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Sivelestat Relaxes Porcine Coronary Arteries via Inhibition of Ca^{2+} Sensitization without Affecting Ca^{2+}-Induced Contraction

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Abstract

Sivelestat is a neutrophil elastase inhibitor and the effects of sivelestat on the contractile regulation of vascular smooth muscle (VSM) have not been reported. This study was designed to determine whether sivelestat affects the contractility of porcine coronary arteries, and if so, to elucidate the underlying mechanism(s) and particularly those involving the Ca^{2+} sensitization of VSM.

Sivelestat induced the concentration-dependent ($3\times10^{-5}-3\times10^{-4}$ M) vasorelaxation of porcine coronary arteries, with or without endothelium, when precontracted with U46619 (G protein-coupled receptor agonist; 100 nM). Simultaneous measurements of tension and $[Ca^{2+}]_i$ demonstrated that sivelestat shifted the $[Ca^{2+}]_i$-tension curve to the right and downward during stimulation with 118 mM K^+ and 100 nM U46619. In β-escin-permeabilized arterial strips, sivelestat abolished contractions induced by GTP plus U46619 at a constant $[Ca^{2+}]_i$, whereas it had no effect on Ca^{2+}-induced contractions. These findings suggested that sivelestat induced vasorelaxation via selective inhibition of the Ca^{2+} sensitization induced by U46619, without affecting Ca^{2+}-induced contractions.

In conclusion, sivelestat relaxes porcine coronary artery smooth muscle via a selective inhibition of Ca^{2+} sensitization induced by a G protein-coupled receptor agonist, without affecting Ca^{2+}-induced contractions.

Key words; smooth muscle, coronary artery, Ca^{2+} sensitivity, vasorelaxation, vasospasm

Introduction

Sivelestat sodium hydrate (sodium *N*-{2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl} aminoacetate tetrahydrate; sivelestat) is a novel synthetic specific inhibitor of neutrophil elastase. Sivelestat reliably attenuates acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[1~3], which are life-threatening diseases characterized by a neutrophilic inflammatory response and commonly associated with sepsis, aspiration pneumonia and trauma. Neutrophils and neutrophil elastase are believed to play key roles in ALI and ARDS[4~6], and this specific neutrophil elastase inhibitor has been approved and is commercially available in Japan as a treatment for ALI associated with systemic inflammatory response syndrome.

Patients with ALI or ARDS often require intensive care, including respiratory and cardiovascular support, to maintain coronary blood flow, stable blood pressure and stable heart rate. It is therefore important to determine how sivelestat affects hemodynamics. However, there are no reports in the literature on the effects of sivelestat on the regulatory mechanisms of

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vascular smooth muscle (VSM).

Coronary vasospasm, involving sustained, abnormal contraction of the coronary artery, plays important roles in a wide variety of ischemic heart diseases[7]. Currently, medical treatments for coronary vasospasm include beta-adrenergic agonists, nitroglycerin or Ca^{2+} antagonists. Although these drugs inhibit vasospasm, they also decrease the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and exert a Ca^{2+}-desensitizing effect on Ca^{2+}-induced contractions, leading to a decrease in normal blood pressure. Thus, hypotension is an adverse reaction to coronary vasospasm treatment. Selective inhibition of Ca^{2+} sensitization (contraction not dependent on increased $[Ca^{2+}]_i$) but not Ca^{2+}-induced contraction is required to prevent hypotension during treatment of coronary vasospasm.

The mechanism of Ca^{2+} sensitization has attracted attention as a possible molecular target to prevent vasospasm. In the past few years we have investigated a number of drugs to determine if they selectively inhibit Ca^{2+} sensitization. Results indicated that sivelestat induces vasorelaxation via selective inhibition of Ca^{2+} sensitization.

The present study was designed to determine whether sivelestat affects the contractility of porcine coronary arteries, and if so, to elucidate the underlying mechanism(s), particularly that of Ca^{2+} sensitization of VSM.

Methods

A. Tissue preparation of vascular smooth muscle strips

All procedures were approved by the Committee on Ethics in Animal Experiments of Saga Medical School and were conducted in conformity with institutional guidelines.

Porcine coronary arteries were prepared as described previously[8,9]. In brief, porcine hearts were obtained from a local abattoir. The left anterior descending artery (2–3 cm from the origin) was excised and immediately placed in ice-cold physiologic salt solution (PSS; in mM concentration: NaCl, 123; KCl, 4.7; $CaCl_2$, 1.25; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 15.5; glucose, 11.5) aerated with 95% O_2 and 5% CO_2. The arteries were dissected from surrounding tissues and cut into strips (1×4 mm)[8]. In all experiments, the endothelium was carefully removed, and complete denudation of the endothelium was confirmed by the lack of relaxation induced by 1 μM bradykinin[8,9].

B. Isometric tension recording

Tension recording of VSM strips was performed as described previously[8]. In brief, the prepared strips were mounted vertically in an organ bath filled with PSS (aerated with 95% O_2 and 5% CO_2) and maintained at 37°C. A force transducer (TB-612T; Nihon Kohden, Tokyo, Japan) was used to measure isometric tension. The strips were stretched in a stepwise manner to the optimal point of their length-tension relation (~300 mg). The developed tension was expressed as a percentage, assuming the values at steady state in normal PSS and in response to U46619 precontraction to be 0% and 100%, respectively. Various concentrations of sivelestat were applied cumulatively under conditions of steady state contraction induced by U46619. Concentrations of sivelestat greater than 3×10^{-4} M were not tested because of the limitation of its solubility.

C. Simultaneous measurements of tension and $[Ca^{2+}]_i$

Strips lacking endothelium were loaded with the $[Ca^{2+}]_i$ indicator dye fura-2 AM as described previously[8,10,11]. In brief, strips were incubated for 4 hr at 37°C in normal PSS gassed with 95% O_2 and 5% CO_2 with a resulting pH of 7.4 and containing 12.5 μM fura-2 AM plus 5% fetal calf serum. The fura-2-loaded strips were mounted vertically in a quartz organ bath, and tension was measured with a force transducer (TB-612T; Nihon Kohden). For purposes of equilibration, during 1 h of incubation in normal PSS, the strips were stimulated repeatedly with 118 mM K^+ in PSS every 15 min, and the resting tension was adjusted to an optimal level (~300 mg). To assess $[Ca^{2+}]_i$ levels, the ratio of fura-2 fluorescence intensity at 500 nm at alternating (400 Hz) excitation (340 and 380 nm; F340/F380) was monitored with a fura-2 fluorometer (CAM-230; Japan Spectroscopic Co., To-

kyo, Japan) as described previously[8,10,11]. Changes in tension and the fluorescence ratio were expressed as percentages, assuming the values at steady state at normal 5.9 mM K^+ and 118 mM K^+ to be 0% and 100%, respectively.

D. Membrane permeabilization and tension measurement

Membrane permeabilization of VSM with β-escin was performed as described previously[8,12]. In brief, small strips (100–150 μm×2 mm) of porcine left circumflex coronary artery smooth muscle were dissected. Isometric tension was measured with a force transducer (UL-2g; Minebea, Tokyo, Japan) in a well on a 'bubble' plate at 28℃[8,9,12]. The solution was changed by sliding the bubble plate to an adjacent well. When steady responses to high K^+ were observed, the strips were incubated for 10 min in normal relaxing solution (in mM concentration: potassium methanesulfonate, 74.1; magnesium methanesulfonate, 2; MgATP, 4.5; EGTA, 1; creatine phosphate, 10; 1,4 piperazinediethane sulfonic acid, 30) containing 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) as a mitochondria blocker and 1 μM leupeptin as a protease inhibitor. For β-escin permeabilization, the strips were incubated with 30 μM β-escin for 20–30 min in normal relaxing solution[8,9,12]. Calmodulin (1 μM) was added to the activating solution. Tension was expressed as a percentage, assigning the values in normal relaxing solution (pCa > > 8, nominally zero Ca^{2+} with 10 mM EGTA) and activating solution (pCa 4.5, buffered with 10 mM EGTA) as 0% and 100%, respectively.

E. Solutions and drugs

Sivelestat was a generous gift from Ono Pharmaceutical Co. (Osaka, Japan). U46619 (G protein-coupled receptor agonist) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Fura-2 AM (acetoxymethyl ester form of fura-2) and ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) were purchased from Dojindo Laboratories (Kumamoto, Japan). Calmodulin was purchased from Biomol (Plymouth Meeting, PA, USA). Methanesulphonic acid and creatine phosphate disodium were from Wako Pure Chemical Industries (Osaka, Japan). ATP and GTP were from Boehringer Mannheim (Mannheim, Germany). Bradykinin and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were from Katayama Chemical Industries (Osaka, Japan). U46619 was dissolved in dimethyl sulphoxide (DMSO). Other drugs were dissolved in distilled water.

F. Statistical analysis

All values are expressed as mean±SEM. The number of vascular strips from different pigs (n) is indicated for each experiment. Student's t-test was used to analyze results, and $P<0.05$ was accepted as statistically significant.

Results

A. Effect of sivelestat on tension

Sivelestat elicited concentration-dependent (3×10^{-5} -3×10^{-4} M) relaxation of U46619-precontracted arterial strips (**Fig. 1**).

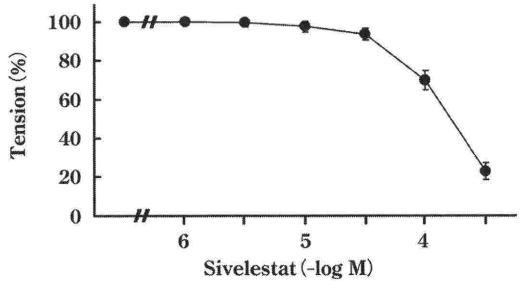

Figure 1 Effects of cumulative application (1×10^{-6}–3×10^{-4} M) of sivelestat on tension in porcine coronary artery strips.

Summary of experiments on the effects of sivelestat on contractions induced by U46619 ($n=5$, endothelium-denuded). Data are shown as mean±SEM.

B. Effects of sivelestat on $[Ca^{2+}]_i$ and tension during contractions induced by U46619

When vascular strips were exposed to 118 mM K^+, the fluorescence ratio and tension reached maximum levels within 30 sec and 10 min, respectively. These levels were either sustained or slightly reduced during depolarization. When 100 nM U46619 was added in normal PSS, the fluorescence ratio rapidly rose to a peak and then decreased to a sustained value ($59.8 \pm 3.8\%$, $n=5$). The tension also developed rapidly, reached a maximum, and plateaued ($84.4 \pm 7.0\%$, $n=5$).

Cumulative application of sivelestat (3×10^{-5} — 3×10^{-4} M) induced a concentration-dependent decrease in the contraction induced by U46619 (100 nM), without affecting the levels of $[Ca^{2+}]_i$.

C. Effect of sivelestat on the $[Ca^{2+}]_i$-tension relation

To evaluate the effects of sivelestat on the $[Ca^{2+}]_i$-tension relation of U46619-induced contractions, changes in $[Ca^{2+}]_i$ and tension obtained by cumulative application of extracellular $CaCl_2$ during stimulation with 118 mM K^+ and 100 nM U46619 were investigated in the presence or absence of 3×10^{-4} M sivelestat (**Fig. 2A, B**). In response to stepwise increments of extracellular Ca^{2+} concentration (0.025–1.25 mM), $[Ca^{2+}]_i$ and tension increased in a concentration-dependent manner. The $[Ca^{2+}]_i$-tension relation shifted to the left in response to 100 nM U46619 during stimulation with 118 mM K^+, and sivelestat significantly shifted the $[Ca^{2+}]_i$-tension relation to the right and downward during stimulation with 118 mM K^+ and 100 nM U46619 (**Fig. 2C**). These findings showed that sivelestat almost completely inhibited the Ca^{2+} sensitization induced by U46619.

D. Inhibitory effect of sivelestat on Ca^{2+} sensitization induced by U46619 plus GTP in β-escin-permeabilized VSM

At a constant $[Ca^{2+}]_i$ (pCa 6.3) in β-escin-permeabilized VSM of porcine coronary arteries, U46619 (30 nM) induced rapid contractions in the presence of 10 μM GTP but not in its absence. This Ca^{2+}-independent contraction was abolished by 3×10^{-4} M sivelestat (**Fig. 3**). In contrast, the tension at pCa 6.3 (=Ca^{2+}-induced contraction) was not affected by 3×10^{-4} M sivelestat. These results suggested that sivelestat mediated vasorelaxation via selective inhibition of the Ca^{2+} sensitization induced by the receptor agonist U46619, without affecting the Ca^{2+}-induced contraction.

Discussion

In the present study, we found that sivelestat induces vasorelaxation of the coronary artery via selective inhibition of Ca^{2+} sensitization induced by a G protein-coupled receptor agonist, without affecting the Ca^{2+}-induced contraction.

Changes in $[Ca^{2+}]_i$ play a central role in the excitation-contraction coupling in VSM. The contraction of VSM is initiated by an increase in $[Ca^{2+}]_i$ and subsequent phosphorylation of the myosin light chain by a complex of Ca^{2+}-calmodulin and the myosin light chain kinase. Although it is generally accepted that smooth muscle tone is primarily regulated by $[Ca^{2+}]_i$, the development and maintenance of tension does not simply depend on $[Ca^{2+}]_i$. Recently, it was shown that in membrane-permeabilized VSMs, some agonists induce enhanced sensitivity of myofilaments to Ca^{2+} through a G protein-mediated pathway[12,13].

Vascular contraction as a result of Ca^{2+} sensitization of VSM has attracted attention as a cause of vasospasm[14], and coronary artery spasm plays an important role in the pathogenesis of a wide variety of ischemic heart diseases including variant angina and unstable angina, myocardial infarction, ventricular arrhythmias and sudden death[7,15~17]. Meanwhile, sphingosylphosphorylcholine (a sphingolipid), and Src family tyrosine kinases were recently identified as upstream mediators of Rho-kinase-mediated Ca^{2+} sensitization of porcine VSM contractions[8,18]. Additionally, eicosapentaenoic acid (EPA), a Src family tyrosine kinase translocation inhibitor, selectively inhibits Ca^{2+} sensitization of VSM contractions[18]. There are currently no reports of drugs that can selectively inhibit Ca^{2+} sensitization without affecting

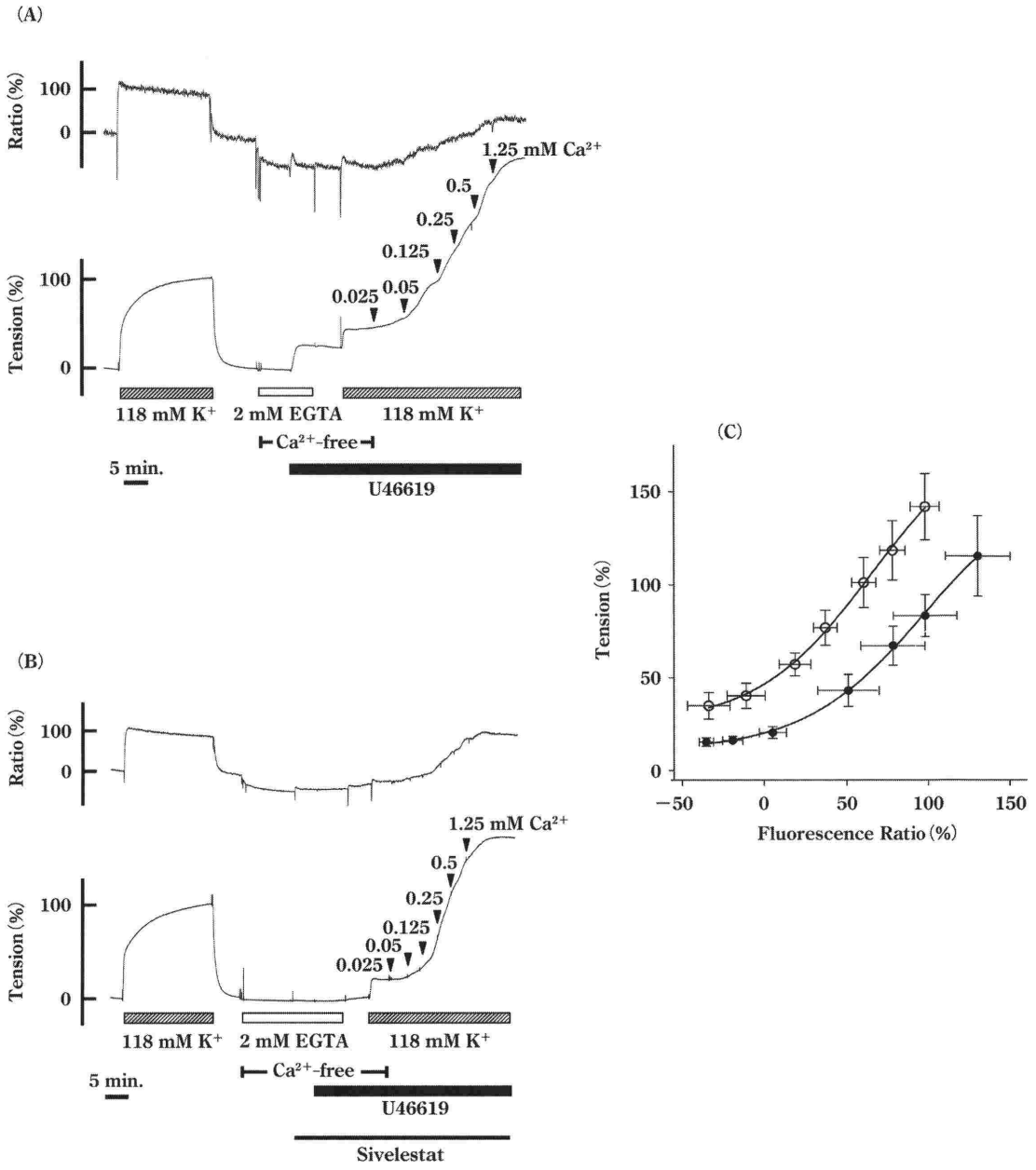

Figure 2 Effects of sivelestat on changes in $[Ca^{2+}]_i$ and tension induced by U46619 in porcine coronary arteries without endothelium.

Various concentrations of sivelestat ($3\times10^{-5}-3\times10^{-4}$ M) were applied cumulatively during steady state contraction. Changes in $[Ca^{2+}]_i$ and tension were monitored simultaneously, and the levels of $[Ca^{2+}]_i$ (F340/F380) and tension were normalized to the responses to 118 mM K^+ depolarization (=100%). Transient noise in the fluorescence ratio was recorded during exchange and mixing of solutions containing sivelestat. (**A** and **B**) Representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of extracellular $CaCl_2$ in Ca^{2+}-free 118 mM K^+ in the presence of 100 nM U46619, without (**A**) and with (**B**) 3×10^{-4} M sivelestat. (**C**) $[Ca^{2+}]_i$–tension relation of contractions induced by the cumulative application of extracellular $CaCl_2$. The $[Ca^{2+}]_i$–tension relation in response to 100 nM U46619 during 118 mM K^+ depolarization indicates Ca^{2+} sensitization induced by U46619 (**open circle**). Sivelestat shifted the $[Ca^{2+}]_i$–tension relation to the right and downward during stimulation with U46619 plus high K^+ depolarization (**closed circle**). Data are shown as mean±SEM of four independent experiments.

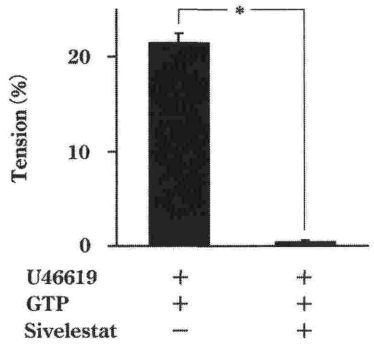

Figure 3 Inhibitory effect of sivelestat on Ca^{2+} sensitization induced by U46619 and GTP in β-escin-permeabilized vascular smooth muscle of porcine coronary arteries.

Summary of repeated experiments of the relaxing effect of 3×10^{-4} M sivelestat on contraction induced by 30 nM U46619+10 μM GTP under constant $[Ca^{2+}]_i$ (pCa 6.3 buffered with 10 mM EGTA). Tension levels are expressed as a percentage of the values in normal (pCa 6.3 buffered with 10 mM EGTA; 0%) and activating (pCa 4.5 buffered with 10 mM EGTA; 100%) solutions. Data are shown as mean±SEM. $n=4$. *; $P<0.05$.

Ca^{2+}-induced contraction, with the exception of EPA. In the present study, we determined whether sivelestat directly and selectively inhibits Ca^{2+} sensitization induced by a G protein coupled receptor agonist by measuring $[Ca^{2+}]_i$ and tension simultaneously in intact artery strips loaded with fura-2 AM and by membrane permeabilization of VSM with β-escin. We showed that sivelestat induced vasorelaxation via selective inhibition of Ca^{2+} sensitization without affecting the Ca^{2+}-induced normal physiological contraction, which is similar to the vasorelaxation induced by EPA.

For the treatment or prevention of vasospasm, vasorelaxant agents have been used that act via cAMP or cGMP, such as beta-adrenergic agonists or nitroglycerin, and that decrease $[Ca^{2+}]_i$, such as Ca^{2+} antagonists,. Although these drugs inhibit vasospasm[19], they also decrease $[Ca^{2+}]_i$ and have a Ca^{2+}-desensitizing effect on the Ca^{2+}-induced contraction, leading to a decrease in normal blood pressure. In the present study, sivelestat, like EPA, selectively inhibited Ca^{2+} sensitization induced by the agonist U46619 and did not affect the native Ca^{2+} sensitivity (without agonists). These findings are compatible with the notion that sivelestat can inhibit Ca^{2+} sensitization without affecting $[Ca^{2+}]_i$ or Ca^{2+}-induced contraction. Indeed, systemic hypotension has never been reported as an adverse effect in the clinical use of sivelestat for ALI and ARDS.

Taken together with the essential role of Ca^{2+} sensitization in the pathogenesis of vasospasm, our data suggest that sivelestat may be used for the prevention of these critical cardiovascular events and to maintain normal blood pressure by selective inhibition of the Ca^{2+} sensitization induced by a G protein coupled receptor agonist. The molecular mechanism(s) by which sivelestat inhibits the Ca^{2+} sensitization of VSM is not clear, and further studies are needed.

Conclusion

Our data indicate that sivelestat relaxes porcine coronary artery VSM via selective inhibition of Ca^{2+} sensitization induced by a G protein-coupled receptor agonist, without affecting the normal Ca^{2+}-induced contraction. Although the molecular mechanism(s) by which sivelestat selectively inhibits Ca^{2+} sensitization was not investigated in the present study, further investigation may provide insight into possible molecular targets for the prevention of coronary vasospastic diseases.

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