Relationship between Cyclic Nucleotide Levels and 5-Methyl-6-(4-pyridyl)-2H-
1,4-thiazin-3(4H)-one (ZSY-27), a New Positive Inotropic Agent with a Vaso-
dilatory Action, -Induced Relaxation of Rabbit Thoracic Aorta

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The aim of this investigation was to substantiate the hypothesis that the vasorelaxant effects of 5-methyl-6-(4-pyridyl)-2H- 1,4-thiazin-3(4H)-one (ZSY-27) are mediated by accumulation of intracellular cyclic nucleotides as a consequence of inhibition of cyclic nucleotide phosphodiesterase activity. Both activities of adenosine 3',5'-monophosphate-phosphodiesterase (cAMP-PDE) in the presence of ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and guanosine 3',5'-
monophosphate-phosphodiesterase (cGMP-PDE) in the presence of calcium-calcmodulin from rabbit thoracic aorta were inhibited in a concentration-dependent manner by ZSY-27 (10^{-8} to 10^{-5} M). The IC_{50} values for ZSY-27 on cAMP- and cGMP-PDE activity were 2.1 \times 10^{-4} and 8.8 \times 10^{-4} M, respectively. Furthermore, ZSY-27 antagonized competitively cAMP-PDE (K_i = 1.9 \times 10^{-4} M). On the other hand, ZSY-27 exhibited a mixed-type inhibitory pattern, with reduction of both maximum velocity and affinity for the substrate of the cGMP-PDE, with a K_i value of 1.0 \times 10^{-3} M. Spontaneous myogenic tone of rabbit thoracic aorta was significantly attenuated from 1 min after addition of ZSY-27 (3 \times 10^{-4} M). Contents of cAMP and cGMP were significantly increased from 1 and 3 min after addition of ZSY-27, respectively. Temporally, relaxant effects of ZSY-27 were associated with increases of cAMP content, but not with that of cGMP content. Furthermore, the relaxant effect of ZSY-27 on the phenylephrine-induced contraction was not affected by the pretreatment with nitroprusside. These results support the hypothesis that elevation of cAMP level via inhibition of cAMP-PDE activity is intimately involved in the mechanism of vasorelaxation produced by ZSY-27.

Keywords — 5-methyl-6-(4-pyridyl)-2H- 1,4-thiazin-3(4H)-one (ZSY-27); pyridyl thiazin-
one derivative; cyclic AMP; cyclic GMP; phosphodiesterase; vasorelaxation; rabbit thoracic aorta

Introduction

Vasodilator therapy is currently being applied to patients with heart failure in order to reduce ventricular afterload and improve cardiac performance.\textsuperscript{1-5} More recently, it has been reported that combined therapy of a vasodilator and an inotropic drug, and the positive inotropic drugs with a vasodilatory action provide the more beneficial efficiency for patients with severe congestive heart failure.\textsuperscript{4-6} 5-Methyl-6-(4-pyridyl)-
2H- 1,4-thiazin-3(4H)-one (ZSY-27) has been shown to have potent positive inotropic and vasodilatory effects.\textsuperscript{7,8} Accordingly, the vasodilatory activity of ZSY-27 is an important component in the therapy of congestive heart failure. It is thought that the increase in intracellular adenosine 3',5'-monophosphate (cAMP)
content via inhibition of cAMP-
phosphodiesterase (PDE) activity might be expected not only to produce positive inotropic effects, but also to induce a relaxation of vascular smooth muscle.\textsuperscript{9-12} Vasodilatory effects of ZSY-27 were considered as a consequence of increase in cAMP via inhibition of cAMP-PDE, because those of ZSY-27 were potentiated by forskolin, an activator of adenylyl cyclase in membranes (our unpublished observation). Moreover, ZSY-27 stimulated exocrine secretion in the dog pancreas via an increase of intracellular cAMP level.\textsuperscript{13} However, it is not known whether vasorelaxation produced by ZSY-27 corresponds with the increase in intracellular cyclic nucleotides as a consequence of inhibition of cyclic nucleotide-PDE activity in the vascular smooth muscles.

Therefore, the present study was designed to determine the relationship between the relaxant

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action and accumulation of cyclic nucleotides via inhibition of cyclic nucleotide-PDE activity by ZSY-27.

Materials and Methods

Preparation of Crude Cyclic Nucleotide PDE — Thoracic aortae were removed rapidly from decapitated adult male white rabbits (weighing 2.0—2.5 kg) and placed in ice-cold 20 mM Tris–HCl buffer (pH 7.5). After removal of adhering fatty and connective tissues, the aortae were cut into helical strips, and then, stripped of adventitia and endothelium. The media obtained were finely minced with scissors and suspended in 10 volumes of ice-cold 20 mM Tris–HCl buffer (pH 7.5). The mince was then sonicated in the ice-cold solution using a Kinematica Polytron PT 10/35 (3 bursts of 10 s duration at a rheostat setting of 5.0). The homogenate was centrifuged at 105000 x g for 20 min at 4 °C and the resulting supernatant fraction was taken as a crude cAMP- and guanosine 3',5'-monophosphate (cGMP)-PDE fraction. The subdivided crude PDE fractions were stored at −80 °C until use.

Protein was determined by the method of Lowry et al. with the use of bovine serum albumin as standard.

The Effects of ZSY-27 on cAMP- and cGMP-PDE Activity — The effects of ZSY-27 on cAMP- and cGMP-PDE activities were measured according to the method described by Bauer and Schwabe. The standard reaction medium contained 40 mM Tris–HCl buffer (pH 8.0), 5 mM MgCl₂, [³H]cAMP (1 μM, 150000 cpm) or [³H]cGMP (1 μM, 150000 cpm) as a substrate, ZSY-27 or vehicle, and diluted crude PDE in a final volume of 0.2 ml. Crude PDE was suitably diluted with 0.5% bovine serum albumin solution, because the substrate conversion was always kept below 20%. Furthermore, cAMP- and cGMP-PDE of multiple forms were assayed in the presence and absence of 0.5 mM ethylene glycol-bis (β-aminoethyl ether) N,N',N''-tetraacetic acid (EGTA) or 0.1 mM CaCl₂ plus 20 nM calmodulin (Ca-CaM). The assay was initiated by addition of 20 μl crude PDE solution to the assay mixture which was preincubated at 30 °C for 3 min. The reaction tubes were maintained at 30 °C for 10 min and terminated by transferring the reaction tubes to a preheated water bath of 93 °C for 3 min followed by immediate cooling in ice-cold water. Following reequilibration at 30 °C for 3 min, 50 μl of Crotalus atrox snake venom (2 mg/ml) was added and maintained at 30 °C for 40 min. After addition of 10 μl adenosine (6.5 mM) or guanosine (2.6 mM) as a carrier of the corresponding nucleotides, the reaction was terminated by applying an aliquot of 0.2 ml to the QAE A-25 Sephadex column (7 x 11 mm) previously equilibrated with 30 mM ammonium formate (pH 6.0) and the eluate was collected directly into scintillation vials. Two ml of ammonium formate (pH 6.0) was applied to each column in order to elute completely adenosine or guanosine produced as a consequence of reaction and the eluates were also collected in the same scintillation vials. After addition of 10 ml of scintillation fluid for aqueous samples, radioactivities were counted using a liquid scintillation counter (Aloka LSC-900). The IC₅₀ values (concentration which produced 50% inhibition of substrate hydrolysis) for ZSY-27 were determined from concentration-response curves. As mentioned above, the assay for cAMP- and cGMP-PDE activity consists of a two step isotopic procedure. In the first step cyclic nucleotides are hydrolyzed to the corresponding 5'-monophosphates by the PDE reaction. In the second step the 5'-monophosphates are then further hydrolyzed to the corresponding nucleosides with snake venom 5' nucleotidase. Therefore, in the same procedure, the effect of ZSY-27 on the 5'-nucleotidase was tested by addition of ZSY-27 (2.4 x 10⁻³ M) between inactivation of PDE by heat and addition of Crotalus atrox snake venom.

Furthermore, the inhibitory modes of ZSY-27 (2 x 10⁻⁴ M) on cAMP- or cGMP-PDE were determined using a wide range of substrate concentration (cAMP, 0.5–5.0 μM and cGMP 1–10 μM). The data were analyzed using Lineweaver–Burke analyses.

The Effects of ZSY-27 on Spontaneous Myogenic Tone, and cAMP and cGMP Accumulation in Rabbit Thoracic Aorta — The
rabbit thoracic aortae were cleaned and cut into spiral strips of about 3 mm in width and 3.5 cm in length. Following, these were stripped of adventitia and endothelium. The preparations were suspended in an organ bath filled with 20 ml of modified Krebs solution. The composition of this solution was as follows (mM): NaCl 118, KCl 4.75, CaCl$_2$·2H$_2$O 2.5, MgCl$_2$·6H$_2$O 1.2, NaHCO$_3$ 25 and glucose 11. The solution was aerated with 95% O$_2$ and 5% CO$_2$, and the pH was 7.2—7.3 and kept at 37 °C. The preparations were equilibrated for 3 h under 2 g tension. Contractile responses were measured isotonically by means of isometric transducers (M. E. Commercial, ME-4013T) and recorded on an ink-writing recorder (Rikadenki, R-02). ZSY-27 (2 × 10$^{-4}$ M) was added to the bathing solution when spontaneous tone reached a steady level. Papaverine (3 × 10$^{-4}$ M) was added after 45 min of ZSY-27 addition in order to define 100% relaxation of each preparations.

cAMP and cGMP were measured by radioimmunoassay. Immediately after the smooth muscle preparations were removed at various times after exposure to ZSY-27 (3 × 10$^{-4}$ M), the reactions were stopped by freezing in liquid nitrogen, and then the frozen tissue was homogenized in 6% trichloroacetic acid using a glass-glass homogenizer. The samples were centrifuged twice at 1000 × g for 15 min at 4 °C. The combined supernatant was used for radioimmunoassay using a cyclic nucleotide assay kit, and the pellet was determined for protein content by the method of Lowry et al. After addition of 1 N HCl to the consolidations of the acid-soluble fraction, they were freed of trichloroacetic acid by extracting four times with 5 ml of water-saturated ether. The extracts were freeze-dried. The freeze-dried samples were dissolved in 50 mM acetic acid buffer (pH 6.2) and radioactive iodine-labeled cAMP or cGMP and antibody corresponding to cAMP or cGMP added to that. The mixtures were incubated for 16 to 20 h at 0 °C. Following, the excess unbound radioactivity was absorbed on activated charcoal. The radioactivity remaining in the supernatant was measured on a gamma counter (Aloka ARC-501). Differences between values were evaluated by Duncan’s new multiple range test and taken to be significant when $p$ values were less than 0.05.

The Effect of Nitroprusside on the Relaxant Action of ZSY-27 — Preparations were incubated with nitroprusside (10$^{-8}$ M) for 30 min before the addition of phenylephrine (5 × 10$^{-7}$ M). ZSY-27 (10$^{-5}$—10$^{-4}$ M) was added to the bathing solution in a cumulative manner, after the contraction by the agonist reached a steady-state level. To determine whether relaxant actions of ZSY-27 were affected in the presence and absence of nitroprusside, the pIC$_{50}$ (negative logarithm of the concentration of antagonist required for 50% inhibition of the stimulant-induced precontraction) of each values was compared by Student’s $t$-test.

Drugs — The following drugs were used: ZSY-27 (Zenyaku), papaverine HCl (Wako), phenylephrine HCl (Sigma), sodium nitroprusside dihydrate (Wako), Crotalus atrox snake venom (Sigma), cAMP and cGMP assay kit (Yamasa Shoyu). CaM was purified to homogeneity from bovine brain after the procedure of Dedman and Kaetzel (1983).16

Results

The Effects of ZSY-27 on cAMP- and cGMP-PDE Activity

Five'-nucleotidase activities in Crotalus atrox snake venom on corresponding nucleotides with cAMP or cGMP as a substrate were not af-

![Graph](image)

Fig. 1. Effects of EGTA and Ca-CaM on cAMP-PDE in Rabbit Thoracic Aorta

Each point represents the mean value of the two experiments. ○, vehicle; ●, 0.5 mM EGTA; △, 0.1 mM CaCl$_2$ plus 20 nM CaM.
Vasodilatory Action of ZSY-27

Fig. 2. Inhibition of cAMP-PDE by ZSY-27
The concentrations of cAMP and PDE were 0.2 nmol and 30 μg/ml, respectively. Each point represents the mean ± S.E. of the six experiments. ●, in the presence of EGTA; ○, in the presence of Ca-CaM.

Inhibited by ZSY-27 (2.4 × 10⁻³ M) either in the presence of EGTA or Ca-CaM (data not shown).

Activity of cAMP-PDE from rabbit thoracic aorta was not affected by addition of EGTA or Ca-CaM (Fig. 1). AMP-PDE activity in either EGTA or Ca-CaM medium was inhibited in a concentration dependent manner by ZSY-27. Then, the IC₅₀ values for ZSY-27 on cAMP-PDE activity in media containing EGTA and Ca-CaM were (2.1 ± 0.3) × 10⁻⁴ M or (2.4 ± 0.2) × 10⁻⁴ M, respectively, and these values were much the same (Fig. 2). On the other hand, activity of cGMP-PDE was stimulated by Ca-CaM, while that of cGMP-PDE was inhibited by EGTA (Fig. 3). Accordingly, subsequent experiments with cAMP- or cGMP-PDE were done in the presence of Ca-CaM. Activity of cGMP-PDE in Ca-CaM medium was inhibited in a concentration dependent manner by ZSY-27, when the IC₅₀ value was (8.8 ± 2.3) × 10⁻⁴ M (Fig. 4). The Kₘ and Kᵢ values for cAMP- and cGMP-PDE were analyzed using Lineweaver–Burk plot (Fig. 5 and 6). When cAMP and cGMP were used as a substrate, the Kₘ values were 1.84 and 1.83 μM, respectively. ZSY-27 exhibited a competitive inhibitory effect on cAMP-PDE (Kᵢ = 1.93 × 10⁻⁴ M). On the other hand, ZSY-27 exhibited reduction of both maximum velocity and affinity for substrate of the cGMP-PDE, namely a mixed-type inhibitory pattern (Kᵢ = 1.0 × 10⁻³ M).

The Effects of ZSY-27 on Spontaneous
Fig. 5. Double-Reciprocal Plot of Adenosine Formation by Rabbit Aorta PDE in the Presence of Ca-CaM
Velocity is expressed as nmol of cAMP hydrolyzed/min/mg of protein. The concentration of ZSY-27 (●) was $2 \times 10^{-4}$ M. Open circles show the control values. Each point represents the mean value of the two experiments. ○: $1/v = 0.9/s \pm 0.5$ ($r = 0.993$). ●: $1/v = 2.0/s \pm 0.6$ ($r = 0.995$).

Fig. 6. Double-Reciprocal Plot of Guanosine Formation by Rabbit Aorta PDE in the Presence of Ca-CaM
Velocity is expressed as nmol of cGMP hydrolyzed/min/mg of protein. The concentration of ZSY-27 (●) was $10^{-3}$ M. Open circles show the control values. Each point represents the mean value of the two experiments. ○: $1/v = 1.63/s \pm 0.62$ ($r = 0.992$). ●: $1/v = 3.16/s \pm 1.07$ ($r = 0.994$).

Fig. 7. The Effects of ZSY-27 on Spontaneous Myogenic Tone, and cAMP and cGMP Accumulation in Rabbit Thoracic Aorta
The contractile response was measured by isotonic mean, furthermore, cAMP (○) and cGMP (●) levels were measured by radioimmunoassay. Papaverine ($3 \times 10^{-4}$ M) was added to the bath at 45 min after administration of ZSY-27 in order to define 100% relaxation of each preparation. Each point represents the mean ± S.E. of 6 experiments.

Myogenic Tone, and cAMP and cGMP Accumulation in Rabbit Thoracic Aorta
The effects of a single concentration ($2 \times 10^{-4}$ M, nearly $K_m$ value concentrations of cAMP- and cGMP-PDEs for the respective substrates) of ZSY-27 on temporal increases of cAMP and cGMP contents, and relaxation on spontaneous myogenic tone in rabbit thoracic aorta are shown in Fig. 7. This concentration of ZSY-27 was sufficient to relax vascular smooth muscle; spontaneous myogenic tone was significantly reduced from 1 min after addition of ZSY-27. Furthermore, ZSY-27 produced temporally a marked increase in cAMP and cGMP contents from basal values of 22.36 ± 2.27 ($n = 6$) and 7.22 ± 1.44 fmol/mg wet weight ($n = 6$), respectively. However, to cause significant increases in cAMP and cGMP contents 1 and 3
**Vasodilatory Action of ZSY-27**

![Graph showing effects of ZSY-27 on relaxation](image)

Fig. 8. Effects of Nitroprusside on the ZSY-27-Induced Inhibition of Phenylephrine-Induced Constrictions

Nitroprusside (●; $10^{-9}$ M) and vehicle (○) were added 30 min before the addition of phenylephrine ($5 \times 10^{-7}$ M). ZSY-27 was added to the bath in a cumulative manner when the contractile response of agonist reached a steady level. Papaverine ($3 \times 10^{-4}$ M) was added at 45 min after the injection of ZSY-27 in order to define 100% relaxation of each preparation. Each point represents the mean ± S.E. of 8 experiments.

min after addition of ZSY-27 were required, respectively.

**The Effect of Nitroprusside on the ZSY-27 Relaxant Action of ZSY-27**

The pIC$_{50}$ values of ZSY-27 in the presence and absence of nitroprusside were 4.73 ± 0.07 ($n = 8$) and 4.58 ± 0.05 ($n = 8$), respectively. The difference between values was not significant (Fig. 8).

**Discussion**

In this study, crude cyclic nucleotide-PDE from rabbit thoracic aorta was used to test the hypothesis that the vasorelaxant effects of ZSY-27 are mediated by accumulation of intracellular cyclic nucleotides as a consequence of inhibition of cyclic nucleotide-PDE activity. Activity of crude cAMP-PDE was not affected by Ca-CaM or EGTA, while that of crude cGMP-PDE was potentiated by Ca-CaM, and inhibited by EGTA. Hagiwara et al. 17) reported that cyclic nucleotide PDE exists in multiple forms in rabbit vascular smooth muscle, namely cGMP-PDE (Fr. III with high affinity for cGMP and stimulation by CaM), calcium-dependent PDE (Fr. II with low affinity for cAMP and cGMP) and cAMP-PDE (Fr. I with high affinity for cAMP). Accordingly, crude cAMP- and cGMP-PDE used in this experiment seem to fit the criteria for Fr. III and I, respectively, of the three enzyme types as described by Hagiwara et al. 17) Activity of crude cAMP-PDE was antagonized competitively by ZSY-27 ($K_i = 2.2 \times 10^{-4}$ M). However, ZSY-27 exerted a mixed-type inhibitory effect, reduction of maximum velocity and affinity for substrate of the cGMP-PDE, with a $K_i$ of $7.1 \times 10^{-4}$ M. Consistent with the $K_i$ values, the IC$_{50}$ value of ZSY-27 on cAMP-PDE activity ($2.2 \times 10^{-4}$ M) was lower than that on cGMP-PDE activity ($8.8 \times 10^{-4}$ M). These results demonstrated that ZSY-27 is a fairly selective inhibitor of the cAMP-PDE. Furthermore, this result shows that the concentrations of ZSY-27 which cause the inhibition on cAMP-PDE activity were about the same with those which cause the vasodilation on high K (34.5 mM)- and phenylephrine (1 × 10$^{-6}$ M)-induced precontractions in isolated rabbit thoracic aorta (pIC$_{50}$ = 3.62 and 3.54, respectively, our unpublished observation). It seems that the inhibitory effect on cAMP-PDE activity in this study is weaker than the positive inotropic effects. 8) The reason for diversity between them is not clear. However, in the dog whole body ZSY-27 (0.03-1 mg/kg, i.v.) increased the cardiac contractile force and decreased the blood pressure dose-dependently. Both actions appeared within 30 s after injection of ZSY-27 and reached maximum within 1 min. 9) Therefore, this diversity may be attributed to the difference of animals used. In addition, the relaxant effects of ZSY-27 was temporally associated with increases of cAMP content, but not with that of cGMP content. In this study, the vasorelaxant effect of ZSY-27 on phenylephrine-induced contraction was not affected by nitroprusside, a stimulator of guanylate cyclase. 18,19) Furthermore, that of ZSY-27 for phenylephrine-induced contraction was potentiated by forskolin, an ac-
tivator of adenylate cyclase, but not affected by methylene blue, an inhibitor of guanylate cyclase (our unpublished observation). These results suggest that ZSY-27 produced the arterial smooth muscle relaxation via cAMP-, but not cGMP-dependent processes, and further support the hypothesis that elevation of cAMP level via inhibition of cAMP-PDE activity is intimately involved in vasorelaxation produced by ZSY-27.

References