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Effects of a Novel 1,4-Benzothiazepine Derivative, K201, on Cytosolic Ca^{2+} and Contraction in Isolated Smooth Muscle of Rat Aorta

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The 1, 4-benzothiazepine derivative K201 (synonym JTV519) was newly synthesized for use as a cardioprotective agent. This study was conducted to examine the effects of K201 on rat aortic smooth muscle contraction and the mobilization of intracellular Ca^{2+}. Intracellular Ca^{2+} level was determined using a fluorescent Ca^{2+} indicator furaPE3 and $^{45}Ca^{2+}$. Norepinephrine induced transient contraction and an increase in calcium transient ($[Ca^{2+}]i$) under Ca^{2+}-free conditions in isolated rat aorta smooth muscle samples. Pretreatment with K201 at 10^{-5} M inhibited the contraction and increase of $[Ca^{2+}]i$ induced by norepinephrine at 10^{-6} M. K201 at 10^{-5} M almost completely inhibited the vascular smooth muscle contraction induced by high potassium (K^+), although approximately 20% of calcium transient remained. Addition of 10^{-5} M verapamil almost completely inhibited the resting $[Ca^{2+}]i$. $[Ca^{2+}]i$-tension relationship was examined at various doses of K201 and diltiazem in rat aorta stimulated by high K^+. Both muscle tension and $[Ca^{2+}]i$ decreased by K201 dose-dependently, greater relaxation was induced by K201 than by diltiazem at the same resting level of $[Ca^{2+}]i$. In high K^+-induced depolarization, pretreatment with K201 at 10^{-4} M inhibited $^{45}Ca^{2+}$ influx by 34%, while diltiazem at 10^{-5} M inhibited the influx by 85%. In addition to a mild α-adrenoceptor and Ca^{2+} -channel blocking activity, K201 may alter the Ca^{2+} sensitivity of intracellular contractile elements.

Key words: K201 (JTV519), Ca^{2+}-channel blocker, Ca^{2+}sensitivity, smooth muscle, furaPE3/AM

Introduction

The novel 1,4-benzothiazepine derivative, K201 (4-[3-{1-(4-benzyl) piperidinyl} propionyl]-7-methoxy-2, 3, 4, 5-tetrahydro-1, 4-benzothiazepine), was synthesized for use as a cardioprotective agent (Fig. 1).

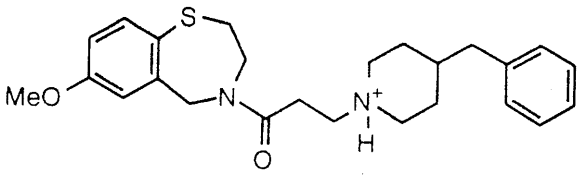

Fig. 1 Chemical structure of K201

In a rat model of myocardial injury produced by an adrenaline-caffeine load (sudden cardiac death due to myofibril overcontraction), K201 has been shown to have a significantly greater cardioprotective effect than Ca^{2+}-channel blockers (verapamil, diltiazem, and KT362), a β-adrenoceptor blocker (propranolol) and an α-adrenoceptor blocker (prazosin)[1]. K201 also has a strong cardioprotective effect against ischemia-reperfusion myocardial injury and an anti-ischemic effect. A beneficial effect of

K201 on ischemia-induced myocardial damage is attributed, at least in part, to the activation of delta isoform of protein kinase C (δ-PKC)[2]. K201 is a nonspecific blocker of sodium, potassium and calcium channels and has potential use as an antiarrhythmic[3)4)].

K201 inhibits high potassium (K^+)-and norepinephrine (NE)-induced rat aortic smooth muscle contraction at essentially the same concentrations, in contrast to the 1,5-benzothiazepine derivative, diltiazem, which is more potent in relaxing high K^+-induced than NE-induced vascular smooth muscle contraction [K201 IC_{50} : high K^+ (40 mM), $(6.5 \pm 1.5) \times 10^{-7}$ M; NE (1μM), $(1.5 \pm 0.9) \times 10^{-6}$ M vs. diltiazem IC_{50} : high K^+, $(9.1 \pm 1.4) \times 10^{-8}$ M ; NE, $(1.0 \pm 0.6) \times 10^{-5}$ M][1)]. These features of K201 are similar to those of KT362, a 1,5-benzothiazepine derivative previously shown to be an intracellular Ca^{2+} antagonist[5)].

In this study, the relationship between the K201-induced inhibition of vascular smooth muscle contraction and the decrease of cytosolic Ca^{2+} level ($[Ca^{2+}]i$) was investigated, using $^{45}Ca^{2+}$ and a fluorescent Ca^{2+} indicator furaPE3.

Materials and Methods

Male Wistar rats (Sankyo Labo-service, Tokyo, Japan) at 8 to 9 weeks of age and weighing 250 to 300 g were used. Animal care and experimentation were approved by the Animal Experimentation Ethical Committee of the Tokyo Women's Medical University.

The thoracic aorta was removed and cut into helical strips (1～2 mm wide and 7～8 mm long). The tissue strips were immersed in physiological saline solution (PSS) containing 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 0.01 mM ethylenediamine tetraacetic acid (EDTA) and 5.5 mM glucose. Endothelial cells were removed by gently rubbing the intimal surface in PSS. An equimolar high K^+ solution was prepared by substituting NaCl with KCl (69.6 mM NaCl, 72.7 mM KCl). The solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37 ℃ and maintained at pH 7.4. Muscle tension was recorded isometrically using a strain gauge transducer (Type T7-30-240, Orientec, Japan). The specimen was attached to a holder at the bottom of a 10 ml muscle bath and maintained under resting tension of 0.5 to 1.0 g for about 60 min until high K^+-induced contraction was stabilized.

Simultaneous to muscle tension recording, $[Ca^{2+}]i$ was measured in furaPE3-loaded thoracic aorta muscle sample according to the modified method of Karaki et al. The aortic muscle specimen was treated with 5μM furaPE3/AM at room temperature for 5 to 6 h and then at 4 ℃ for 8 to 12 h. Cremophor EL (0.02%), a noncytotoxic detergent, was added to increase the solubility of furaPE3/AM. The immobilized specimen was illuminated with light from a xenon high-pressure lamp (75 W) transmitted alternately through 340 nm and 380 nm interference filters mounted on a rotating filter wheel (48 Hz). The intensity of fluorescence emitted as result of excitation by the two wavelengths was monitored with a photomultiplier equipped with a 500 nm filter (CAF100, Japan Spectroscopic, Tokyo, Japan).

Ca^{2+} influx was measured using $^{45}Ca^{2+}$ according to the modified lanthanum method. Rat thoracic aortic rings were incubated in 10^{-4} M K201 or 10^{-5} M diltiazem solution for 20 min. Both solutions were then replaced with a high K^+ (80 mM) nutrient solution (36.7 mM NaCl, 80 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 4.0 mM glucose) and $^{45}Ca^{2+}$ at 37 kBq (1 mCi) /ml was added. The specimen was incubated for 10 min and washed with ice-cooled nutrient solution containing La^{3+} for 60 min. After washing, $^{45}Ca^{2+}$ in the tissue was measured with a liquid scintillation counter.

Results are expressed as mean ± SEM. Differences were considered to be statistically significant when the p values were less than 0.05 using two-way analysis of variance in combination with Dunnet's test.

The following compounds were used in this study: K201 (JT Labo, Japan), fura PE3/AM (Wako Pure Chemicals, Tokyo, Japan), cremophor EL (Nacalai Tesque, Kyoto, Japan), DMSO (Wako Pure Chemicals), verapamil (Eisai, Tokyo, Japan), diltiazem (Tanabe, Osaka, Japan), norepinephrine (Sigma Chemicals, St. Louis, MO), KCl (Sigma),

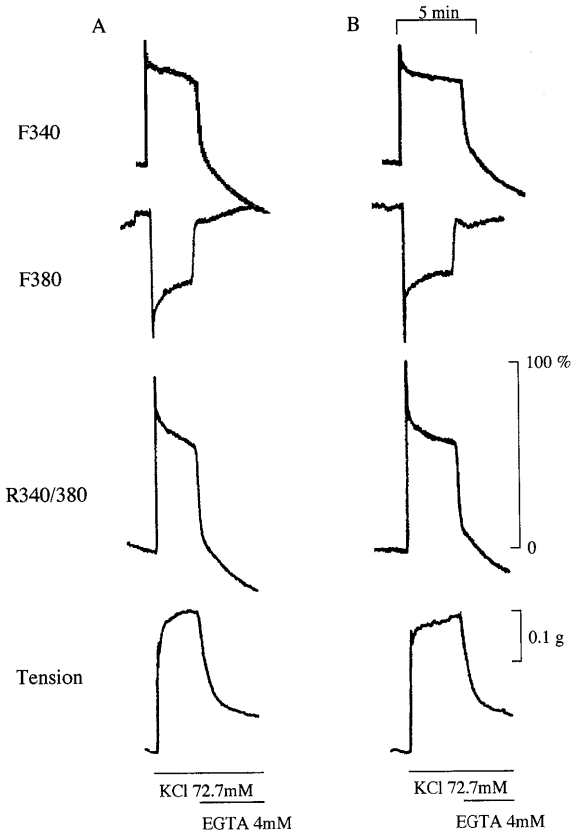

Fig. 2 Change in furaPE3-Ca^{2+} fluorescence during contraction of rat aortic muscle samples induced by high potassium (72.7 mM)
A: control, B: after 30 min, the same specimen as A.
F340: fluorescence emission (500 nm) at 340 nm excitation.
F380: fluorescence emission at 380 nm excitation.
R340/380: ratio of F340 to F380, an indicator of $[Ca^{2+}]i$; 100 % represents the maximal increase in $[Ca^{2+}]i$ induced by high potassium.
EGTA: glycoletherdiamine-N, N, N', N'-tetraacetic acid.

EDTA and glycoletherdiamine-N, N, N', N'-tetraacetic acid (EGTA) (Dojindo Labo, Kumamoto, Japan).

All compounds used in the furaPE3 experiment were dissolved in deionized water, and 10^{-4} M K201 and 10^{-5} M diltiazem used with $^{45}Ca^{2+}$ were dissolved in DMSO in each case to a final concentration of 0.03%. K201 had no direct effect on the pH of PSS or fluorescence of furaPE3.

Results

FuraPE3 leaks out of the cell more slowly than fura2, and allows a longer period of fluorescence recording. As shown in Fig. 2, a high K^+ load caused an enantiomorphic change in emissions (at 500 nm) at excitation wavelengths of 340 (F340) and 380 (F 380) nm, and increased the ratio of F340/F380 (R 340/380). Attenuation of the signal was slight even after a period of 30 min.

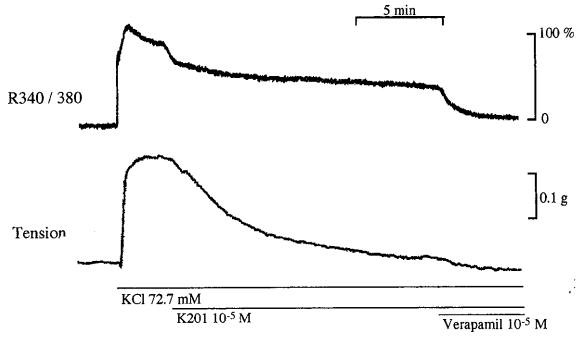

Fig. 3 Effects of sequential additions of K201 at 10^{-5} M and verapamil at 10^{-5} M on $[Ca^{2+}]i$ (upper trace) and contraction (lower trace) in rat aortic muscle stimulated by high potassium at 72.7 mM

Figure 3 shows a typical example of the relaxation of high K^+-induced contraction by K201. 10^{-5} M K201 almost completely relaxed the contraction to resting level, but K201 incompletely reversed the increase in $[Ca^{2+}]i$, and the residual $[Ca^{2+}]i$ (approximately 20%) decreased to baseline following the addition of 10^{-5} M verapamil. Figure 4 shows the relationship between relaxation of high K^+-induced contraction and change in $[Ca^{2+}]i$ by treatment with K201 or diltiazem. In K201-treated vascular smooth muscle, both muscle tension and $[Ca^{2+}]i$ decreased dose-dependently, but the change in muscle tension was greater than the change in $[Ca^{2+}]i$.

The inhibition of high K^+-induced intracellular $^{45}Ca^{2+}$ influx by K201 or diltiazem is shown in Fig. 5. K201 inhibited only 34% of $^{45}Ca^{2+}$ influx even at 10^{-4} M, a concentration that completely inhibited high K^+-induced contraction. Diltiazem inhibited 85% of the high K^+-stimulated $^{45}Ca^{2+}$ influx at a concentration of 10^{-5} M, which also totally inhibited high K^+-induced contraction.

Figure 6 shows the effect of K201 pretreatment on NE-induced contraction and change of $[Ca^{2+}]i$ under Ca^{2+}-free condition. The specimens were first loaded with high K^+. Then, K201 was added to the test samples and high K^+ was removed from the controls. Fifteen min later, 4 mM EGTA was added, followed by 10^{-6} M NE after $[Ca^{2+}]i$ had decreased to lower than the baseline. Addition of EGTA de-

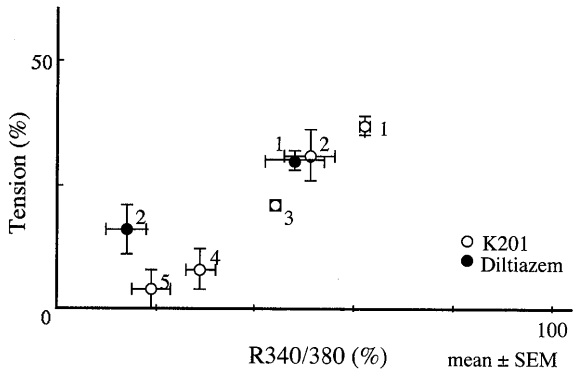

Fig. 4 Relationship between changes in high K^+-induced $[Ca^{2+}]i$ and aortic muscle contraction after treatment with K201 (○) or diltiazem (●)
The high K^+ concentration was 72.7 mM, and furaPE3 was used to measure $[Ca^{2+}]i$. 100% represents high K^+-induced contraction and increase in $[Ca^{2+}]i$ before the addition of K201 or diltiazem. Each point represents mean ± SEM of 4 or 5 experiments. ○: 1 denotes 3×10^{-7} M K201, 2 is 1×10^{-6} M, 3 is 3×10^{-6} M; 4 is 5×10^{-6} M, and 5 is 1×10^{-5} M K201. ●: 1 denotes 1×10^{-7} M diltiazem, and 2 is 1×10^{-6} M.

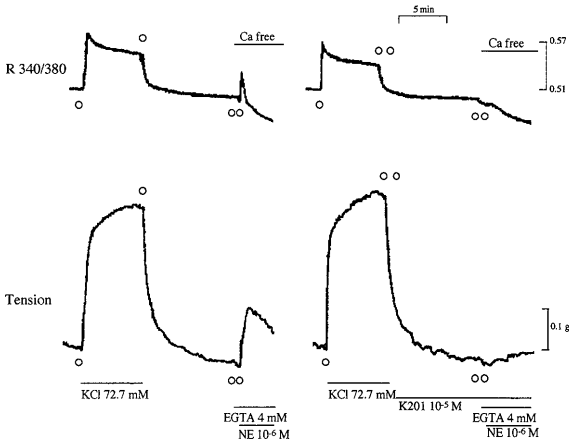

Fig. 6 Effects of pretreatment with 10^{-5} M K201 on transient increase in $[Ca^{2+}]i$ (upper trace, indicated by R340/380) and muscle tension (lower trace) induced by 10^{-6} M norepinephrine under Ca^{2+}-free condition (Ca^{2+} depleted by addition of 4 mM EGTA)
EGTA: glycoletherdiamine-N, N, N', N'-tetraacetic acid, NE: norepinephrine.

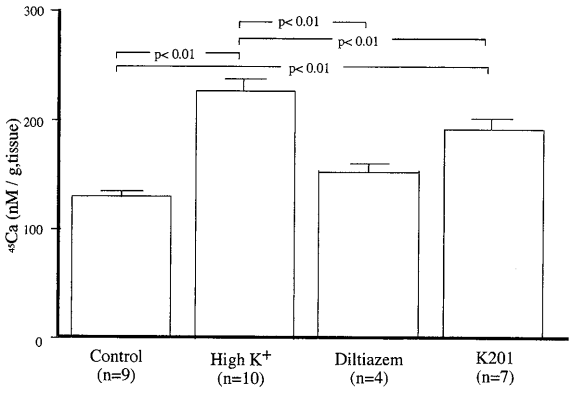

Fig. 5 Effects of K201 (10^{-4} M) and diltiazem (10^{-5} M) on $^{45}Ca^{2+}$ influx induced by high K^+
Data are mean ± SEM of 4 to 10 experiments.

creased $[Ca^{2+}]i$ to below the resting level but did not change the resting tone of the muscle. The application of NE induced only a sustained but only weak contraction and a transient increase of $[Ca^{2+}]i$ in the controls. These change could not be seen in samples pretreated with K201.

Discussion

Contractions of smooth muscle are regulated mainly by changes in $[Ca^{2+}]i$[6]. High K^+-induced smooth muscle contraction is attributed to Ca^{2+} influx through the voltage-operated Ca^{2+}-channel. The $[Ca^{2+}]i$ is proportional to the levels of myosin light chain (MLC) phosphorylation and smooth muscle contraction. High K^+-induced contraction is inhibited by Ca^{2+}-channel blockers in proportion to the decrease in $[Ca^{2+}]i$. High K^+does not increase the Ca^{2+}sensitivity of contractile elements in general. When furaPE3 was used as an indicator of $[Ca^{2+}]i$, K201 nearly completely relaxed the high K^+-induced contraction while intracellular $[Ca^{2+}]i$ was maintained. In the $^{45}Ca^{2+}$ study, K201 relaxed smooth muscle contraction sufficiently, although K201 was less effective than diltiazem in inhibiting the high K^+-induced $^{45}Ca^{2+}$ influx. Thus, K201 relaxes high K^+-induced smooth muscle contraction to a greater degree than is generally expected in spite of a residual level of Ca^{2+} in the cell.

MLC phosphorylation and Ca^{2+} sensitivity

Receptor activation increases Ca^{2+} sensitivity of contractile elements. Receptor is coupled to a Ca^{2+} mobilizing pathway to increase $[Ca^{2+}]i$ mainly by opening the voltage-operated Ca^{2+}-channels (L type) and partly by releasing Ca^{2+} from the sarcoplasmic reticulam. Receptor also is coupled to a signaling pathway that activates PKC and tyrosine kinase. PKC and tyrosine kinase inhibit MLC phosphatase and activate MLC kinase.

The myosin-phosphorylation theory is widely ac-

cepted as the mechanism by which $[Ca^{2+}]i$ activates smooth muscle contractile elements. Increase in $[Ca^{2+}]i$ activates MLC kinase, phosphorylates MLC, and induces contraction. Ca^{2+} sensitization of contractile elements may be due to modulation of the relationship among $[Ca^{2+}]i$, MLC phosphorylation and tension development. Some mechanisms are proposed to explain changes in Ca^{2+} sensitivity of myosin phosphorylation. The first mechanism is activation of Ca^{2+}-calmodulin-dependent protein kinase II by $[Ca^{2+}]i$ increase, which phosphorylates MLC kinase with consequent decrease in activity[7)8)]. The second mechanism is inhibition of MLC phosphatase. Arachidonic acid produced by receptor-mediated activation of phospholipase A_2 may directly inhibit MLC phosphatase[9)]. Receptors are coupled to a pathway that activates PKC and tyrosine kinase. PKC and tyrosine kinase may also inhibit the MLC phosphatase and augment MLC phosphorylation[10)11)]. The third mechanism is the availability of calmodulin. The concentration of the Ca^{2+}-calmodulin complex may regulate MLC kinase activity. The degree of Ca^{2+}sensitivity is determined by the balance between MLC kinase and MLC phosphatase activity[7)12)]. NE-induced Ca^{2+} sensitization in smooth muscle is explained by these mechanisms.

The NE-induced increase in Ca^{2+} sensitivity may also result from activation of actin, independent of MLC phosphorylation[13)~17)]. This actin-linked regulatory mechanism is activated by NE in the presence of a resting level of $[Ca^{2+}]i$ and may be more sensitive to Ca^{2+} than MLC kinase[17)]. Actin-binding proteins such as calponin and caldesmon have been identified in smooth muscle and proposed as possible thin filament-based regulatory proteins[18)~21)].

Recent reports have presented evidence of the involvement of the small GTPase Rho in Ca^{2+} sensitivity in smooth muscle contraction. Rho regulates MLC phosphorylation through Rho kinase and MLC phosphatase. Upregulated Rho-kinase inhibits MLC phosphatase, resulting in vascular smooth muscle hypercontraction[22)23)]. In the receptor mediated increase in Ca^{2+} sensitivity, the PKC/Rho-kinase pathway may play an important role. The relationship between PKC and Rho-kinase remains to be elucidated. Recent studies support the hypothesis that high K^+ may also increase Ca^{2+} sensitivity via Rho-kinase activation[24)].

Effects of pharmacological agents

Ca^{2+}-channel blockers, when used at concentrations effecting sufficient relaxation of high K^+-induced vascular smooth muscle contraction, generally achieve only partial relaxation of NE-induced contraction. However, Ca^{2+}-channel blockers are known to have multiple sites of action in addition to voltage-operated Ca^{2+}-channels, including ion channels, exchangers and enzymes[25)]. Diltiazem at 10^{-4} to 10^{-3} M relaxes NE-induced vascular smooth muscle contraction to resting level. Diltiazem possibly acts on the Ca^{2+} mobilizing pathway. KT362, 1, 5-benzothiazepine derivative, is considered an intracellular Ca^{2+} antagonist since it inhibits caffeine-and NE-induced intracellular Ca^{2+} release[5)]. KT362 also inhibits high K^+-and NE-induced vascular smooth muscle contraction at approximately the same concentration. Verapamil inhibits not only voltage-operated Ca^{2+}-channels but also Na^+-channels, α_1-adrenoceptors and α_2-adrenoceptors. In the rat aorta, semotiadil fumarate has been reported to inhibit transient contraction induced by NE in the absence of external Ca^{2+} and does not inhibit the late phase increase in $[Ca^{2+}]i$ induced by NE, despite significant decrease in contraction. Semotiadil may inhibit vascular smooth muscle contraction not only through blockade of the voltage-operated Ca^{2+}-channel but also by inhibition of Ca^{2+} release and Ca^{2+} sensitivity[26)]. Agents that increase cyclic AMP and cyclic GMP relax smooth muscle contracted by high $K^+$with slight inhibiton of $[Ca^{2+}]i$, suggesting that both cyclic nucleotides decrease Ca^{2+} sensitivity of contractile elements[27)]. Cytochalasines and mycalolide B (a specific inhibitor of actomyosin ATPase) putatively depolymerize actin and inhibit contraction in smooth muscle[28)]. These agents are thought to be actin inhibitors. Calyculin A (an inhibitor of phosphatases, including MLC phosphatase) and CPI 17 (PKC-potentiated inhibitor of MLC phosphatase) induce MLC phosphorylation in smooth muscle without an increase in $[Ca^{2+}]i$[29)]. Hy-

droxyfasudil and Y-27632 (specific inhibitor of Rho-kinase) inhibit MLC phosphorylation and contraction in smooth muscle[24)30)]. Trifluoperazine (a K^+-channel opener), hypoxia, adrenomedullin, insulin and volatile anesthetics have also been reported to decrease Ca^{2+} sensitivity[17)].

Various effects of K201

K201 inhibits the binding of annexin V to actin *in vitro*[1)]. Annexin V is a non-EF hand protein that binds to negatively charged (acidic) phospholipid and actin in a Ca^{2+}-dependent manner[31)]. This protein has been suggested to exhibit anticoagulant and anti-inflammatory activities by inhibiting phospholipase A_2 activity *in vitro*. Annexin V has been shown to penetrate membranes and form ion channels[32)33)]. K201 inhibits annexin-dependent Ca^{2+} influx through artificial lipid membrane[34)]. K201 binds allosterically to annexin V and inhibit annexin V dependent Ca^{2+} entry[35)]. The action of K201 may be related to unknown Ca^{2+} mobilization. Annexin V is present in rat smooth muscle, but its exact physiological functions are not yet known. The fact that K201 inhibits binding of annexin V to actin indicates its possible participation in the thin filament regulatory system. Recently, the anti-ischemic effect of K201 is known to be mediated through activation of δ-PKC, which induces ischemic preconditioning[2)]. K201 may have intracellular actions.

Conclusions

K201 relaxed high K^+-induced vascular smooth muscle contraction while maintaining $[Ca^{2+}]i$. Previous study has shown that the K201 relaxes high K^+-induced contraction at almost the same concentration necessary to relax NE-induced contraction[1)]. Thus K201 may inhibit muscle contraction by blocking L-type Ca^{2+}-channels and α-adrenoceptors.

In Ca^{2+}-free solution, K201 completely inhibited a transient $[Ca^{2+}]i$ increase and a sustained but weak muscle contraction induced by NE. These changes are not usually affected by L-type Ca^{2+}-channel blockers. Thus, K201 may inhibit receptor-mediated signaling pathways and/or Ca^{2+} release from the Ca^{2+} store. However, α-adrenoceptor blockers generally exert a weak inhibitory action on high K^+-induced contraction. Therefore K201 may be capable of altering the Ca^{2+} sensitivity of intracellular contractile elements.

These results suggest that K201 inhibits contraction of vascular smooth muscle not only through blockade of the L-type Ca^{2+}-channels and α-adrenoceptors, but also via inhibition of Ca^{2+} release and decrease of Ca^{2+} sensitivity.

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新規1,4ベンゾチアゼピン誘導体K201のラット大動脈平滑筋の細胞内Caと収縮への効果

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1,4ベンゾチアゼピン誘導体K201（JTV519）は心筋保護を目的に新規合成された．心筋ではナトリウム，カリウム，カルシウムチャンネルの阻害作用があり，抗虚血，抗不整脈作用を有する．この研究ではラット大動脈を用いて，K201の平滑筋の細胞内カルシウムと収縮への作用を検討した．細胞内カルシウム測定には蛍光指示薬furaPE3/AMと放射性カルシウム45（^{45}Ca）を用いた．カルシウム除去液では，10^{-6} Mノルエピネフリンによる収縮は持続性であるが，カルシウムトランジエント（$[Ca^{2+}]i$）は一過性であった．この収縮と$[Ca^{2+}]i$は10^{-5}M K201の前投与により消失した．72.7mM高濃度カリウムによる収縮を10^{-5} M K201は100%抑制するが，$[Ca^{2+}]i$は約20%が残存した．この残存した$[Ca^{2+}]i$は10^{-5}Mベラパミルで消失した．高濃度カリウムによる脱分極では，10^{-4}M K201の前投与による^{45}Caの流入の抑制は34%で，10^{-5} Mジルチアゼムによる抑制は85%であった．10^{-4}M K201と10^{-5}Mジルチアゼムはほぼ完全に高濃度カリウムによる収縮を抑制した．K201の血管平滑筋収縮抑制への作用機序としてCaチャンネル拮抗作用，α受容体阻害作用では十分に説明できず，Ca感受性を変える細胞内Ca拮抗作用が推測される．