 5 inhibitory activity, produced a similar, moderate, relaxation at 10^{-10} to 10^{-7} M (sildenafil: 66.8 ± 13.7%; T-1032: 77.9 ± 10.8% at 10^{-7} M). However, sildenafil, but not T-1032, produced further relaxation at the higher concentrations (sildenafil: 102.0 ± 0.6%; T-1032: 81.0 ± 7.2% at 10^{-4} M, $P < 0.05$). Sildenafil also produced a more potent relaxation than did T-1032 at the high concentrations (10^{-5} and 10^{-4} M) in endothelium-denuded aortic rings and in the presence of N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor (3×10^{-4} M). Moreover, the high concentrations of sildenafil, but not of T-1032, caused a rightward shift of the concentration-response curve for calcium chloride in K^+-depolarized endothelium-denuded preparations. In the ligand binding assay for the L-type Ca^{2+} channels, the affinities of sildenafil at 10^{-5} M for binding sites of nitrendipine and (−)-desmethoxyverapamil [(−)-D888] (35.2 ± 3.3% and 35.8 ± 1.9%, respectively) were higher than those of T-1032 (11.8 ± 4.0% and −13.1 ± 1.3%, respectively, $P < 0.05$). Regarding cyclic nucleotide levels, both phosphodiesterase type 5 inhibitors increased cGMP levels at 10^{-6} M. However, sildenafil, but not T-1032, further increased cGMP levels at the higher concentrations (sildenafil: 15.7 ± 2.7 pmol/mg protein; T-1032: 5.6 ± 0.6 pmol/mg protein at 10^{-4} M, $P < 0.05$). These results suggested that high concentrations of sildenafil had additional vasorelaxant properties through mechanisms other than phosphodiesterase type 5 inhibition. Sildenafil-induced relaxation appears to be due to inhibition of the external Ca^{2+}-dependent cascade for contraction and/or to an increase in cGMP levels. In contrast, T-1032 only showed a vasorelaxant property due to phosphodiesterase type 5 inhibition. In conclusion, T-1032 appears to be a specific phosphodiesterase type 5 inhibitor compared with sildenafil and a useful compound to examine the physiological function of phosphodiesterase type 5.

Keywords: Phosphodiesterase type 5 inhibitor; Aorta, rat; Relaxation; Ca^{2+} channel antagonist; Cyclic nucleotide

1. Introduction

Sildenafil is a selective inhibitor of phosphodiesterase type 5, a cyclic guanosine monophosphate (cGMP)-specific hydrolytic enzyme (Boolell et al., 1996; Ballard et al., 1998) used for the treatment of erectile dysfunction (Goldstein et al., 1998). Penile erection depends on relaxation of the corpus cavernosum. In response to sexual stimulation, cavernous nerves release nitric oxide (NO), which stimulates the formation of cGMP by guanylate cyclase, and sildenafil enhances the increase of cGMP levels by phosphodiesterase type 5 inhibition in corpus cavernosum smooth muscle cells. The increase of cGMP levels results in relaxation of the corpus cavernosum. Phosphodiesterase type 5 was also found in vascular smooth muscle (Beavo, 1995; Boolell et al., 1996; Sampson et al., 2001; Wallis et al., 1999). It has been shown that sildenafil amplifies the vasorelaxation induced by NO donors, (Wallis et al., 1999; Omote, 1999; Medina et al., 2000a) and by neurogenic NO (Medina et al., 2000b). These data indicate that a phosphodiesterase type 5 inhibitor potentiates the vasorelaxation induced by NO by enhancement of the increase of cGMP levels. In addition, it has been reported that sildenafil increases the cGMP levels without exogenous NO in canine coronary artery (Wallis et al., 1999) and shows a direct relaxant effect in isolated human vessels (Medina et al., 2000a,b). It seems that these properties of sildenafil were dependent on activation of the preexisting NO/cGMP system in these vessels.

T-1032 [methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoqui-

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noline carboxylate sulfate], a newly synthesized isoquinolone derivative, shows selective phosphodiesterase type 5 inhibition in an enzyme assay and its potency and selectivity are similar to those of sildenafil (Kotera et al., 2000). It has been reported that T-1032 showed the pharmacological properties of a phosphodiesterase type 5 inhibitor both in vitro and in vivo (Takagi et al., 2001; Noto et al., 2000). Therefore, it is presumed that the vasorelaxant property of T-1032 is similar to that of sildenafil in isolated arteries. However, we obtained the unexpected result that sildenafil caused a more potent vasorelaxation than did T-1032 at the high concentrations.

We now examined the vasorelaxant properties of sildenafil and T-1032 in the rat aorta, and explored the mechanism underlying the difference in vasorelaxant effect between the two phosphodiesterase type 5 inhibitors.

2. Materials and methods

This study was approved by the Animal Research Committee of Tanabe Seiyaku and all efforts were made to minimize animal suffering and to reduce the number of animals used.

2.1. Animals and tissue preparations

Male Wistar rats (190–280 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and exsanguinated. The thoracic aorta was carefully removed and stored in physiological salt solution (PSS) at 4 °C. After removal of adherent adventitial tissue, the aorta was cut into ring segments 5 mm in length. In certain rings, the endothelium was removed by rubbing the intimal surface.

The ring segment was suspended in a 10-ml organ bath chamber containing a 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 1.5 g by gradual incremental stretching. After equilibration for 60 min, potassium chloride (KCl, 4×10^{-2} M) or phenylephrine (3×10^{-6} M) was added to the preparation for characterization of contractility. The preservation of endothelial function or the effectiveness of endothelial removal was tested routinely by checking the responsiveness to acetyl-β-methylcholine chloride (10^{-6} M), which induces vasorelaxation through endothelial NO. The composition of PSS was as follows (mM): KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaCl 118, D-(+)-glucose 11, $NaHCO_3$ 25.

2.2. Concentration-response curves for sildenafil and T-1032

Phenylephrine (3×10^{-6} M) was added to the preparation to obtain a tonic contraction. After the phenylephrine contractile response was stabilized, sildenafil or T-1032 was added to the preparation at an interval of 30 min. Papaverine (10^{-4} M) was added to the preparation to confirm the maximal relaxation of the preparation at the end of the experiment. When the influence of N^G-nitro-L-arginine methyl ester (L-NAME) on sildenafil and T-1032-induced relaxation was to be evaluated, L-NAME (3×10^{-4} M) was added to the preparation 20 min before addition of phenylephrine. The relaxations were expressed as percentages of the relaxation induced by papaverine.

2.3. Calcium chloride ($CaCl_2$)-induced contraction

The endothelium-denuded preparation was exposed to Ca^{2+}-free and K^+-depolarizing PSS after check of the contractility and absence of endothelial function. Concentration-response curves for $CaCl_2$ (3×10^{-5} to 3×10^{-2} M) were obtained in a cumulative fashion. Sildenafil, T-1032 or diltiazem was added to the preparation 30 min before the first addition of $CaCl_2$. The contractile responses were expressed as percentages of the contraction induced by $CaCl_2$ (3×10^{-3} M) obtained in advance. The composition of Ca^{2+}-free and K^+-depolarizing PSS was as follows (mM): KCl 80, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 45.2, D-(+)-glucose 11, $NaHCO_3$ 25.

2.4. Phorbol 12,13-dibutyrate (PDB)-induced contraction

The endothelium-denuded preparation was exposed to Ca^{2+}-free PSS after check of the contractility and absence of endothelial function. Concentration-response curves for PDB (3×10^{-9} to 3×10^{-6} M) were obtained in a cumulative fashion. Sildenafil or T-1032 was added to the preparation 30 min before the first addition of PDB. The contractile responses were expressed as percentages of the contraction induced by KCl (4×10^{-2} M) obtained in advance with normal PSS. The composition of Ca^{2+}-free PSS was as follows (mM): KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 120.5, D-(+)-glucose 11, $NaHCO_3$ 25, EGTA 0.5.

2.5. Cyclic nucleotide levels

In the study of cyclic nucleotides levels (cGMP and cAMP), the endothelium-intact preparation was suspended in the organ bath and phenylephrine (3×10^{-6} M)-induced contraction was obtained as described above. Then, sildenafil or T-1032 (10^{-6}, 10^{-5} and 10^{-4} M) was added to the preparation. After the maximal relaxant response to test compounds was obtained, the preparation was immediately frozen with liquid N_2. The frozen preparation was homogenized in 1 ml of 6% trichloroacetic acid containing disodium EDTA (10^{-3} M). 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 5×10^{-2} M sodium acetate buffer (pH 6.2). The pellet was dissolved in 0.5 ml of 2 N NaOH, and used in the measure-

ment of protein content. The cyclic nucleotide and protein contents in each solution were measured with commercially available cGMP and cAMP immunoassay kits (Amersham, UK) and BCA protein assay kit (pierce, Rochford, IL, USA), respectively. The cyclic nucleotide levels were expressed in picomoles per milligram protein.

2.6. Radioligand binding assays

Radioligand binding assays were performed according to modifications of the methods of Ehlert et al. (1982), Reynolds et al. (1986) and Schoemaker and Langer (1985). Male Wistar rats (150–250 g) were killed by stunning and exsanguination and the brain was rapidly removed. In the [^{3}H]diltiazem binding assay, the cerebral cortex was homogenized with a Polytron homogenizer in cold 50 mM Tris–HCl buffer containing 0.1% bovine serum albumin (pH 7.4). The homogenate was washed three times by centrifugation at 48 000 × *g* for 10 min followed by resuspension of the pellet in fresh 50 mM Tris–HCl buffer containing 0.1% bovine serum albumin. The final pellet was resuspended to an original tissue concentration of 100 mg/ml in Tris–HCl buffer containing 0.1% bovine serum albumin. Aliquots (0.2 ml) of this homogenate were incubated with [^{3}H]diltiazem (2×10^{-9} M) with or without the test compounds (10^{-6} or 10^{-5} M) in 50 mM Tris–HCl buffer containing 0.1% bovine serum albumin. Incubation was carried out at 37 °C for 60 min. Membrane-bound [^{3}H]diltiazem was trapped at the end of the incubation period by rapid vacuum filtration of the incubation mixture over a Whatman GF/B glass fiber filter. The filter was washed with three 5-ml aliquots of cold Tris–HCl buffer, and trapped radioactivity was measured subsequently by liquid scintillation spectrometry. Binding in the presence of diltiazem (10^{-5} M) was defined as non-specific.

The [^{3}H]nitrendipine and (−)-[*N*-methyl-^{3}H]desmethoxyverapamil ([^{3}H](−)-D888) binding assays were performed according to almost the same protocol as for the [^{3}H]diltiazem binding assay. In the [^{3}H]nitrendipine binding assay, 50 mM Tris–HCl buffer (pH 7.7) was used instead of Tris–HCl buffer containing 0.1% bovine serum albumin. Incubation of the homogenate with [^{3}H]nitrendipine (10^{-6} M) was carried out at 25 °C for 90 min. Nifedipine (10^{-6} M) was used as non-specific ligand. The [^{3}H](−)-D888 binding assay was performed using the homogenate of brain and 50 mM HEPES buffer (pH 7.4). Incubation of the homogenate with [^{3}H](−)-D888 (4×10^{-10} M) was carried out at 25 °C for 60 min. Methoxyverapamil (D600) (10^{-5} M) was used as non-specific ligand.

2.7. Chemicals

Sildenafil citrate, T-1032 and diltiazem were synthesized by Discovery Research Laboratory, Tanabe Seiyaku (Saitama, Japan). L-Phenylephrine hydrochloride, acetyl-β-methylcholine chloride, papaverine hydrochloride, phorbol 12,13-dibutyrate, N^G-nitro-L-arginine methyl ester, nifedipine and D600 were obtained from Sigma (St. Louis, MO,

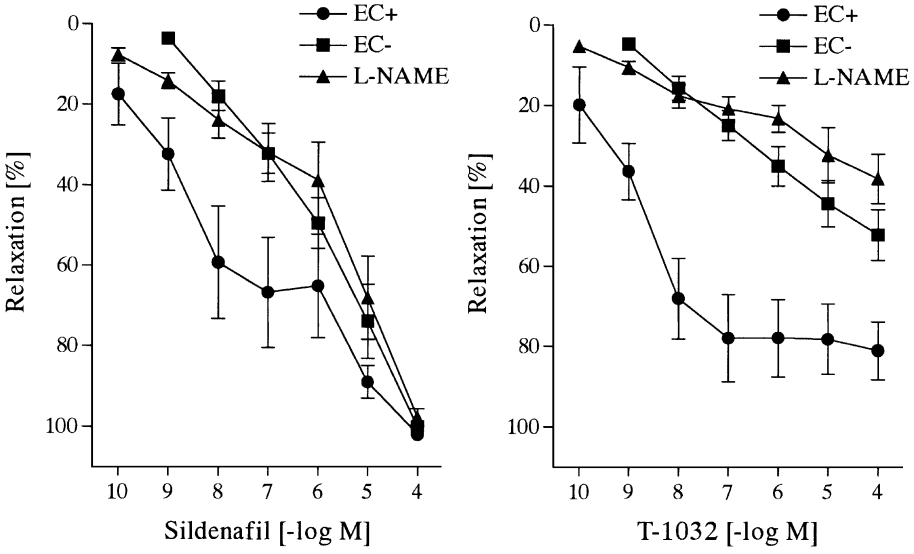

Fig. 1. Effects of endothelium denudation and L-NAME on sildenafil and T-1032-induced relaxation in the isolated rat aorta precontracted with phenylephrine. Sildenafil and T-1032 (10^{-10} to 10^{-4} M in the endothelium-intact and L-NAME-pretreated preparations, and 10^{-9} to 10^{-4} M in the endothelium-denuded preparations) were added to preparations precontracted with phenylephrine (3×10^{-6} M). L-NAME (3×10^{-4} M) was added to the preparation 20 min before addition of phenylephrine. Relaxations induced by papaverine (10^{-4} M) were taken as 100%. Data are shown as means ± S.E.M. for four or five preparations. In this study, phenylephrine (3×10^{-6} M) induced a submaximal contraction in the rat aortic rings. The magnitude of the contractile response to phenylephrine in the endothelium-intact, endothelium-denuded and L-NAME-pretreated preparation was 1.22 ± 0.12, 1.32 ± 0.05 and 1.86 ± 0.08 g, respectively. In each preparation, the reduction of the isometric tension was similar when hydrochloric acid (0.0005 N or less) was used as vehicle. The mean value of the maximal reduction of isometric tension by hydrochloric acid was 27.6 ± 4.8% of papaverine-induced relaxation. L-NAME: nitro-L-arginine methyl ester. EC+: endothelium-intact preparation. EC −: endothelium-denuded preparation. L-NAME: L-NAME-pretreated preparation.

Table 1
Maximal vasorelaxations induced by sildenafil and T-1032 at each concentration in isolated rat aorta

Concentration (M)	Endothelium-intact preparation		Endothelium-denuded preparation		L-NAME-pretreated preparation	
	Sildenafil	T-1032	Sildenafil	T-1032	Sildenafil	T-1032
10^{-10}	17.5 ± 7.7	19.9 ± 9.5	–	–	7.7 ± 1.6	5.4 ± 1.1
10^{-9}	32.4 ± 9.0	36.5 ± 7.0	3.6 ± 0.7	4.7 ± 1.5	14.2 ± 1.9	10.6 ± 1.6
10^{-8}	59.3 ± 14.0	68.0 ± 10.0	17.9 ± 3.6	15.7 ± 3.0	23.9 ± 4.4	17.7 ± 2.9
10^{-7}	66.8 ± 13.7	77.9 ± 10.8	32.1 ± 5.0	24.9 ± 3.7	32.0 ± 7.2	20.8 ± 3.0
10^{-6}	65.1 ± 12.8	77.9 ± 9.6	49.4 ± 6.3	35.0 ± 4.9	38.8 ± 9.6	23.3 ± 3.4
10^{-5}	88.9 ± 4.1	78.2 ± 8.7	73.8 ± 9.2[a]	44.3 ± 5.8	68.0 ± 10.3[a]	32.3 ± 6.9
10^{-4}	102.0 ± 0.6[a]	81.0 ± 7.2	100.1 ± 0.3[a]	52.1 ± 6.3	97.7 ± 2.1[a]	38.2 ± 6.1

The relaxations were expressed as a percentage of the relaxation induced by papaverine. Statistical analysis was performed using repeated measures ANOVA followed by Student's *t*-test (unpaired values). Values are means ± S.E.M. for four or five rats.

[a] $P < 0.05$, compared with T-1032-treated group.

USA). [^{3}H]Diltiazem and [^{3}H]nitrendipine were obtained from New England Nuclear (Boston, MA, USA). [^{3}H](−)-D888 was obtained from Amersham (Buckinghamshire UK). All other chemicals were of analytical grade. Sildenafil and T-1032 were dissolved in hydrochloric acid (0.05 N) to obtain the concentration of 10^{-2} M following dilution with distilled water. PDB and nifedipine were dissolved in ethanol and all other chemicals were dissolved in distilled water.

2.8. Statistical analysis

Statistical analysis was performed with a repeated measures two-way analysis of variance (ANOVA) followed by Student's *t*-test (unpaired values, sildenafil vs. T-1032 in Table 1 and Fig. 4) and Dunnett's test (vs. vehicle in Fig. 4). The affinity of sildenafil and of T-1032 for the L-type Ca^{2+} channel (Table 2) were compared using a *t*-test with Bonferroni correction. *P* values <0.05 were considered as statistically significant. The pA_2 values were determined by the method of Arunlakshana and Schild (1959). Data are presented as means ± S.E.M.

3. Results

3.1. Relaxant effects of sildenafil and T-1032

Sildenafil and T-1032 induced relaxation in the aortic rings, and the difference in relaxant effects between sildenafil and T-1032 was observed in the endothelium-intact, endothelium-denuded and L-NAME-pretreated preparations (Fig. 1). In the endothelium-intact preparations, the sildenafil-induced vasorelaxation was biphasic: the first phase at 10^{-10} to 10^{-7} M and the second phase at 10^{-6} to 10^{-4} M. In the case of T-1032, relaxation reached a plateau at a concentration of 10^{-7} M without further relaxation. The relaxation induced by sildenafil at 10^{-4} M was significantly different from that by T-1032 ($P < 0.05$) (Table 1). In the

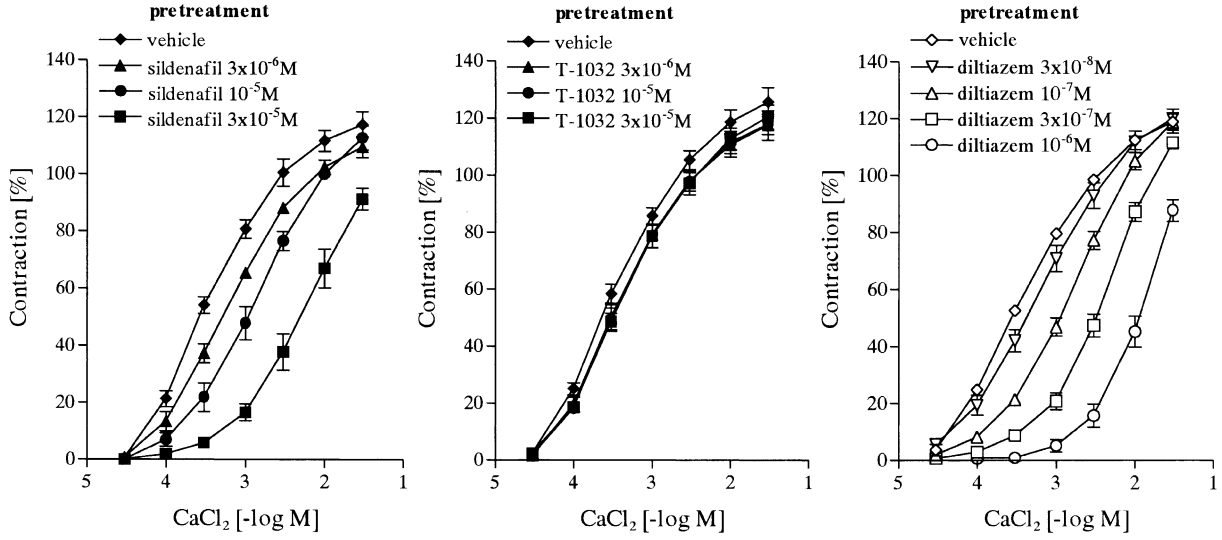

Fig. 2. Concentration-response curves for $CaCl_2$ in endothelium-denuded rat aorta and effects of several concentrations of sildenafil, T-1032 and diltiazem. $CaCl_2$ (3×10^{-4} to 3×10^{-1} M) was added to the preparations in Ca^{2+}-free and K^+-depolarizing PSS. Sildenafil, T-1032 (3×10^{-6}, 10^{-5}, 3×10^{-5} M) and diltiazem (3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} M) were added to the preparation 30 min before the first addition of $CaCl_2$. Contractions induced by $CaCl_2$ (3×10^{-3} M) obtained in advance were taken as 100%. Data are shown as means ± S.E.M. for four preparations. PSS: physiological salt solution.

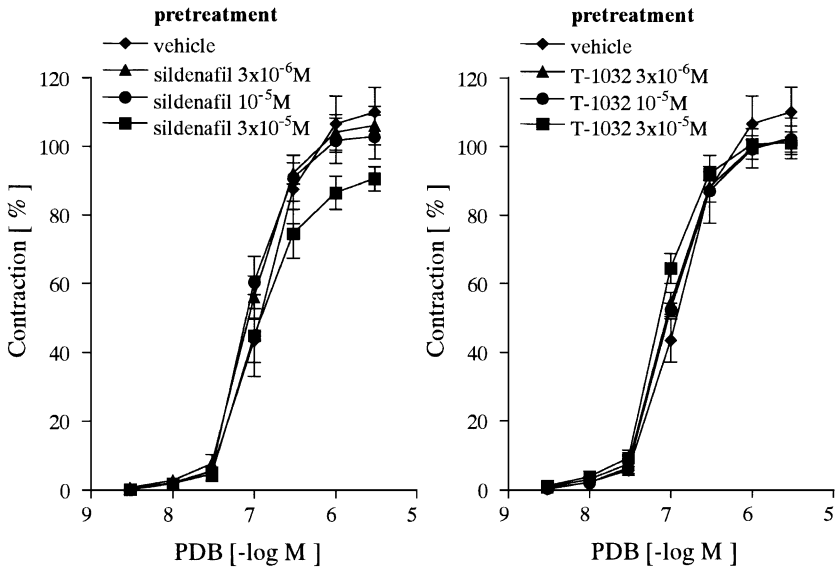

Fig. 3. Concentration-response curves for PDB in endothelium-denuded aortic rings and effects of several concentrations of sildenafil and T-1032. PDB (3×10^{-9} to 3×10^{-6} M) was added to the preparations in Ca^{2+}-free PSS. Sildenafil and T-1032 (3×10^{-6}, 10^{-5}, 3×10^{-5} M) were added to the preparation 30 min before the first addition of PDB. Contractions induced by KCl (4×10^{-2} M) obtained in advance in normal PSS were taken as 100%. Data are shown as means ± S.E.M. for four preparations. PSS: physiological salt solution.

endothelium-denuded and L-NAME-pretreated preparations, T-1032 at 10^{-8} to 10^{-4} M exerted a moderate relaxation. In contrast, sildenafil produced a more potent relaxation than did T-1032, especially at the high concentrations. The relaxation induced by sildenafil at 10^{-5} M or more was significantly different from that by T-1032 ($P < 0.05$) (Table 1). There are no significant differences between sildenafil and T-1032 at 10^{-6} M or less in any of the preparations.

3.2. Effects of sildenafil and T-1032 on the contractile response to $CaCl_2$ and PDB in the endothelium-denuded aortic rings

Pretreatment with sildenafil (3×10^{-6} to 3×10^{-5} M), but not T-1032, caused a dose-dependent rightward shift of the concentration-response curve for $CaCl_2$ in the high K^+-depolarized preparation (Fig. 2). The pA_2 value of sildenafil for the $CaCl_2$ contraction was 5.4. Diltiazem also caused a rightward shift of the concentration-response curve for $CaCl_2$ with the pA_2 value of 7.5 with the same experimental protocol. In contrast, neither of the phosphodiesterase type 5 inhibitors (3×10^{-6} to 3×10^{-5} M) influenced the concentration-response curve for PDB (3×10^{-8} to 3×10^{-5}M) under Ca^{2+}-free conditions (Fig. 3).

3.3. Binding affinities of sildenafil and T-1032 for the L-type Ca^{2+} channel

Sildenafil (10^{-6} and 10^{-5} M) showed affinity for the binding sites of diltiazem, nitrendipine and (−)-desmethoxyverapamil [(−)-D888] on the L-type Ca^{2+} channel (Table 2). Compared to that of T-1032, the affinity of sildenafil at 10^{-5} M for binding sites of nitrendipine and (−)-D888 was significantly higher than that of T-1032.

3.4. Effects of sildenafil and T-1032 on cyclic nucleotide levels

Fig. 4 shows the influence of sildenafil and T-1032 on cGMP and cAMP levels in the isolated rat aorta. T-1032 (10^{-6} to 10^{-4} M) increased cGMP significantly to almost the same levels. In contrast, sildenafil (10^{-6} to 10^{-4} M)

Table 2
Affinity of sildenafil and T-1032 for the L-type Ca^{2+} channel

Binding site	Ligand	Percentage inhibition			
		Sildenafil		T-1032	
		10^{-6} M	10^{-5} M	10^{-6} M	10^{-5} M
Benzothiazepine	[^{3}H]Diltiazem	13.5 ± 4.1	17.0 ± 3.3	− 1.2 ± 2.5	12.1 ± 7.5
Dihydropyridine	[^{3}H]Nitrendipine	11.9 ± 2.0	35.2 ± 3.3[a]	4.8 ± 5.1	11.8 ± 4.0
Phenylalkylamine	[^{3}H](−)-D888	12.5 ± 5.5	35.8 ± 1.9[a]	− 0.1 ± 5.7	− 13.1 ± 1.3

Results were expressed as the percentage inhibition of specific radioligand binding. Values are means ± S.E.M. for three experiments. Statistical analysis was performed using a t-test with Bonferroni correction.

[a] $P < 0.05$, compared with T-1032-treated group.

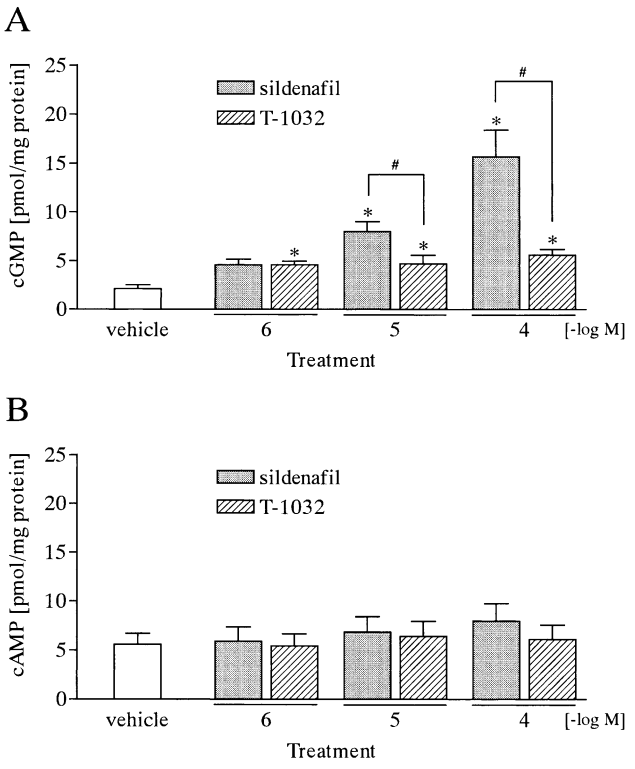

Fig. 4. Changes in cyclic nucleotide levels on treatment with sildenafil and T-1032 in endothelium-intact rat aorta. (A) cGMP levels. (B) cAMP levels. Both compounds (10^{-6}, 10^{-5} and 10^{-4} M) were added to preparations precontracted with phenylephrine (3×10^{-6} M). After the maximal change in tension was obtained, the preparation was immediately frozen in liquid N_2. Cyclic nucleotide (cGMP and cAMP) levels were measured with a commercially available kit. Data are shown as means ± S.E.M. for 10 preparations.

showed a concentration-dependent increase in cGMP levels, and cGMP levels at 10^{-5} and 10^{-4} M were significantly higher than those with the same concentrations of T-1032. Neither of the phosphodiesterase type 5 inhibitors (10^{-6} to 10^{-4} M) influenced the cAMP levels in the isolated rat aorta.

4. Discussion

In the present study, we examined the vasorelaxant property of sildenafil and T-1032, and showed a difference in the actions of these compounds on isolated rat aorta. Our data indicated that sildenafil at high concentrations had vasorelaxant properties not due to phosphodiesterase type 5 inhibition in the isolated rat aorta.

Sildenafil caused a biphasic relaxation, but T-1032 did not. The first phase of relaxation, which was similar to the T-1032-induced relaxation, reached a plateau at the concentration of 10^{-7} M. The sildenafil-induced first phase relaxation was attenuated by endothelium denudation as well as by treatment with L-NAME, a NO synthase inhibitor. These results suggested that the relaxation was related to the NO/cGMP signaling pathway. It has been reported that sildenafil (Wallis et al., 1999; Omote, 1999; Medina et al., 2000a,b) and T-1032 (Takagi et al., 2001) at 10^{-7} M or less potentiate the vasorelaxation induced by NO donors in isolated tissues, probably through blockade of phosphodiesterase type 5. Therefore, the relaxations induced by sildenafil and T-1032 at 10^{-7} M or less were suggested to be attributable to their inhibitory effects on phosphodiesterase type 5. Sildenafil at 10^{-5} M or more, but not T-1032, caused a further (second phase) relaxation in the isolated rat aorta. The sildenafil-induced second phase relaxation was not attenuated by endothelium-denudation and L-NAME, suggesting that this relaxation was independent of the inhibition of phosphodiesterase type 5.

Vascular tone is regulated by the $[Ca^{2+}]_i$ and the sensitivity to Ca^{2+} of the contractile elements in the smooth muscle cell (Karaki et al., 1997). Ca^{2+} influx via voltage-dependent L-type Ca^{2+} channels is a major pathway to increase $[Ca^{2+}]_i$ (Vogalis et al., 1991; Ganitkevich and Isenberg, 1991; Kuriyama et al., 1995) and its blockade causes the cells to relax or inhibits the contraction. Therefore, we examined the influence of sildenafil and T-1032 on Ca^{2+}-induced contraction in K^+-depolarized preparations. Interestingly, sildenafil at 3×10^{-6} M or more, but not T-1032, caused a rightward shift of the concentration-response curves for $CaCl_2$. The inhibitory effect of sildenafil on Ca^{2+}-induced contraction was approximately 100-fold weaker than that of diltiazem, an L-type Ca^{2+} channel blocker. It seems that this effect of sildenafil was not caused by phosphodiesterase type 5 inhibition, because of an inactive NO/cGMP system due to absence of endothelial NO from these preparations. Moreover, sildenafil and T-1032 did not influence the concentration-response curves for PDB under Ca^{2+}-free conditions. PDB is reported to evoke vasocontraction through the activation of protein kinase C (Kanashiro and Khalil, 2001; Ohanian et al., 1996; Karaki et al., 1997) in the presence and absence of Ca^{2+} (Whitney et al., 1995). Therefore, neither of the phosphodiesterase type 5 inhibitors would have any influence on the protein kinase C-related and external Ca^{2+}-independent cascade for contraction. These results suggested that sildenafil at 10^{-5} M or more has an additional vasodilator effect(s) such as a Ca^{2+} channel antagonistic-like effect. Sildenafil has been reported to show specific binding affinity for the L-type Ca^{2+} channel (affinity for the binding site of nitrendipine: 51% at 10^{-5} M [Barry et al., 1998]). When we examined the binding affinities of sildenafil and T-1032 for the binding sites of diltiazem, nitrendipine and (−)-D888 in the present study, sildenafil showed higher affinities for binding sites of nitrendipine and (−)-D888 than did T-1032. These results suggested that the Ca^{2+} channel antagonistic-like effect of sildenafil might be due in part to a direct blocking action on the L-type Ca^{2+} channel. To clarify the precise mechanism of this action, electrophysiological study will be essential.

We also considered the possibility of an inhibitory effect of sildenafil on some other phosphodiesterase isozyme(s) for the mechanism of sildenafil-induced second phase relaxa-

tion. Therefore, we measured cyclic nucleotide levels in the presence and absence of sildenafil or T-1032. Both phosphodiesterase type 5 inhibitors at 10^{-6} M caused an increase in cGMP levels without changes in cAMP. The change in cGMP levels would result in their phosphodiesterase type 5 inhibition, because the concentration at 10^{-6} M is high enough to inhibit phosphodiesterase type 5 for accumulation of cGMP in endothelium-intact preparations. Interestingly, sildenafil at 10^{-5} M or more, but not T-1032, caused a large increase in cGMP levels in a concentration-dependent manner. We did not examine the detailed mechanism in this study. However, circumstantial evidence indicates that the phosphodiesterase type 1 inhibitory effect of sildenafil may be related to the relaxation. Phosphodiesterase type 1 is an important phosphodiesterase for hydrolysis of cGMP in vessels (Wallis et al., 1999; Saeki and Saito, 1993; Miyahara et al., 1995). Although sildenafil is classified as a phosphodiesterase type 5 inhibitor, the concentrations at submicromolar ranges also inhibit phosphodiesterase type 1. Recently, sildenafil was reported to inhibit phosphodiesterase type 1 and type 5 at 50% inhibitory concentrations (IC_{50} values) of 2.7×10^{-7} and 3.6×10^{-9} M, while T-1032 inhibits phosphodiesterase type 1 and type 5 with IC_{50} values of 3.0×10^{-6} and 1.0×10^{-9} M (Kotera et al., 2000). Thus, the inhibitory effect of sildenafil on phosphodiesterase type 1 is approximately 10-fold more potent than that of T-1032. Furthermore, it was reported that zaprinast (10^{-5} M), a concentration that selectively inhibits phosphodiesterase type 5, did not potentiate the sodium nitroprusside-induced rise of cGMP, but zaprinast (10^{-4} M), a concentration that inhibits both phosphodiesterase type 1 and type 5, potentiated the rise of cGMP in vascular smooth muscle cells (Mercapide et al., 1999). Therefore, the blockade of both phosphodiesterase type 1 and type 5 by sildenafil, but not by T-1032, would cause a marked increase in cGMP levels.

In conclusion, results of this study indicated clearly that sildenafil at high concentrations had additional vasorelaxant properties, other than phosphodiesterase type 5 inhibition, in the isolated rat aorta. The sildenafil-induced relaxation appeared to be related to mechanisms such as inhibition of the external Ca^{2+}-dependent cascade for contraction and/or a marked increase in cGMP levels. As T-1032 did not show the additional vasorelaxant properties, T-1032 is considered to be the better tool as a specific phosphodiesterase type 5 inhibitor.

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