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Opioid receptor antagonists increase $[Ca^{2+}]_i$ in rat arterial smooth muscle cells in hemorrhagic shock¹

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KEY WORDS hemorrhagic shock; vascular smooth muscle; opioid receptors; calcium

ABSTRACT

AIM: To examine the effects of opioid receptor antagonists and norepinephrine on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mesenteric arterial (MA) smooth muscle cells (SMC) isolated from normal and hemorrhagic shocked rats in the vascular hyporesponse stage. **METHODS:** The rat model of hemorrhagic shock was made by withdrawing blood to decrease the artery mean blood pressure to 3.73-4.26 kPa and keeping at the level for 3 h. $[Ca^{2+}]_i$ of vascular smooth muscle cells (VSMC) were detected by the laser scan confocal microscopy. **RESULTS:** In the hyporesponse VSMC of rats in hemorrhagic shock, selective δ -, κ -, and μ -opioid receptor antagonists (naltrindole, nor-binaltorphimine, and β -funaltrexamine, 100 nmol/L) as well as norepinephrine 5 μ mol/L significantly increased $[Ca^{2+}]_i$ by 47% \pm 13%, 37% \pm 14%, 33% \pm 10%, and 54% \pm 17%, respectively, although their effects were lower than those in the normal rat cells (the increased values were 148% \pm 54%, 130% \pm 44%, 63% \pm 17% and 110% \pm 38%, respectively); and the norepinephrine-induced increase in $[Ca^{2+}]_i$ was further augmented by three δ -, κ -, and μ -opioid receptor antagonists (50 nmol/L, respectively) application (from 52% \pm 16% to 99% \pm 29%, 146% \pm 54% and 137% \pm 47%, respectively). **CONCLUSION:** The disorder of $[Ca^{2+}]_i$ regulation induced by hemorrhagic shock was mediated by opioid receptor and α -adrenoceptor, which may be partly responsible for the vascular hyporesponse, and the opioid receptor antagonists improved the response of resistance arteries to vascular stimulants in decompensatory stage of hemorrhagic shock.

INTRODUCTION

Circulation hypotension induced by hemorrhagic shock is one of the main causes of death after severe wounds or trauma. When the hemorrhagic shock has developed to a decompensatory stage, it is difficult to

reverse the hypotension using usual vasoconstrictor, such as norepinephrine. One of the explanations of hypotension is due to the hyposensitivity of arterial smooth muscle cells (SMC) to vascular constrictor stimuli^[1,2]. Previous studies showed that the vascular hyporesponse was related to many factors, such as a reduction in the sensitivity of α -adrenoceptor^[3], as well as an increase of the production of nitric oxide and tumor necrosis factor^[4,5].

Ca^{2+} is an important second messenger involved in exciting-contraction coupling and plays a key role in regulation of vascular smooth muscle tone^[6,7]. It was

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reported that the activities of large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) and ATP-sensitive K^+ channels were increased in vascular smooth muscle cells (VSMC) during the decompensatory phase of severe hemorrhagic shock^[2,8]. The activation of K^+ channels stabilizes the membrane potential, causing reduction of cell excitability and Ca^{2+} influx. Our recent work showed that Ca^{2+} channels were inhibited in mesenteric arterial (MA) VSMC of shocked rats^[9]. These results suggest that the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and its regulation could be disordered in VSMC during the decompensatory stage of severe hemorrhagic shock. Further studies found that the opioid receptor antagonists increased Ca^{2+} currents and decreased BK_{Ca} currents in MA SMC of shocked rat^[9,10], indicating that opioid receptor antagonists could have a modulation effects on $[\text{Ca}^{2+}]_i$. The present work was undertaken to observe and compare the effects of norepinephrine and opioid receptor antagonists on the $[\text{Ca}^{2+}]_i$ in MA SMC isolated from normal and hemorrhagic shocked rats in decompensatory stage.

MATERIALS AND METHODS

Hemorrhagic shock model and measurement of vascular responsiveness A hemorrhagic shocked rat model was reproduced according to the method described previously^[1,2]. In brief, Wistar rats (provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China), weighing 220-260 g, were anesthetized with urethane 0.8 g/kg and chloralose 30 mg/kg intramuscularly and a constant level of anesthesia was maintained with repeated bolus intravenous infusions of a mixture of urethane (0.2 g/kg) and chloralose (6 mg/kg)^[11]. Body temperature was kept at 37 °C using a heating pad and heat lamp. Both femoral arteries were cannulated, one for continuous measurement of blood pressure and the other for the sampling of blood and to facilitate the controlled hemorrhage. One femoral vein was cannulated for giving drugs. After completion of the surgical preparation, the rats were allowed to stabilize for 30 min. The artery mean blood pressure was then decreased to 3.73-4.26 kPa and kept for 3 h by withdrawing blood from a femoral artery gradually. Previously, under our experimental conditions, a hyporesponse of the artery SMC to norepinephrine (6 $\mu\text{g}/\text{kg}$) was observed when the hemorrhagic shock developed for more than 1 h^[2,9,10]. Sham-shocked rats underwent all surgical procedures suffered by the hem-

orrhagic-shocked animals except for bleeding.

All experiments conformed to the guidelines of the National Institutes of Health on the ethical use of animals and all experimental procedures were reviewed and approved by the Animal Care and Use Committee of Shanghai Institute of Physiology. All efforts were made to minimize the animal suffering and to reduce the number of animals used.

Preparation of single vascular smooth muscle cells The freshly dispersed VSMC were dissociated from MA of the normal rats or the rats in hemorrhagic shock as described previously^[2,12] and modified slightly. In brief, after the completion of 3-h shock or sham shock procedure, the rats were decapitated and the second- and third-order branches of MA (diameter <300 μm) were dissected and immersed in a Ca^{2+} -free isolation solution (in mmol/L: NaCl 145, KCl 6, glucose 10, HEPES 10, pH 7.3 adjusted with NaOH) oxygenated with 100 % O_2 . After the connective tissue was carefully removed, the endothelium was stripped using a dissection microscopy. The cleaned arteries were incubated for 15 min at 36 °C in the Ca^{2+} -free isolation solution bubbled with 100 % O_2 , then transferred to Ca^{2+} -free isolation solution containing 0.3 % collagenase (type I) and dithiothreitol 1 mmol/L to digest at 36 °C for 45 min. The digested tissue was washed several times and resuspended in the solution without collagenase. In a Krebs's solution containing 0.2 % bovine serum albumin, the tissue was cut into small pieces and was gently agitated using fire-polished Pasteur pipettes with a 100-400 μm tip diameter to disperse cells. The Krebs's solution consists of (in mmol/L) NaCl 119, NaHCO_3 24, KCl 4.7, KH_2PO_4 1.18, MgSO_4 1.17, CaCl_2 1.6, and glucose 5.5 (pH 7.4).

Fluorescence measurement of intracellular free Ca^{2+} concentrations The cell suspension was dripped onto a poly-L-lysine coated glass coverslip attached on the bottom of a recording chamber (0.2 mL volume). After adhered to the glass coverslip, the cells were loaded with Fluo 3-AM 10 $\mu\text{mol}/\text{L}$ (acetoxymethyl-ester) in darkness min at 37 °C for 30 min. Then the chamber was mounted on the stage of an inverted microscopy (Zeiss Axiovert 100M, Carl Zeiss Co, Germany). The cells were continuously perfused with dye-free Krebs's solution for 3 min to complete hydrolysis of dye. The cell density used to measure $[\text{Ca}^{2+}]_i$ was 7-10 cells per microscopic visual field.

Fluo 3-AM loaded VSMC were imaged with a Zeiss LSM-510 laser scanning confocal microscopy

(Germany). The change in fluorescence excited by 488 nm of argon laser was measured at 525 nm in the intracellularly loaded Fluo 3-AM cells, and was taken to estimate the changes in $[Ca^{2+}]_i$ upon administration of drugs. The fluorescence images were acquired by a photomultiplier detector (LSM-510, Version 2.01, Carl Zeiss Co, Germany) at a rate of 0.3 Hz and the fluorescence intensity was stored digitally. Time course of fluorescence changes of the cells was obtained automatically with analysis software (TimeSeries, Carl Zeiss Co, Germany). The fluorescence signal of Ca^{2+} represented Ca^{2+} concentration^[13,14]. The fluorescence signals were normalized as $\Delta F/F_0$ (%), where F_0 was the resting fluorescence and ΔF was Ca^{2+} elevation over the resting fluorescence.

The test drugs were dissolved in the Krebs's solution and continuously perfused.

Drugs and reagents The stock solution of Fluo 3-AM (Molecular Probes Inc, USA) was prepared by dissolving 50 μ g of the fluorescent dye into 50 μ L dimethyl sulfoxide and kept frozen in aliquots until use. Collagenase, poly-*L*-lysine, naltrindole, and nor-binaltorphimine were all Sigma products (St Louis, MO, USA). Norepinephrine and dithiothreitol were purchased from Serva (Heidelberg, Germany) and β -funaltrexamine from Research Biochemicals International (Natick, MA, USA). All other reagents were of analytical grade.

Statistic analysis All data were expressed as mean \pm SD (n =number of cells) and evaluated by *t*-test. *P* values less than 0.05 were considered to be statistically significant. The cells used in present work were isolated from 18 rats (6 for the sham shock group and 12 for the shock group).

RESULTS

Effects of δ -, κ -, and μ -opioid receptor antagonists on $[Ca^{2+}]_i$ of VSMC isolated from MA of normal and shocked rats The changes of $[Ca^{2+}]_i$ induced by opioid receptor antagonists in MA SMC isolated from the normal rats were detected. Opioid receptor antagonists 100 nmol/L could block opioid receptor agonist-induced inhibition of Ca^{2+} current and increase K^+ current in other cells was chosen^[15,16]. After perfusing the cells with the antagonists-contained Krebs's solution, a transient increase of $[Ca^{2+}]_i$ was observed. The onset of the effect was in 10-15 s, and a maximal response occurred in 30-60 s of the continuous application of drugs. At a concentration of 100 nmol/L, the selective

δ -, κ -, and μ -opioid receptor antagonists, naltrindole, nor-binaltorphimine and β -funaltrexamine resulted in a significant increase of the $\Delta F/F_0$ by 148 % \pm 54 % (n =38), 130 % \pm 44 % (n =49), and 63 % \pm 17 % (n =39), respectively. The effect of β -funaltrexamine was weaker than that of naltrindole or nor-binaltorphimine. A typical change of fluorescence intensity of $[Ca^{2+}]_i$ induced by nor-binaltorphimine was shown in Fig 1. In a few cells, $[Ca^{2+}]_i$ oscillation was observed after application of the antagonists (data not shown). In order to examine the response of the cells accurately, the responses of $[Ca^{2+}]_i$ of the same cells 2-3 times were measured. It was found that the cell responses to repeated stimulation were almost equal in amplitude (data not shown).

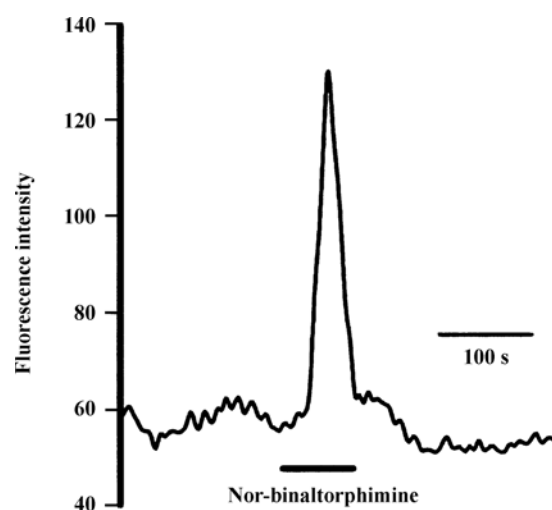


Fig 1. $[Ca^{2+}]_i$ increase induced by nor-binaltorphimine 100 nmol/L in mesenteric artery smooth muscle cells isolated from the normal rats. The cells were loaded with Fluo 3-AM and $[Ca^{2+}]_i$ were measured by laser scan confocal microscope. The increase of $[Ca^{2+}]_i$ was represented as a change of dye fluorescence intensity in arbitrary unit (au).

The opioid receptor antagonists also induced an increase of $[Ca^{2+}]_i$ in the cells isolated from the rats in hemorrhagic shock. However, the increased values of $[Ca^{2+}]_i$ were markedly lower than those in the normal cells. After application of naltrindole, nor-binaltorphimine and β -funaltrexamine at the same concentration of 100 nmol/L, $\Delta F/F_0$ were 47 % \pm 13 % (n =17), 37 % \pm 14 % (n =21) and 33 % \pm 10 % (n =18), respectively (Fig 2). When these data were compared with those obtained in the normal cells mentioned in above paragraph, a significant difference was found either for naltrindole and nor-binaltorphimine (both P <0.01) or for β -funaltrexamine (P <0.05). Meantime, compared with

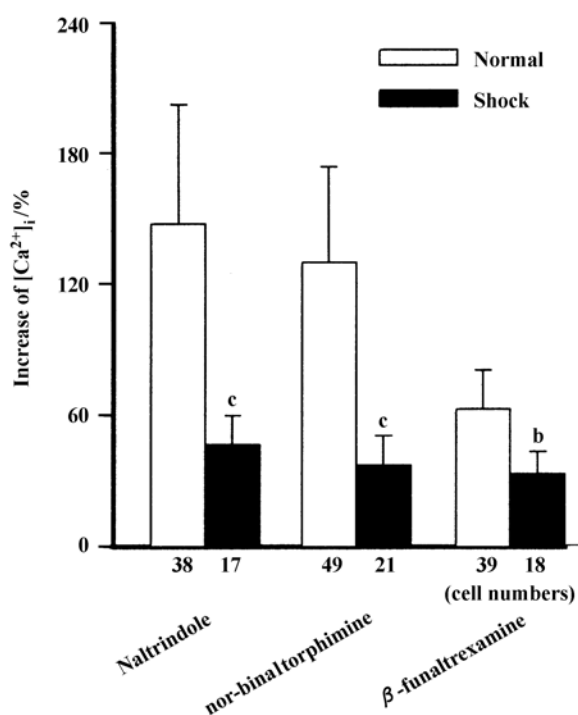


Fig 2. Effects of naltrindole, nor-binaltorphimine, β -funaltrexamine 100 nmol/L on $[Ca^{2+}]_i$ in mesenteric artery smooth muscle cells isolated from the normal rats and the rats in hemorrhagic shock. The fluorescence signal changes were normalized as $\Delta F/F_0$ (%), where F_0 was the resting fluorescence and ΔF was $[Ca^{2+}]_i$ elevation over the resting fluorescence. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs normal group. The number under each column indicates the cell numbers tested.

those of normal cells, the time from onset to peak of $[Ca^{2+}]_i$ elevation after nor-binaltorphimine and β -funaltrexamine application was prolonged, but did not obviously change after naltrindole application (data not shown).

Effects of norepinephrine on $[Ca^{2+}]_i$ of VSMC isolated from MA of normal and shocked rats After perfusing the cells with norepinephrine 5 μ mol/L, an α -adrenoceptor agonist, a transient rise of $[Ca^{2+}]_i$ in MA SMC of the normal rats and the rats in hemorrhagic shock was found. The $\Delta F/F_0$ 110 \pm 38 % ($n=31$) in the cells of normal rats was higher than those 54 \pm 17 % ($n=23$) in hemorrhagic shocked rats ($P < 0.01$).

Effects of δ -, κ -, and μ -opioid receptor antagonists on norepinephrine-induced $[Ca^{2+}]_i$ elevation of VSMC isolated from MA of shocked rats To determine whether the $[Ca^{2+}]_i$ elevation induced by norepinephrine from the cells of hemorrhagic shocked rats was affected by opioid receptor antagonists, the cells

were perfused with Krebs's solution containing norepinephrine and opioid receptor antagonists after the fluorescence intensity returned to basal level by washout.

It was found that three opioid receptor antagonists augmented the norepinephrine-induced $[Ca^{2+}]_i$ transient in MA SMC of hemorrhagic shocked rats. The change of Ca^{2+} transient ($\Delta F/F_0$) evoked by norepinephrine 5 μ mol/L was increased by naltrindole, nor-binaltorphimine, and β -funaltrexamine 50 nmol/L from 52 \pm 16 % ($n=76$) to 99 \pm 29 % ($n=15$), 146 \pm 54 % ($n=17$), and 137 \pm 47 % ($n=44$), respectively ($P < 0.01$, Fig 3).

DISCUSSION

The present study showed that in the hyporesponsive VSMC of rats in hemorrhagic shock, selective δ -, κ -, and μ -opioid receptor antagonists as well as norepinephrine significantly increased $[Ca^{2+}]_i$, but their effects were lower than those in the cells of normal rats. The results demonstrated that during the vascular hyporesponsive stage of hemorrhagic shock, the regulation of $[Ca^{2+}]_i$ was mediated by δ -, κ -, μ -opioid receptors, and α -adrenoceptor in MA SMC. Moreover, the opioid receptor antagonists promoted norepinephrine-induced $[Ca^{2+}]_i$ elevation, indicating that besides antagonizing the effects of opioid receptor agonist, increase of $[Ca^{2+}]_i$ may be one of the mechanisms of the opioid receptor antagonists improving the response of resistance arteries to vasoactive stimulants.

Many works demonstrated that the opioid peptides, acting via the opioid receptors, down-regulated the $[Ca^{2+}]_i$ of the neurons through inhibiting Ca^{2+} channels, suggestive of tight receptor-channel coupling^[15-17]. It was reported that opioid receptors were expressed in human internal mammary arterial and aortic vascular smooth muscle cells^[18,19]. Although no reports directly demonstrated the presence of the 3 opioid receptors in MA SMC of rats, based on the present and our previous studies^[9,10], we deduced that the opioid receptors might be expressed in the cells and the opioid receptor antagonists-induced increase of $[Ca^{2+}]_i$ was mediated by opioid receptors. For the observations that the opioid receptor antagonists induced the increase of $[Ca^{2+}]_i$ in the absence of opioid receptor agonist in the present study, this may result from a constitutive activity of the opioid receptors. It is now widely accepted that these G-protein-coupled receptors can exist in equilibrium between an active and an inactive state of the receptors^[20]. It has been suggested that even in the absence of

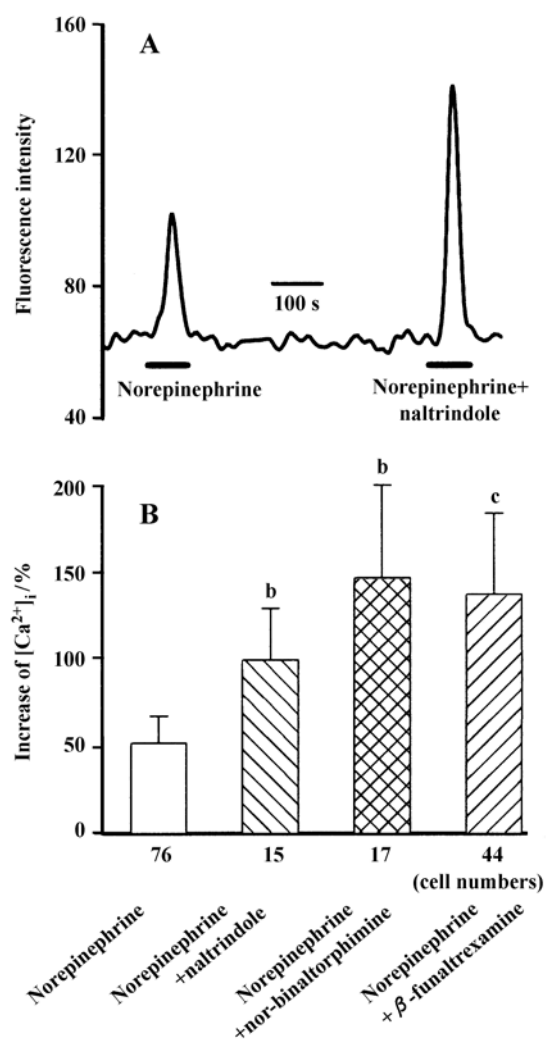


Fig 3. Augmentation of the norepinephrine-induced [Ca²⁺]_i elevation by opioid receptor antagonists in mesenteric artery smooth muscle cells isolated from the rats in hemorrhagic shock. A: an original record showing [Ca²⁺]_i increase induced by norepinephrine 5 μmol/L as well as naltrindole 50 nmol/L and norepinephrine 5 μmol/L. The increase of [Ca²⁺]_i in Fluo 3-AM loaded cells was represented as an increase of dye fluorescence intensity shown in arbitrary unit (au). **B:** naltrindole, nor-binaltorphimine, and β-funaltrexamine 50 nmol/L increased norepinephrine-induced [Ca²⁺]_i transient. The fluorescence signal changes were normalized as $\Delta F/F_0$ (%), where F_0 was the resting fluorescence and ΔF was [Ca²⁺]_i elevation over the resting fluorescence. Mean±SD. ^b $P < 0.05$, ^c $P < 0.01$ vs norepinephrine group. The number under each column indicates the cell numbers tested.

receptor agonist, these receptors can maintain a conformation that can activate G-protein and so display constitutive activity^[21-23]. Another possibility may be that these opioid receptors were activated to a certain extent by remaining endogenous agonists *in vitro*.

Certainly, further elucidation is necessary.

Many studies reported that the concentration of endogenous opioid peptides in blood plasma was increased in trauma-induced hemorrhagic shocked animal^[24]. Injection of anti-endorphine serum antagonized the decrease of blood pressure induced by opioid peptides. Opioid receptor antagonists improved the circulation function and increased the survival time and survival rate of hemorrhagic shocked rats^[25,26]. These data implied that the increase of opioid peptides in shocked animals contributed to systemic vasodilation of resistance arteries, and that the opioid receptor antagonists could be one of therapeutic approaches for improving the circulation function. Indeed, the effects of opioid receptor antagonists were mainly through acting on central nerve system and then the cardiovascular functions were regulated. However, our present results demonstrated that direct action on opioid receptors in VSMC might be one of the mechanisms for opioid receptor antagonists to antagonize shock. Certainly, the antishock effects of the three opioid receptor antagonists were limited. Combination of various antishock approaches should be considered in clinic.

In conclusion, this study demonstrated that the hemorrhagic shock resulted in the disorder of [Ca²⁺]_i regulation was mediated by opioid receptor and α-adrenoceptor, which may be partly responsible for vascular hyporesponse, and to increase [Ca²⁺]_i may be one of the mechanisms of the opioid receptor antagonists to improve the response of resistance arteries to vasoactive stimulants in decompensatory stage of hemorrhagic shock.

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