

Mechanisms of Direct Inhibitory Action of Ketamine on Vascular Smooth Muscle in Mesenteric Resistance Arteries

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Background: Ketamine was previously suggested to relax vascular smooth muscle by reducing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, no direct evidence is available to indicate that ketamine reduces the $[Ca^{2+}]_i$ in vascular smooth muscle of systemic resistance arteries.

Methods: Endothelium-intact or -denuded smooth muscle strips were prepared from rat small mesenteric arteries. Isometric force and $[Ca^{2+}]_i$ were measured simultaneously in the fura-2-loaded, endothelium-denuded strips. In some experiments, only isometric force was measured in either the endothelium-intact or β -escin-treated, endothelium-denuded strips.

Results: In the endothelium-intact strips, lower concentrations ($\leq 30 \mu M$) of ketamine slightly enhanced norepinephrine-induced contraction, whereas higher concentrations ($\geq 100 \mu M$) of ketamine inhibited both norepinephrine- and KCl-induced contractions. In the fura-2-loaded strips, ketamine ($\geq 100 \mu M$) inhibited the increases in both $[Ca^{2+}]_i$ and force induced by either norepinephrine or KCl. Ketamine also inhibited the norepinephrine-induced increase in $[Ca^{2+}]_i$ after treatment with ryanodine. In the absence of extracellular Ca^{2+} , ketamine notably inhibited the norepinephrine-induced increase in $[Ca^{2+}]_i$, whereas it only minimally inhibited caffeine-induced increase in $[Ca^{2+}]_i$. Ketamine had little influence on the $[Ca^{2+}]_i$ -force relation during force development to stepwise increment of extracellular Ca^{2+} concentration during either KCl depolarization or norepinephrine stimulation. Ketamine did not affect Ca^{2+} -activated contractions in the β -escin membrane-permeabilized strips.

Conclusions: The action of ketamine on contractile response to norepinephrine consists of endothelium-dependent vasoconstricting and endothelium-independent vasodilating components. The direct vasorelaxation is largely a result of reduction of $[Ca^{2+}]_i$ in vascular smooth muscle cells. The $[Ca^{2+}]_i$ -reducing effects are caused by inhibitions of both voltage-gated Ca^{2+} influx and norepinephrine-induced Ca^{2+} release from the intracellular stores.

INDUCTION of anesthesia with ketamine is often associated with increases in cardiac output, arterial blood pressure, and heart rate.¹ It is generally believed that direct stimulation of the central nervous system leading

to increased sympathetic nervous system outflow is a primary mechanism for the cardiovascular stimulation.¹⁻³ In addition, inhibition of norepinephrine uptake into postganglionic sympathetic nerve endings, depression of baroreceptor reflex activity, and adrenocortical stimulation may also be involved.^{1,3-5}

In support of the aforementioned contention, ketamine fails to produce the stimulatory cardiovascular effects or even causes severe systemic hypotension if stimulation of the sympathetic nervous system is inhibited (e.g., during general or epidural anesthesia^{2,3,6}) or, conversely, already maximized (e.g., in critically ill conditions⁷). The systemic hypotension was associated with significant decreases in both systemic vascular resistance and cardiac index.⁷ Because clinically relevant concentrations (up to approximately $100 \mu M$ ^{8,9}) of ketamine cause both myocardial depression and vasodilation *in vitro*,¹⁰⁻¹³ the direct inhibitory actions on both myocardium and vascular smooth muscle (VSM) may contribute to the systemic hypotension.

The mechanisms for the direct vasodilating action of ketamine are not entirely clear. Earlier studies in the 1980s suggested that the ketamine-induced direct vasodilation was caused by a reduction of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSM cells.^{11,14} In support of this idea, Ratz *et al.*¹⁵ reported in 1993 that ketamine reduces the $[Ca^{2+}]_i$ in a conduit artery (i.e., femoral artery). In addition, ketamine was more recently suggested to inhibit Ca^{2+} mobilization in single VSM cells.^{16,17} However, no direct evidence is presently available to indicate that ketamine reduces $[Ca^{2+}]_i$ in systemic resistance arteries, which have been reported to be significantly different from the conduit arteries in many of their physiologic and pharmacologic properties, including Ca^{2+} -mobilization processes.¹⁸⁻²⁰ In addition, to date, the effects of ketamine on the Ca^{2+} -force relation (i.e., myofilament Ca^{2+} sensitivity) have not yet been studied in cell membrane-intact VSM cells. Thus, in this study, using isolated mesenteric resistance arteries, we evaluated the effects of ketamine on Ca^{2+} mobilization caused by either norepinephrine or KCl as well as its effects on the Ca^{2+} -force relation in fura-2-loaded, cell membrane-intact VSM cells. To further investigate the action of ketamine on myofilament Ca^{2+} sensitivity, we also evaluated the effects of ketamine on Ca^{2+} -activated contractions in β -escin-treated, membrane-permeabilized VSM cells. In addition, this study provides novel information regarding the influence of endothelium over

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the action of ketamine on contractile response to norepinephrine in the resistance arteries.

Materials and Methods

Force and Ca^{2+} Measurements

After obtaining approval from the Kyushu University Animal Care and Use Committee (Fukuoka, Japan), an endothelium-intact or -denuded VSM strip was prepared from the third-order branch of male Sprague-Dawley rat (weight, 250–350 g) mesenteric artery (approximately 150–200 μm in diameter), and isometric force was measured by attaching the strip to a strain gauge transducer as previously described.^{21,22} Briefly, the strip was horizontally mounted in a chamber attached to the stage of a microscope, and the resting tension was adjusted to obtain a maximal response to KCl. The solution was changed by infusing it into one end while aspirating simultaneously from the other end. Endothelial intactness was verified by the ability of acetylcholine (1 μM) to cause complete ($\geq 90\%$) relaxation during contractions induced by norepinephrine (10 μM). Conversely, functional removal of endothelium was verified by the inability of acetylcholine (10 μM) to cause significant ($\geq 10\%$) relaxation during contractions induced by norepinephrine (10 μM). All experiments were conducted in the presence of guanethidine (3 μM) to prevent norepinephrine outflow from the sympathetic nerve terminals.

In the first series of experiments with the endothelium-intact strips, using the previously described force recording method, only the isometric force was measured to examine the effects of ketamine on contractions induced by norepinephrine or KCl (40 mM) in the endothelium-intact strips.

In the next series of experiments with the endothelium-denuded strips, changes in the $[Ca^{2+}]_i$ were measured simultaneously with those in force, using fura-2, a fluorescent Ca^{2+} -indicator dye.²³ Our method on the fura-2 fluorometry was also previously detailed.^{24,25} Briefly, to enable loading of the fura-2 into the VSM cells, the strips were incubated in normal physiologic salt solution (PSS) containing 10 μM acetoxymethyl ester of fura-2 (fura-2-AM) and 2% albumin for approximately 2 h at approximately 35°C. After this period, the solution containing fura-2-AM was washed out with normal PSS for approximately 1 h to ensure sufficient esterification of fura-2-AM in the cells and to equilibrate the strips before the measurements.^{24,25} Changes in the fluorescence intensity of the fura-2- Ca^{2+} complex were measured by a fluorometer equipped with a dual-wavelength excitation device (CAM-230, Japan Spectroscopic, Tokyo, Japan) connected to the microscope with optical fibers. The microscope was focused on the VSM layers, and the tissue was illuminated with ultraviolet lights of the wavelengths of 340 and 380 nm alternatively limited to a frequency of 1,000 Hz. The fura-2 fluorescence

signals induced by excitation at 340 and 380 nm were collected through the 10-times objective lens (Plan Fluor, Nikon, Tokyo, Japan) and measured through a 500-nm filter with a photomultiplier. The background fluorescence (including autofluorescence of the strip) as excited by 340 and 380 nm ultraviolet light was obtained after completion of each experiment by breaking the cell membranes with Triton-X-100 (1%) and subsequently quenching the fura-2 fluorescence signals with $MnCl_2$ (20 mM) as reported previously.^{25–27} The ratio ($R_{340/380}$) of fura-2 fluorescence intensities excited by 340 nm (F_{340}) to those excited by 380 nm (F_{380}) was calculated after subtracting the background fluorescence. None of the agents used during Ca^{2+} measurements influenced the fluorescence signals. In all of the Ca^{2+} measurements, as we previously showed,²⁵ changes in F_{340} and F_{380} were constantly in opposite directions. Therefore, the observed changes in F_{340} and F_{380} would reflect changes in the $[Ca^{2+}]_i$ but not motion artifacts. All experiments with the fura-2-loaded strips were conducted during the period in which constant vascular responses were obtained, *i.e.*, for approximately 3 h.²⁵

In the final series of experiments, only isometric force was measured in the fura-2-nonloaded endothelium-denuded strips, the smooth muscle membrane of which was permeabilized with β -escin (saponin ester). To achieve the membrane-permeabilization, the smooth muscle strips were incubated with β -escin (50 μM for 25 min) at room temperature (approximately 22°C) in relaxing solution after measuring steady contractions induced by 40 mM K^+ .^{21,22} Ionomycin (0.3 μM) was present throughout the β -escin-permeabilized muscle experiments to eliminate the influence of intracellular Ca^{2+} stores.

As previously reported,^{21,22} to prevent early deterioration of the thin vascular strips, the aforementioned experiments with membrane-intact and β -escin membrane-permeabilized strips were conducted at 35°C and room temperature (approximately 22°C), respectively.

Solutions and Drugs

The ionic concentrations of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-buffered PSS were as follows: 138 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose. The pH was adjusted with NaOH to 7.35 at 35°C. The high K^+ (40 mM) solutions were prepared by replacing NaCl with KCl isoosmotically. The Ca^{2+} -free solution was prepared by removing $CaCl_2$ and adding 2 mM EGTA.

The compositions of relaxing or activating solutions used in the β -escin-permeabilized muscle experiments were determined by solving multiequilibrium equations using a hydrogen ion activity coefficient of 0.75 and association constants for the various ions as detailed previously.²² The composition of the relaxing solution was 80 mM potassium methanesulfonate, 20 mM PIPES,

7 mM Mg(MS)₂, 5 mM adenosine 5'-triphosphate, 10 mM creatinine phosphate, and 4 mM EGTA. The 4 mM EGTA-containing activating solution was prepared by adding a specific amount of Ca(MS)₂ to obtain the desired concentration of free Ca²⁺ ions based on the calculations previously reported.²² The pH was adjusted with KOH to 7.00 at 22°C, and the ionic strength was kept constant at 0.2 M by adjusting the concentration of potassium methanesulfonate. Guanosine 5'-triphosphate (50 μM) was present throughout the experimental periods to minimize rundown of contractile responses in the β-escin-permeabilized strips.²⁸

Ketamine, adenosine 5'-triphosphate, creatinine phosphate, guanosine 5'-triphosphate, HEPES, ionomycin, β-escin, norepinephrine, acetylcholine, guanethidine, and albumin (bovine) were obtained from Sigma Chemical Co. (St. Louis, MO). EGTA, PIPES-K₂, and methanesulfonic acid were obtained from Fluka Chemie AG (Buchs, Switzerland). Fura-2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Ryanodine was purchased from Agri Systems International (Wind Gap, PA). All other reagents were of the highest grade commercially available.

Experimental Design

The effects of ketamine were examined in both fura-2-nonloaded and -loaded strips, in the latter of which isometric force and [Ca²⁺]_i were simultaneously measured.

In the first series of experiments, the effects of ketamine (0.001~1 mM) were examined on contractile responses to either norepinephrine (10 μM) or KCl (40 mM) in the fura-2-nonloaded, endothelium-intact strips. In this experiment, ketamine was applied to the strip precontracted with either norepinephrine or KCl after contractile response to either stimulant reached a plateau.

In the next series of experiments, we examined the effects of ketamine (0.001~3 mM) on increases in [Ca²⁺]_i and force caused by norepinephrine (10 μM) or KCl (40 mM) in the fura-2-loaded, endothelium-denuded strips, using protocols identical to those used in the aforementioned experiments. In other words, ketamine was applied to the strips precontracted with either norepinephrine or 40 mM K⁺ after vascular response (both [Ca²⁺]_i and force) to either stimulant reached a plateau. In some of these experiments, ketamine (0.1~1 mM) was applied before and during application of either norepinephrine or KCl. Specifically, each stimulant (norepinephrine or high K⁺) was applied for 3 min at 7-min intervals so as to obtain reproducible responses, and then ketamine was applied for 5 min before and during the subsequent applications of either stimulant.

In the following several series of experiments, we attempted to clarify mechanisms of direct inhibitory action of ketamine on contractile response to norepineph-

rine observed in the aforementioned control experiments with the endothelium-denuded strips. The concentrations of ketamine used in these experiments were 0.3~3 mM, concentrations at which ketamine distinctly inhibited the norepinephrine response in these experiments. The obtained IC₅₀ values for the inhibitory actions of ketamine on norepinephrine response were approximately 0.3 mM, whereas concentrations of ketamine that almost completely inhibited the norepinephrine response were 1 or 3 mM. Because the clinically relevant concentrations (≤ 100 μM) of ketamine did not consistently inhibit the norepinephrine response in the aforementioned experiments, they were not used in these experiments. However, because intracellular concentrations of ketamine are presumed to be lower than its extracellular concentrations, the concentrations of ketamine used in β-escin membrane-permeabilized muscle experiments were 0.1~1.0 mM, lower than those used in other series of experiments to investigate the mechanism of the direct vasodilating action.

To investigate mechanisms of the observed [Ca²⁺]_i-reducing effect of ketamine in the presence of norepinephrine, we examined the effects of ketamine on the increases in [Ca²⁺]_i and force caused by norepinephrine after either treatment with ryanodine (10 μM, 20 min) or removal of extracellular Ca²⁺ (*i.e.*, in the 2 mM EGTA-containing Ca²⁺-free solution) in the endothelium-denuded strips. We recently showed that the intracellular Ca²⁺ stores (*i.e.*, sarcoplasmic reticulum) can be depleted by treatment with ryanodine in this artery.²⁹ Therefore, the effect of ketamine on the norepinephrine-induced increase in [Ca²⁺]_i observed after the treatment with ryanodine would reflect its effect on the norepinephrine-induced Ca²⁺ influx. In contrast, the effect of ketamine on the norepinephrine-induced increase in [Ca²⁺]_i observed in the Ca²⁺-free solution would reflect its effect on the norepinephrine-induced, presumably inositol 1,4,5-triphosphate (IP₃)-induced,³⁰ Ca²⁺ release from sarcoplasmic reticulum.

In VSM, Ca²⁺ is stored intracellularly in sarcoplasmic reticulum, which contains at least two types of Ca²⁺-release channels, *i.e.*, IP₃ receptor-Ca²⁺-release channel and ryanodine receptor-Ca²⁺-release channel.^{31,32} The IP₃-receptor channel is believed to play a primary physiologic role in Ca²⁺ mobilization.³³ The Ca²⁺-induced Ca²⁺ release may occur through the ryanodine receptor channel, although its physiologic role is not entirely clear.^{31,32,34} In this study, we also examined the effects of ketamine (0.3~3 mM) on increases in [Ca²⁺]_i and force caused by caffeine, which directly activates the Ca²⁺-induced Ca²⁺ release mechanism,³¹ in the Ca²⁺-free solution.

To investigate the effects of ketamine on myofilament Ca²⁺ sensitivity, we examined the effects of ketamine (0.3~1 mM) on increases in [Ca²⁺]_i and force evoked by stepwise incremental increases in the extracellular Ca²⁺

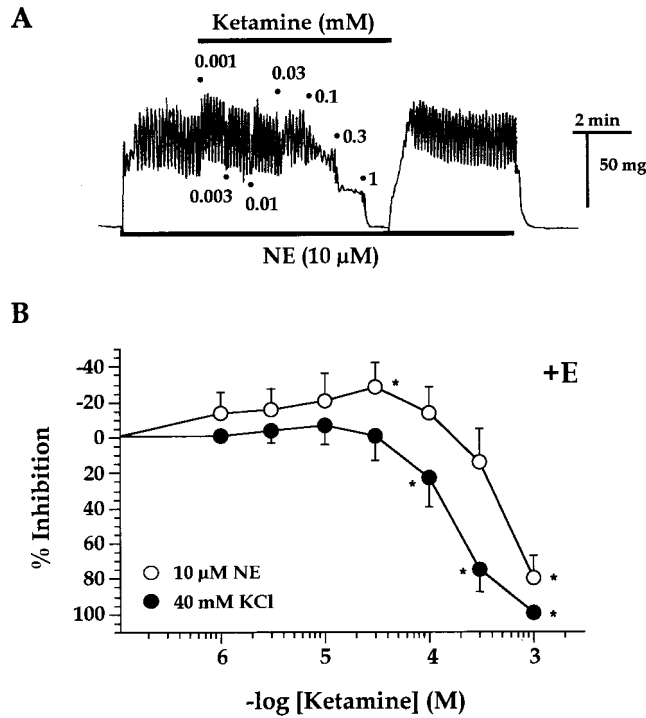


Fig. 1. Effects of ketamine (0.001–1 mM) on norepinephrine (NE; 10 μM)- or KCl (40 mM)-induced contractions in the endothelium-intact (+E), fura-2–nonloaded strips. (A) An example of the effect of ketamine on norepinephrine contraction. (B) Analyzed data. The open and closed circles indicate the data on norepinephrine and KCl contractions, respectively. n = 6. *P < 0.05 versus control (100%) within each group.

concentrations ($[Ca^{2+}]_e$) from 0 to 5 mM during 40 mM K^+ depolarization or those evoked by stepwise incremental increases in the $[Ca^{2+}]_e$ from 0 to 1.5 mM during norepinephrine (10 μM) stimulation without the K^+ -depolarization in the fura-2-loaded strips. In our recent experiments with this artery,²⁵ the stepwise incremental increases in the $[Ca^{2+}]_e$ from 0 to 10 mM during the norepinephrine stimulation in the presence of the K^+ depolarization constantly produced concentration-dependent increases in both $[Ca^{2+}]_i$ and force. However, the increases in the $[Ca^{2+}]_e$ greater than 1.5 mM during the norepinephrine stimulation in the “absence” of the K^+ depolarization, unlike in its presence, often produced somewhat chaotic and sudden decreases in both $[Ca^{2+}]_i$ and force. We therefore did not increase the $[Ca^{2+}]_e$ greater than 1.5 mM in this protocol. Because evidence is accumulating that changes in membrane potential significantly influence the myofilament Ca^{2+} sensitivity or activity of membrane-associated enzymes,^{27,35–37} we decided to examine the action of ketamine on the myofilament Ca^{2+} sensitivity during the norepinephrine stimulation in the “absence” of the K^+ depolarization in this study. In this series of experiments, ketamine was applied to the strip for 5 min before and during subsequent applications of Ca^{2+} .

Finally, we evaluated the effects of ketamine on Ca^{2+} (3 and 30 μM)-activated contractions in the β-escin-

permeabilized, fura-2–nonloaded strips in the absence of norepinephrine; α-adrenergic receptor-coupling was not retained in the β-escin-permeabilized strips prepared from this artery, as previously reported.^{28,38} In this experiment, ketamine (0.1–1 mM) was applied to the strips precontracted with Ca^{2+} after the response to Ca^{2+} reached a plateau.

Calculation and Data Analysis

Although absolute values of $[Ca^{2+}]_i$ have been calculated based on the fura-2 fluorescence ratio and the dissociation constant of fura-2 for Ca^{2+} binding obtained

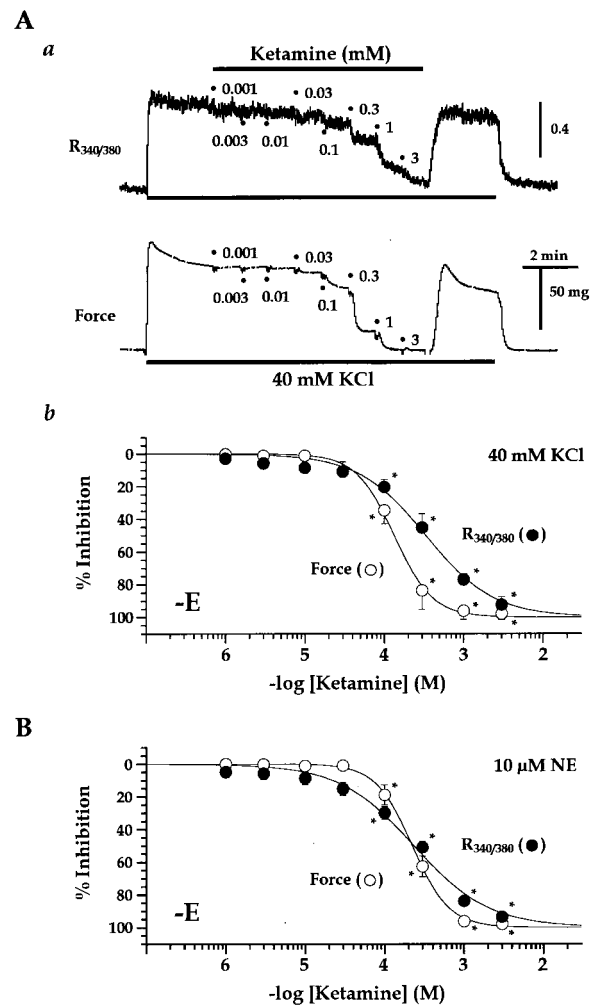


Fig. 2. Effects of ketamine on vascular responses to either KCl or norepinephrine (NE) in the endothelium-denuded (–E), fura-2–loaded strips. (A) Effects of ketamine (0.001–3 mM) on the KCl (40 mM)-induced increases in $R_{340/380}$ ($[Ca^{2+}]_i$) and force. (a) Typical example; (b) the analyzed data (n = 7). The closed and open circles indicate the data on $R_{340/380}$ and force, respectively. The IC_{50} values for the ketamine-induced inhibitions of the increases in $R_{340/380}$ and force caused by KCl are 0.33 and 0.13 mM, respectively. *P < 0.05 versus control (100%) within each group. (B) Effects of ketamine (0.001–3 mM) on increases in $[Ca^{2+}]_i$ ($R_{340/380}$; closed circles) and force (open circles) caused by norepinephrine. The IC_{50} values for the ketamine-induced inhibitions of the increases in $R_{340/380}$ and force caused by norepinephrine are 0.23 and 0.22 mM, respectively. n = 6. *P < 0.05 versus control (100%) within each group.

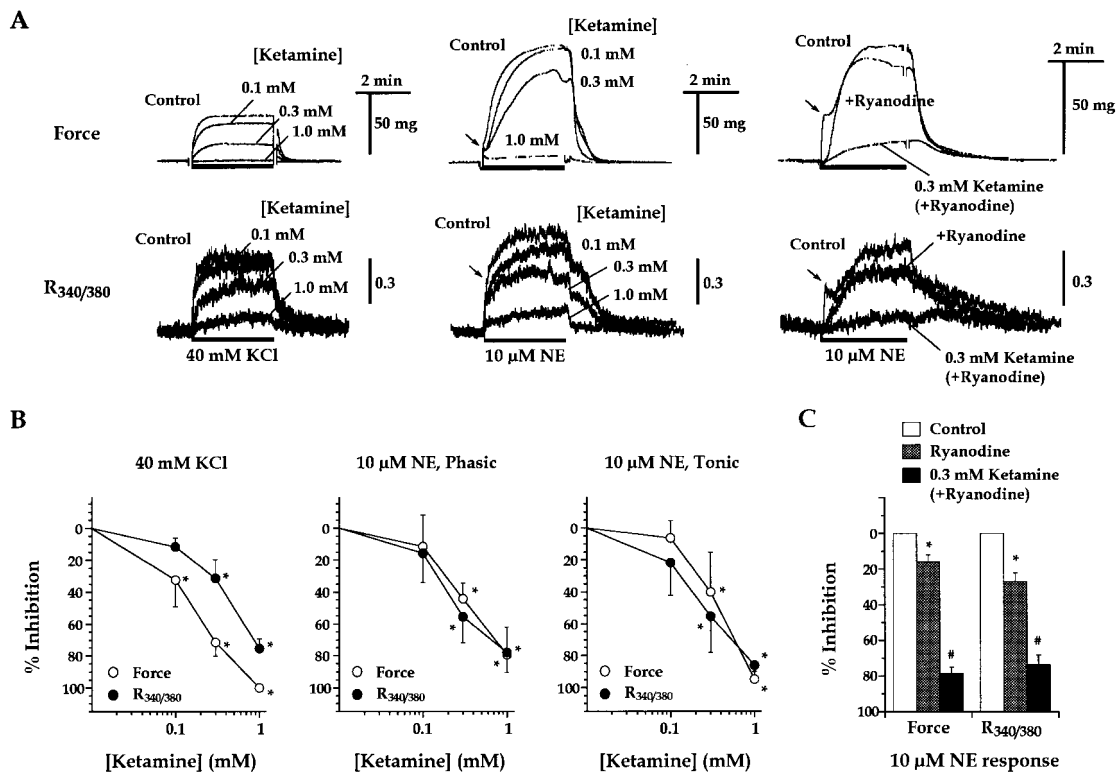


Fig. 3. Effects of ketamine (0.1–1 mM), applied before application of either KCl or norepinephrine (NE), on increases in $[Ca^{2+}]_i$ ($R_{340/380}$) and force caused by either KCl (40 mM) or norepinephrine (10 μ M) in the endothelium-denuded, fura-2-loaded strips. (A) Typical examples of the effects of ketamine on the KCl-induced (left) or norepinephrine-induced (middle, right) induced increases in $R_{340/380}$ and force in either the absence or presence of ryanodine. Arrows indicate the phasic responses to norepinephrine. (B) Analyzed data on the effects of ketamine on increases in $R_{340/380}$ (closed circles) and force (open circles) caused by either KCl or norepinephrine. (C) Analyzed data on the effects of ryanodine on the effects of ketamine on the norepinephrine responses. $n = 4$ or 5. * $P < 0.05$ versus control (100%) within each group.

in vitro,²³ the dissociation constant of fura-2 for Ca^{2+} binding in cytoplasm appears to be significantly different from that measured in the absence of protein because more than half of the fura-2 molecules in cytoplasm are protein-bound.³⁹ Therefore, $R_{340/380}$, calculated after subtracting the background fluorescence, was used as an indicator of $[Ca^{2+}]_i$, as described previously.^{24–27} The concentration–response data for the ketamine-induced inhibitions of the increases in either force or $R_{340/380}$ caused by norepinephrine or KCl were fitted according to a logistic model described by De Lean *et al.*,⁴⁰ and the IC_{50} values were derived from the least-squares fit using the aforementioned model.

Changes in the $R_{340/380}$ and force were expressed as the percent value of the reference. The basal values in normal PSS were assumed to be 0% in all experiments. In experiments where ketamine was applied to the strips precontracted with KCl or norepinephrine, the values immediately before application of ketamine were assumed to be 100%. For the analysis of the effects of ketamine on norepinephrine-induced oscillatory contraction in the endothelium-intact strips, the magnitude of the norepinephrine contraction was defined as the amplitude from the zero line (resting tension level before application of norepinephrine) to the middle of the

oscillations, excluding any erratic peaks. In experiments where the strips were pretreated with ketamine, the maximum values of control (preanesthetic) responses to either norepinephrine or KCl were assumed as 100%. Because the responses to norepinephrine consisted of two distinct components, *i.e.*, an initial phasic and a subsequent tonic component, the effects of ketamine, applied before application of norepinephrine, were assessed on both the phasic and tonic responses to norepinephrine. However, as the phasic response was not necessarily distinct in the response to KCl, the effects of ketamine on the KCl response were evaluated only on the tonic response (*i.e.*, 3 min after application of KCl).

Statistics

All results are expressed as the mean \pm SD. The n denotes the number of preparations. Data were analyzed using analysis of variance, Scheffé F test, and Student t test. Comparisons among groups were performed by two-factor analysis of variance for repeated measures. When overall differences were detected, individual comparisons among groups at each concentration were performed by the Scheffé F test. Comparisons within each group were made by one-factor analysis of variance for repeated measures, and *post hoc* comparisons were

made using the Scheffé F test for multiple comparisons. All other necessary comparisons between two groups were made by the Student *t* test. $P < 0.05$ was considered significant.

Results

Effects of Ketamine on Norepinephrine- or KCl-induced Contraction in the Presence of Endothelium

In the endothelium-intact strips, lower concentrations of ketamine ($\leq 30 \mu\text{M}$) slightly enhanced the norepinephrine ($10 \mu\text{M}$)-induced contraction, whereas a higher concentration of ketamine (1 mM) inhibited the norepinephrine contraction (fig. 1). Higher concentrations ($\geq 30 \mu\text{M}$) of ketamine consistently inhibited rhythmic oscillations observed in the contractile response to norepinephrine (fig. 1). The enhancement of the norepinephrine contraction caused by $30 \mu\text{M}$ ketamine and the inhibition of the norepinephrine contraction caused by 1 mM ketamine were both statistically significant ($P < 0.05$, $n = 6$; fig. 1). In contrast, in the endothelium-intact strips, the low concentrations ($\leq 30 \mu\text{M}$) of ketamine little influenced the KCl-induced contraction, whereas the higher concentrations ($\geq 100 \mu\text{M}$) of ketamine inhibited the KCl contraction ($P < 0.05$, $n = 6$; fig. 1).

Effects of Ketamine on Increases in $R_{340/380}$ and Force Induced by Norepinephrine or KCl in the Fura-2-loaded, Endothelium-denuded Strips

Ketamine ($\geq 100 \mu\text{M}$), applied to the strips precontracted with either KCl or norepinephrine, inhibited the increases in both $R_{340/380}$ and force caused by either KCl or norepinephrine ($P < 0.05$, $n = 6-7$; fig 2). In these experiments, 1 mM ketamine produced complete vasorelaxation ($P > 0.05$ vs. 0%, $n = 6-7$) in the presence of either KCl or norepinephrine without completely inhibiting the increases in $R_{340/380}$ caused by either stimulant, *i.e.*, significant increases in $R_{340/380}$ were still observed after treatment with 1 mM ketamine in the presence of either KCl or norepinephrine ($P < 0.05$, $n = 6-7$; fig 2). Ketamine (0.1~1 mM), applied before application of either KCl or norepinephrine, also significantly inhibited the increases in $R_{340/380}$ and force caused by either KCl or norepinephrine, in a concentration-dependent manner ($P < 0.05$, $n = 5$; fig. 3). Both the phasic and tonic increases in either $R_{340/380}$ or force caused by norepinephrine were inhibited by ketamine (0.3~1 mM; $P < 0.05$; $n = 5$; fig. 3). Also in these experiments, 1 mM ketamine eliminated the contractile response to either norepinephrine or KCl ($P > 0.05$ vs. 0%, $n = 5$) without completely inhibiting the increases in $R_{340/380}$ caused by either stimulant; significant increases in $R_{340/380}$ were still observed after exposure to 1 mM ketamine in the norepinephrine- or KCl-stimulated strips ($P < 0.05$, $n = 5$; fig. 3).

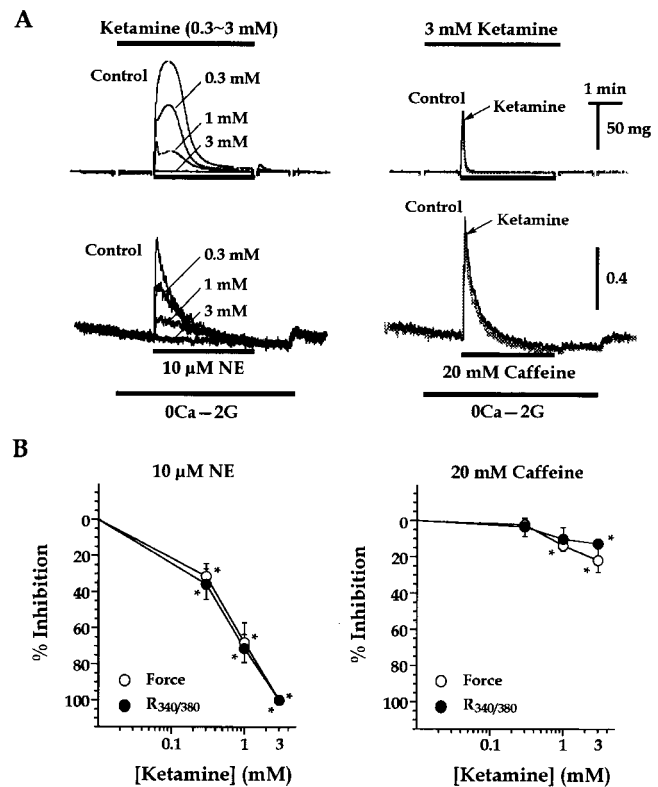


Fig. 4. Effects of ketamine (0.3~3 mM) on norepinephrine (NE)-induced ($10 \mu\text{M}$) or caffeine-induced (20 mM) increases in $R_{340/380}$ and force in 2 mM EGTA-containing Ca^{2+} -free solution (0Ca-2G). (A) Typical examples. Arrows indicate the peak responses to caffeine in the presence of ketamine. (B) Analyzed data. The closed and open circles indicate the data on $R_{340/380}$ and force, respectively. $n = 4$. * $P < 0.05$ versus control (100%) within each group.

Both the phasic and tonic increases in $R_{340/380}$ and force induced by norepinephrine were significantly inhibited by ryanodine ($P < 0.05$; $n = 4$; fig. 3). In particular, the phasic responses to norepinephrine were consistently eliminated by the ryanodine treatment ($P < 0.05$; $n = 4$; fig. 3). Ketamine still inhibited the norepinephrine-induced increases in $R_{340/380}$ and force after the treatment with ryanodine ($P < 0.05$, $n = 4$). The norepinephrine ($10 \mu\text{M}$)-induced transient increases in $R_{340/380}$ and force in the Ca^{2+} -free solution was notably inhibited by ketamine, whereas the caffeine-induced transient increases in $R_{340/380}$ and force in the Ca^{2+} -free solution was only minimally inhibited by ketamine ($P < 0.05$; $n = 4$; fig. 4).

In the fura-2-loaded, K^+ (40 mM) membrane-depolarized or norepinephrine ($10 \mu\text{M}$)-stimulated strips, as shown in figures 5 and 6, the stepwise increment of $[\text{Ca}^{2+}]_i$ produced concentration-dependent increases in both $R_{340/380}$ and force. The increases in both $R_{340/380}$ and force induced by the stepwise increment of $[\text{Ca}^{2+}]_i$ during the K^+ depolarization were both strongly inhibited by 0.3 mM ketamine ($P < 0.05$; $n = 4$; fig. 5) and totally eliminated by 1 mM ketamine ($n = 4$; not shown). The increases in both $R_{340/380}$ and force induced by the

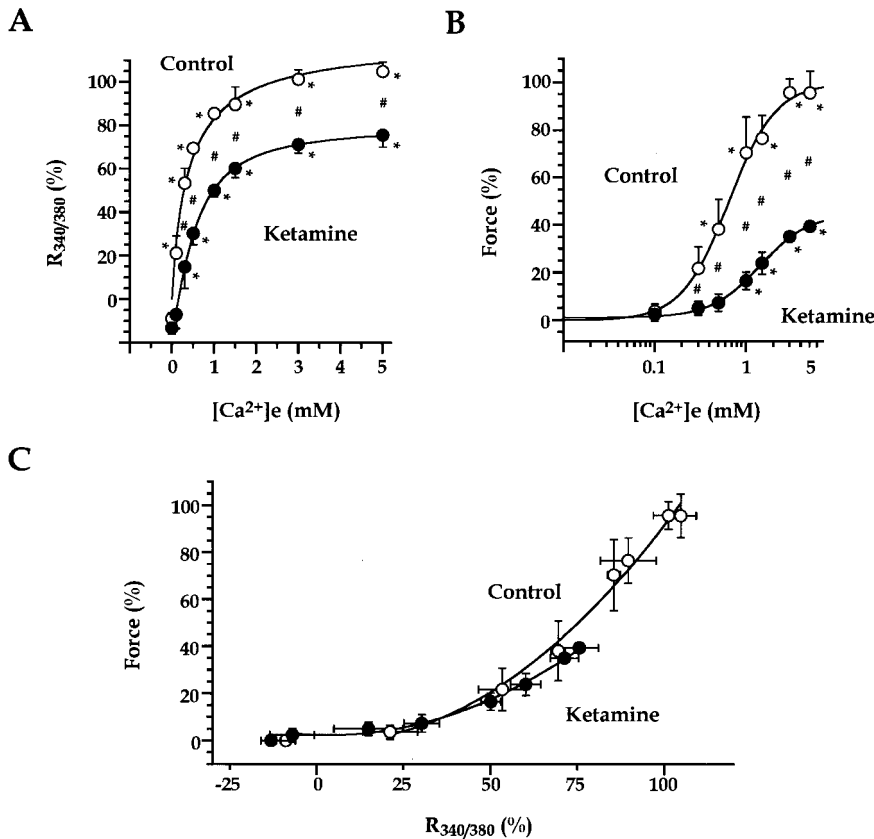


Fig. 5. Effects of ketamine (0.3 mM) on increases in $R_{340/380}$ (A) and force (B) caused by incremental increases in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) during KCl (40 mM) depolarization in the endothelium-denuded, fura-2-loaded strips in the presence of guanethidine. After recording the control responses to KCl (40 mM), the strips were treated with 0.3 mM ketamine for 5 min before and during subsequent cumulative application of various concentrations of Ca^{2+} during the depolarization. In the analyses, the maximal increase in either $R_{340/380}$ or force induced by KCl before exposure to ketamine was assumed to be 100%, whereas basal values in normal physiologic salt solution were assumed to be 0%. $n = 4$. * $P < 0.05$ versus control (100%) within each group. # $P < 0.05$ versus control at each Ca^{2+} concentration. (C) Effects of ketamine on the $R_{340/380}$ -force relation. The $R_{340/380}$ -force relation either in the absence or in the presence of ketamine was constructed from the data shown in (A) and (B).

stepwise increment of $[Ca^{2+}]_e$ during the norepinephrine stimulation were both strongly inhibited by 0.3 and 1 mM ketamine ($P < 0.05$; $n = 4$; fig. 6). Ketamine (0.3 mM) had little influence on the $R_{340/380}$ -force relation during force development to stepwise increment of extracellular Ca^{2+} concentration in the presence of either KCl or norepinephrine (figs. 5 and 6).

Effects of Ketamine on Ca^{2+} -activated Contraction in β -escin Membrane-Permeabilized Muscle

Ketamine (0.1–1 mM) did not significantly influence the Ca^{2+} (3 and 30 μ M)-activated contractions in the β -escin membrane-permeabilized, ionomycin-treated strips ($n = 4$; fig. 7).

Discussion

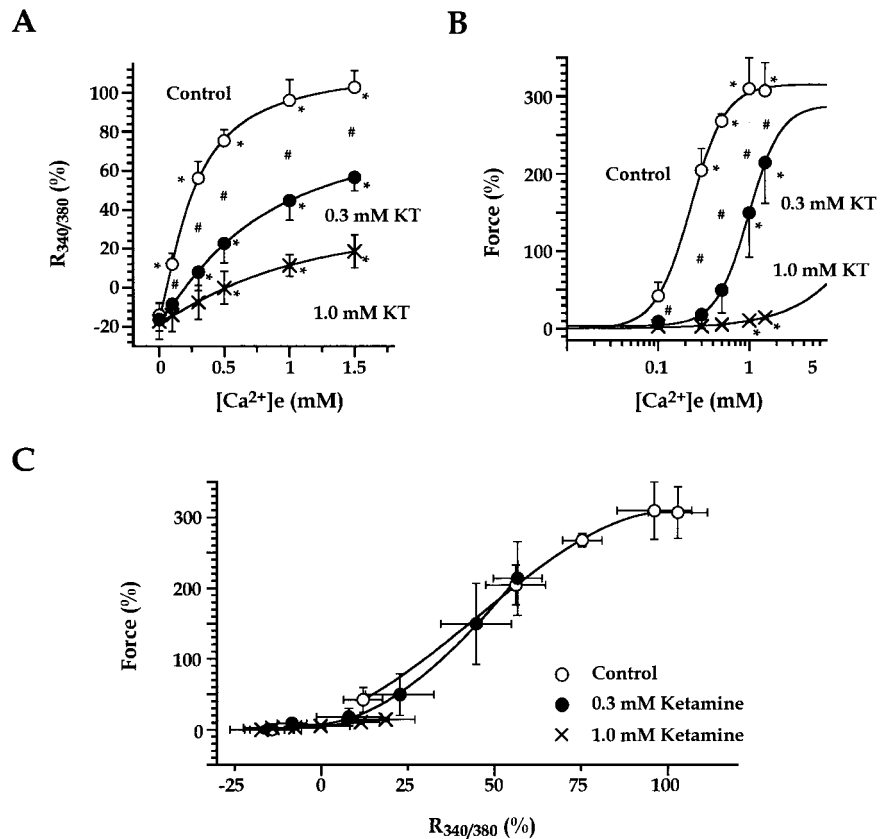
Endothelium-mediated Action of Ketamine on Vascular Smooth Muscle

In previous experiments using the isolated systemic resistance arteries, the effects of ketamine were investigated in the absence of endothelium at low temperature (25°C).^{14,30} In addition, the endothelial intactness or presence was unknown in previous studies.^{10,11,41} Therefore, the current study, for the first time, describes the action of ketamine on endothelium-intact systemic resistance arteries at near-physiologic temperature. A clinically relevant concentration (30 μ M) of ketamine

modestly although significantly enhanced the contractile response to norepinephrine (the sympathetic neurotransmitter) in the presence of endothelium, whereas it did not influence the contractile response to norepinephrine in its absence. In addition, the contractile response to norepinephrine was inhibited by 100 and 300 μ M ketamine only in the absence of endothelium, but not in its presence. These results indicate that the action of ketamine on contractile response to norepinephrine consists of endothelium-dependent vasoconstricting and endothelium-independent vasodilating components. The former action, caused by the clinical concentrations (up to approximately 100 μ M^{8,9}) of ketamine, may contribute slightly to the increase in systemic vascular resistance or arterial pressure associated with intravenous administration of ketamine. In addition, the direct vasodilating action of ketamine (≥ 100 μ M) might possibly contribute to its hypotensive effects observed in the critically ill patients,⁷ in whom the endothelial integrity is presumed to be disturbed by enhanced plasma concentrations of either cytokines⁴² or endotoxin.⁴³

Ketamine was previously suggested to inhibit nitric oxide-signaling pathway in VSM of rat aorta.⁴⁴ We recently showed that the nitric oxide-signaling pathway is involved in the contractile response to norepinephrine in this resistance artery.⁴⁵ Therefore, if ketamine inhibited the nitric oxide pathway in this resistance artery as

Fig. 6. Effects of ketamine (KT; 0.3 and 1 mM) on increases in $R_{340/380}$ (A) and force (B) caused by incremental increases in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) during stimulation with norepinephrine in the endothelium-denuded, fura-2-loaded strips. After recording the control responses to KCl (40 mM), the strips were treated with ketamine for 5 min before and during subsequent cumulative application of various concentrations of Ca^{2+} in the presence of 10 μ M norepinephrine. In the analyses, the maximal increase in either $R_{340/380}$ or force induced by KCl before exposure to ketamine was assumed to be 100%, whereas basal values in normal physiologic salt solution were assumed to be 0%. $n = 4$. KT = ketamine. * $P < 0.05$ versus control (100%) within each group. # $P < 0.05$ versus control at each Ca^{2+} concentration. (C) Effects of ketamine on the $R_{340/380}$ -force relation in the presence of norepinephrine. The $R_{340/380}$ -force relation either in the absence or in the presence of ketamine was constructed from the data shown in (A) and (B).



previously observed in the aorta,⁴⁴ ketamine might possibly enhance the contractile response to norepinephrine by inhibiting the nitric oxide-signaling pathway. As the endothelial function is globally inhibited during the KCl (40 mM) depolarization because of a lack of hyperpolarization caused by K^+ channel opening and a resultant reduced driving force for transmembrane Ca^{2+} influx into endothelial cells, ketamine might fail to enhance the KCl response in the presence of endothelium. Alternatively, endothelium-derived hyperpolarizing factor, the action of which is eliminated by the KCl depolarization, may be involved in the endothelium-dependent vasoconstricting action; the endothelium-derived hyperpolarizing factor-signaling pathway is also involved in the contractile response to norepinephrine in this artery.⁴⁵ Further investigations are necessary to clarify the mechanisms of the observed endothelium-dependent vasoconstricting action.

Rhythmic oscillations were observed in the contractile response to norepinephrine, as recently reported in this artery (fig. 1).⁴⁵ The oscillatory contractile activity, often referred to as vasomotion and regarded as a feature of small vessels, may play an important physiologic role in regulating vascular resistance without disturbing tissue perfusion or oxygen delivery to tissue, or in maintaining normal tissue fluid balance through reduction of net fluid filtration into tissue.^{46,47} The norepinephrine-induced oscillations were inhibited by ketamine (fig. 1),

suggesting that ketamine may interfere with vascular homeostasis such as fine regulation of blood flow or vascular permeability.

Mechanisms of Direct Inhibitory Action of Ketamine on Vascular Smooth Muscle

Using fura-2 fluorometry, the current study, for the first time, provides direct evidence indicating that ketamine reduces $[Ca^{2+}]_i$ of VSM cells in systemic resistance arteries. Ketamine inhibited the KCl-induced increases in $R_{340/380}$. In addition, ketamine inhibited the norepinephrine-induced increases in $R_{340/380}$ after depletion of the intracellular Ca^{2+} stores with ryanodine. Because both KCl- and norepinephrine-induced Ca^{2+} influxes are mediated exclusively by activation of L-type voltage-dependent Ca^{2+} channels in this artery,²⁵ these results indicate that ketamine inhibits the transmembrane Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels. This is consistent with a previous study in which ketamine ($\geq 100 \mu$ M) inhibited whole cell L-type voltage-dependent Ca^{2+} currents in VSM cells.⁴⁸ However, the exact mechanism by which ketamine inhibits the L-type voltage-dependent Ca^{2+} channel activity is currently unknown.

Norepinephrine produced an initial phasic and a subsequent tonic increase in $R_{340/380}$ in the normal PSS containing 1.5 mM Ca^{2+} , whereas it produced only the phasic increase in $R_{340/380}$ in the absence of extracellular Ca^{2+} (*i.e.*, in the Ca^{2+} -free solution). In contrast, norepi-

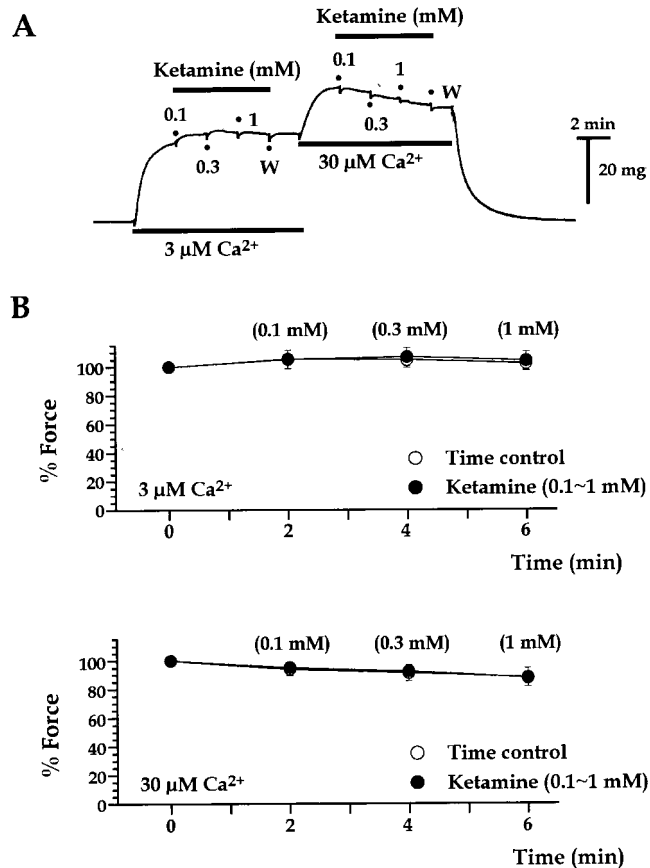


Fig. 7. Effects of ketamine (0.1–1 mM) on the Ca²⁺ (3 and 30 μM)-activated contractions in the endothelium-denuded, fura-2–nonloaded strips. Each concentration of ketamine was applied for 2 min to the strips precontracted with Ca²⁺. (A) A typical example. (B) Analyzed data. The changes in force during application of ketamine (closed circles) were compared with the time control data (open circles). 0 min = time point when ketamine was applied. No significant differences were observed in the time-dependent changes in force during application of either 3 or 30 μM Ca²⁺ between the control and ketamine-treated strips ($P > 0.05$, $n = 4$).

nephrine failed to produce the phasic increase in $R_{340/380}$ after depletion of the intracellular Ca²⁺ stores with ryanodine, and its tonic increase in $R_{340/380}$ was attenuated after the ryanodine treatment. These results indicate that the norepinephrine-induced phasic response is caused by Ca²⁺ release from the intracellular stores, whereas its tonic response is from an interplay between the transmembrane Ca²⁺ influx and the Ca²⁺ release from intracellular stores. High concentrations (≥ 300 μM) of ketamine inhibited the phasic increase in $R_{340/380}$ both in the normal PSS and the EGTA-containing Ca²⁺-free solution, indicating that ketamine inhibits the norepinephrine-induced, presumably IP₃-induced,³⁰ Ca²⁺ release from intracellular stores. Previous studies have shown that ketamine does not influence the Ca²⁺ release evoked by exogenously applied IP₃ during the membrane-permeabilized condition, while it inhibits the norepinephrine-induced IP₃ production or phenylephrine-induced inositol phosphate production.^{14,15,30}

Therefore, ketamine inhibits the norepinephrine-induced Ca²⁺ release from the intracellular stores, presumably by acting on the plasma membrane and thereby interfering with some steps between receptor occupancy and phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (*e.g.*, activation of either α -adrenergic receptor, heterotrimeric G protein, or phospholipase C).

Caffeine is believed to directly activate the Ca²⁺-induced Ca²⁺ release mechanism and thereby cause Ca²⁺ release from the intracellular stores in VSM.³¹ However, ketamine even at higher concentrations (1–3 mM) only minimally inhibited the caffeine-induced increase in $R_{340/380}$ in the Ca²⁺-free solution, suggesting that ketamine has little influence on the Ca²⁺-induced Ca²⁺ release from the intracellular stores. This observation is consistent with a previous finding in isolated rabbit mesenteric arteries.¹⁴

Ketamine (1 mM) was previously shown to have little influence on the Ca²⁺-force relation in saponin membrane-permeabilized VSM cells, suggesting that ketamine does not have an effect on Ca²⁺-induced activation of contractile proteins (*i.e.*, basal myofilament Ca²⁺ sensitivity in the absence of receptor stimulation).¹⁴ However, if the action of ketamine on the myofilament Ca²⁺ sensitivity were mediated by the intact cell membrane or some diffusible cytosolic factors that are lost in the membrane-permeabilized VSM cells, ketamine would fail to influence the myofilament Ca²⁺ sensitivity during the membrane-permeabilized condition. Indeed, we recently observed a significant discrepancy in an anesthetic effect on the myofilament Ca²⁺ sensitivity between the membrane-permeabilized and membrane-intact VSM cells.²⁵ In the current study, we therefore evaluated the effect of ketamine on the myofilament Ca²⁺ sensitivity in the presence of the intact cell membrane. However, as shown in figures 5 and 6, even in the presence of the intact cell membrane, ketamine (0.3 mM) had little influence on the Ca²⁺-force relation either in the absence or in the presence of receptor stimulation. This suggests that ketamine even at the high concentration (0.3 mM) does not have any significant effect on the myofilament Ca²⁺ sensitivity.

In the experiments designed to examine the effects of ketamine on the myofilament Ca²⁺ sensitivity in the fura-2-loaded, membrane-intact strips, however, it was not possible to evaluate the effects of a much higher concentration (1 mM) of ketamine on the Ca²⁺-force relation, because this concentration of ketamine totally or nearly eliminated the increases in either [Ca²⁺]_i or force caused by the stepwise increment of [Ca²⁺]_e during KCl depolarization or norepinephrine stimulation. However, in another series of experiments, 1 mM ketamine eliminated the contractile responses to KCl and norepinephrine without completely inhibiting the KCl- and norepinephrine-induced increases in $R_{340/380}$, re-

spectively (figs. 2 and 3). This may suggest that this high concentration of ketamine inhibits the myofilament Ca^{2+} sensitivity in the presence of the intact cell membrane. However, ketamine (up to 1 mM) failed to inhibit the Ca^{2+} -activated contractions during the membrane-permeabilized condition, consistent with a previous finding.¹⁴ Therefore, the presumed inhibition of myofilament Ca^{2+} sensitivity caused by 1 mM ketamine could be mediated by the intact plasma membrane or caused by an effect on some intracellular regulatory mechanisms of contractile proteins that are impaired in the β -escin-permeabilized VSM cells.

As shown in figures 6 and 7, the force/ $[\text{Ca}^{2+}]_i$ ratio during contractile response to norepinephrine is much higher than that during contractile response to KCl in this artery, indicating that the receptor stimulation increases the myofilament Ca^{2+} sensitivity, as has been well recognized.^{49,50} Although the exact mechanism for the agonist-induced Ca^{2+} sensitization is not entirely clear, protein kinase C (PKC), arachidonic acid, and Rho kinase may be involved in it.^{49,50} As previously discussed, ketamine may interfere with the activation of either receptor, heterotrimeric G protein or phospholipase C. If so, and the activation of PKC were involved in the agonist-induced Ca^{2+} sensitization as previously proposed,⁴⁹ ketamine would inhibit the myofilament Ca^{2+} sensitivity in the presence of receptor stimulation by inhibiting synthesis of diacylglycerol (another product of the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate) and thereby preventing the PKC activation. However, 0.3 mM ketamine, which significantly inhibited the presumed IP_3 -mediated vascular response to norepinephrine in the Ca^{2+} -free solution (fig. 4), had little influence on the Ca^{2+} -force relation in the norepinephrine-stimulated, membrane-intact VSM cells (fig. 6), implying that PKC does not play a major role in the norepinephrine-induced Ca^{2+} sensitization. This is consistent with a recent view that PKCs play only a small and transient role in the receptor agonist-induced, G-protein-coupled Ca^{2+} sensitization.⁵⁰

In conclusion, in rat mesenteric resistance arteries, lower concentrations of ketamine enhance contractile response to norepinephrine in an endothelium-dependent manner, whereas higher concentrations of ketamine inhibit the contractile response to either norepinephrine or KCl through direct effects on VSM. The direct inhibitory action of ketamine on VSM appear to be largely caused by reduction of the $[\text{Ca}^{2+}]_i$ in VSM cells. The $[\text{Ca}^{2+}]_i$ -reducing effects observed in the norepinephrine-stimulated muscle are presumably a result of inhibitions of both voltage-gated Ca^{2+} influx and norepinephrine (or IP_3)-induced Ca^{2+} release from the intracellular stores.

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