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Effects of Sedative Agent JM-1232(-) ((-)-3-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-2-phenyl-3,5,6,7-tetrahydrocyclopenta[f]isoindole-1(2H)-one) on the Carotid Arteries of Rats

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Abstract: In the present study, we investigate whether JM-1232(-) ((-)-3-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-2-phenyl-3,5,6,7-tetrahydrocyclopenta[f]isoindole-1(2H)-one) affects vessels directly or indirectly. We examined the effects of JM-1232(-) with several antagonists on rat carotid arteries using the Magnus method. JM-1232 (-) suppressed contraction non-specifically on norepinephrine, potassium chloride and calcium chloride at a high concentration (E_{max} : 10^{-5} - 10^{-4} M). There were no significant change in each pretreated group consisting of flumazenil, propranolol, atropine, cimetidine, imetit and N(omega)-nitro-L- arginine methyl ester, whereas a significant suppression was observed ($p < 0.05$) in PK11195 (50% inhibition concentration (IC_{50}): $3.2 \pm 0.9 (\times 10^{-5})$ M) and diphenhydramine (IC_{50} : $5.6 \pm 1.7 (\times 10^{-5})$ M). These results suggest that only a high concentration of JM-1232(-) reacts for carotid artery relaxation directly (EC_{50} : about 10^{-5} M). Thus JM-1232 (-) (less than 10^{-6} M) might not directly induce a vessel relaxation that can cause hypotension.

Key words: JM-1232(-), sedative agent, carotid arteries, relaxation

INTRODUCTION

JM-1232(-) ((-)-3-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-2-phenyl-3,5,6,7-tetrahydrocyclopenta[f]isoindole-1(2H)-one) is a non-benzodiazepine (BZ) compound with an isoindoline skeleton, whose application as a new intravenous anesthetic or sedative is being considered and proved to act on BZ receptors (Kanamitsu *et al.*, 2007; Nishiyama *et al.*, 2008; Chiba *et al.*, 2009). The 50% hypnotic dose was 0.69 mg kg^{-1} and the 50% lethal dose was $>90 \text{ mg kg}^{-1}$, when intravenously administered in rats (Kanamitsu *et al.*, 2007). Major side effects of the agents that affect BZ receptors include dependence, anterograde amnesia, respiratory depression, arrhythmia and hypotension through vessel relaxation. It is especially known that anesthetics and sedatives affect the circulatory system (Chamey *et al.*, 2001). There have been many reports that the vessel relaxation is caused not only by central depressant, but also by direct interactions with peripheral blood vessels in drugs acting on the central nervous system (Ishii *et al.*, 1983; French *et al.*, 1989; Pérez-Guerrero *et al.*, 1997; Galindo *et al.*, 2001; Veenman and Gavish, 2006). Hypotension

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is more frequently induced by direct vessel reaction of anesthetic and sedative agents in addition to sympathetic inhibition through central depression. Thus, it is important to evaluate whether the vessel relaxation is direct or indirect for confirming the safety of JM-1232(-).

To investigate whether JM-1232(-) affects vessels directly or indirectly, we examined the effects of JM-1232(-) on rat carotid arteries using the Magnus method.

MATERIALS AND METHODS

Chemicals and Reagents

JM-1232(-) (lot No. 050616) was kindly provided by Maruishi Pharmaceutical, Co., Ltd. (Osaka, Japan). Indomethacin (Sigma-Aldrich, MO, USA), KCl (Nacalai Tesque, Inc., Kyoto, Japan), norepinephrine (NE, NOR-ADRENALIN[®], Daiichi Sankyo Co., Ltd., Tokyo, Japan), acetylcholine (OVISOT[®], Daiichi Sankyo Co., Ltd., Tokyo, Japan), papaverine hydrochloride (Merck Ltd., Tokyo, Japan), calcium chloride (CaCl₂, Nacalai Tesque, Inc., Kyoto, Japan), flumazenil (central BZ receptor antagonist, Sigma-Aldrich, MO, USA), PK11195 (peripheral BZ receptor antagonist, Sigma-Aldrich, MO, USA), (±)-propranolol hydrochloride (beta-adrenergic receptor antagonist, Wako Pure Chemical Industries, Ltd., Osaka, Japan), atropine sulfate hydrate (muscarinic receptor antagonist, Wako Pure Chemical Industries, Ltd., Osaka, Japan), diphenhydramine (H₁ histamine receptor antagonist, Nacalai Tesque, Inc., Kyoto, Japan), cimetidine (H₂ histamine receptor antagonist, Nacalai Tesque, Inc., Kyoto, Japan), imetit dihydrobromide (H₃ histamine receptor antagonist, Sigma-Aldrich, MO, USA) and N(omega)-nitro-L-arginine methyl ester hydrochloride (L-NAME, nitric oxide synthase inhibitor, Sigma-Aldrich, MO, USA) were purchased. Other reagents were commercially available, extra-pure grade chemicals.

Animals

Wistar ST strain male rats (9-11 weeks of age, Japan SLC, Inc., Shizuoka, Japan) were used, with 6-10 of them in each group. The animals were housed in a room maintained at a temperature of 24±1°C, humidity of 55±10% and lighting period from 6:00 to 18:00 and allowed free access to tap water and solid diet (NMF, Oriental yeast Co., LTD., Tokyo, Japan). The animals were acclimatized to the environment for at least one week prior to use in the experiment. All experiment procedures were conducted according to the guidelines for the use of experimental animals and animal facilities established by Osaka University of Pharmaceutical Sciences.

Artery Extraction (Jin *et al.*, 1998)

Rats were anesthetized by intraperitoneal administration of pentobarbital (40 mg kg⁻¹, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), the right femoral vein was exposed and heparin (400 IU, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was injected intravenously. They were then euthanized by exsanguination through cardiac incision, thereafter the carotid artery was immediately isolated and cut into 3-5 mm pieces as ring preparation.

Evaluation of Carotid Artery Reactivity (Jin *et al.*, 1998)

We prepared a Micro Tissue Organ Bath (MTOB-1Z, Serial: 031006_E291006, Primetech Corporation, Tokyo, Japan), which was subsequently filled with 5 mL of Tyrode solution (mM: NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.42, NaHCO₃ 12, glucose 5.7) at 37°C, saturated with 95% O₂ + 5% CO₂. One micromole of indomethacin was also added to the

Tyrode solution, in order to suppress endogenous prostanoid from vascular endothelium in the present study. The artery was mounted and given a 0.25 g tension. The tension measurement in the present study was done on SIGNAL CONDITIONER (SC-20AZ, Primetech Corporation, Tokyo, Japan) and 4ch Data Acquisition System DI-148-U (Primetech Corporation, Tokyo, Japan) and the data were analyzed on the data analysis system WinDaq700 (DATAQ INSTRUMENTS, OH, USA).

After 60-80 min of stabilization in the bath, the artery was contracted with NE (10^{-7} M). Upon reaching the highest point of contractile reaction, JM-1232(-) (10^{-7} - 3×10^{-4} M) was administered. After reaching the maximum relaxation response at the highest concentration, papaverine (10^{-4} M) was administered to relax the artery completely. This complete relaxation response was taken to be the reference point of 100%, along which the artery relaxation response ratio of JM-1232(-) (10^{-7} - 3×10^{-4} M) was calculated and the concentration-contractile response curves were mapped.

Reactivity of JM-1232(-) to NE, KCl and CaCl₂ Contraction

The methods developed by Jin *et al.* (1998) were followed for NE and KCl. For NE, artery contraction reaction with 3×10^{-6} M was observed, which was taken to be the maximum reaction (100%). It was then irrigated with Tyrode solution three times more than 30 min, recovering the artery tension to the baseline. Then JM-1232(-) (3×10^{-6} - 10^{-4} M) was pretreated. The contact time for JM-1232(-) was 15 min, after which NE (3×10^{-9} - 10^{-6} M) was administered. The artery contraction reaction rate was calculated and the concentration-contractile response curves were mapped.

Artery contraction reaction with 5×10^{-2} M of KCl was observed and was taken to be the maximum reaction (100%). After JM-1232(-) (3×10^{-6} - 10^{-4} M) was pretreated, KCl (10^{-2} - 6×10^{-2} M) was then administered. The artery contraction reaction rate was calculated and the concentration-contractile response curves were mapped. A non- pretreated group was designated as a control group.

Experiments for CaCl₂ were performed based on the methods by Chericoni *et al.* (2004) and French *et al.* (1989). After mounting the artery on the device, CaCl₂ was removed from the normal Tyrode solution. Irrigation was performed 8-10 times for 90 minutes with Ca²⁺-free-depolarizing Tyrode solution with KCl (6×10^{-2} M) added, before stabilization. Then, with the remaining Ca²⁺ removed completely, JM-1232(-) (3×10^{-6} - 10^{-4} M) was pretreated. After 15 min, CaCl₂ (10^{-5} - 10^{-2} M) was added. The artery contraction rate was calculated and the concentration-contractile response curves were mapped. A non- pretreated group was defined as the control group, where the artery contraction reaction with CaCl₂ (3×10^{-2} M) was taken to be the maximum reaction (100%).

Reactivity of JM-1232(-) to Various Ligands

Flumazenil, PK11195, propranolol, atropine, diphenhydramine, cimetidine, imetit and L-NAME were each pretreated at 10^{-5} M. The contact time for these was set at 15 min. Similarly, NE, JM-1232(-) and papaverine were administered in that order, after which the artery contraction ratio was calculated. A non-pretreated group was chosen as the control group.

Changes in the Concentration-Contractile Response Curves for JM-1232(-) to Receptor Blockers

The concentration-contractile response curves for JM-1232(-) (10^{-7} - 3×10^{-4} M) were mapped, with the relaxation response for JM-1232(-) (10^{-4} M) at the maximum response

(100%). PK11195 (3×10^{-7} - 10^{-5} M) and diphenhydramine (3×10^{-6} - 10^{-4} M) were pretreated before the concentration-contractile response curves for JM-1232(-) (10^{-7} - 3×10^{-4} M) were mapped and the changes from the pretreatment were measured.

Statistical Analysis

Statistical analysis was carried out with WinNonlin (Pharsight, CA, USA), a nonlinear least-squares program, by which maximal pharmacological effect (E_{max}), pD_2 ($-\log [50\% \text{ effective concentration } (EC_{50})]$) and 50% inhibition concentration (IC_{50}) were calculated. Values in all groups were represented as mean \pm SE. The unpaired Student's t-test was employed in examining two groups, while the Tukey test was used for multi-group examination (StatMate III, Atms Co., Ltd., Tokyo, Japan). The $p < 0.05$ was considered to be significant.

RESULTS

Evaluation of Artery Response

The concentration-contractile response curves for JM-1232(-) were mapped, with the relaxation response of papaverine at 100% (Fig. 1). Our analysis showed E_{max} to be $57.8 \pm 2.4\%$ and pD_2 to be 5.33 ± 0.13 .

Response for NE, KCl and $CaCl_2$ Contractions

In the contractile response by NE with the control group and the JM-1232(-) 3×10^{-6} - 10^{-4} M pretreated groups, E_{max} showed a significant suppression in those groups where JM-1232(-) 10^{-5} M or above was used (Fig. 2). Similarly, in the contractile response by KCl, E_{max} showed a significant suppression where JM-1232(-) 10^{-5} M or above was used (Fig. 3). In the contractile response by $CaCl_2$, E_{max} showed a significant suppression where JM-1232(-) 10^{-5} M or above was used (Fig. 4).

Response of JM-1232(-) for Various Ligands

Relaxation response of JM-1232(-) in control (JM-1232(-) 10^{-4} M) group, flumazenil treated group, PK11195 treated group, propranolol treated group, atropine treated group, diphenhydramine treated group, cimetidine treated group, imetit treated group and L-NAME treated group were 57.6 ± 3.0 , 53.7 ± 3.2 , 43.7 ± 2.7 , 47.0 ± 2.1 , 57.0 ± 3.5 , 38.4 ± 2.7 , 63.8 ± 4.1 , 56.2 ± 4.2

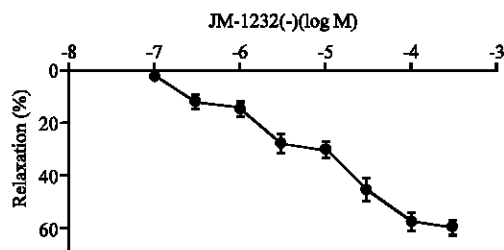


Fig. 1: Concentration-relaxation response curve of JM-1232(-) on norepinephrine (10^{-7} M)-pretreated rat carotid arteries. The maximal relaxation (E_{max}) value obtained by JM-1232(-) was $57.8 \pm 2.4\%$ and pD_2 value was 5.33 ± 0.13 . E_{max} and pD_2 value were determined by a nonlinear least-squares program. The results were given as the percentages of the maximal relaxation for papaverine (10^{-4} M). Each point represents Mean \pm SE ($n = 10$)

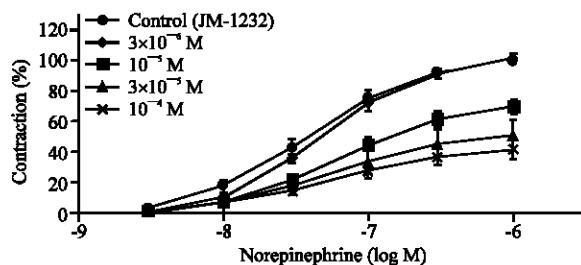


Fig. 2: Effects of JM-1232(-) on the concentration-contractile response curves for norepinephrine in rat carotid arteries. JM-1232(-) dose-dependently inhibited the contractile responses to norepinephrine. The results were given as the percentages of the maximal contraction for norepinephrine (3×10^{-6} M) before addition of the drugs. Each point represents Mean \pm SE (n = 6)

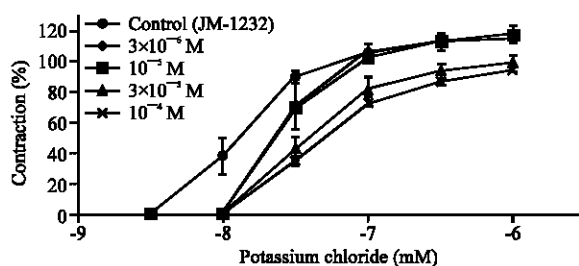


Fig. 3: Effects of JM-1232(-) on the concentration-contractile response curves for potassium chloride (KCl) in rat carotid arteries. JM-1232(-) dose-dependently inhibited the contractile responses to KCl. The results were given as the percentages of the maximal contraction for KCl (60 mM) before addition of the drugs. Each point represents Mean \pm SE (n = 6)

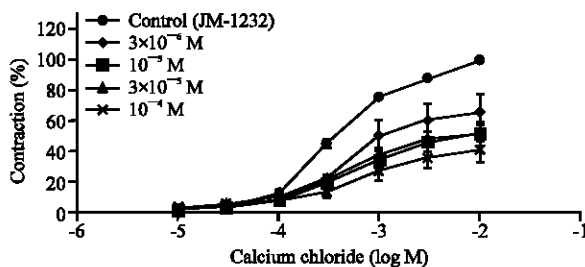


Fig. 4: Effects of JM-1232(-) on the concentration-contractile response curves for calcium chloride (CaCl_2) in rat carotid arteries. JM-1232(-) dose-dependently inhibited the contractile responses to CaCl_2 . The results were given as the percentages of the maximal contraction for CaCl_2 (3×10^{-2} M) before addition of the drugs. Each point represents Mean \pm SE (n = 6)

and $50.5 \pm 1.7\%$, respectively, where the relaxation rate for papaverine is designated as 100%. The significant suppression of relaxation response was observed in the PK11195 treated ($p < 0.05$) and diphenhydramine treated groups ($p < 0.05$) compared with the control group. There was no significant suppression in other groups (Fig. 5).

Changes in the Concentration-contractile Response Curves for JM-1232(-) to Receptor Blockers (3×10^{-7} - 10^{-5} M)

In all the PK11195 pretreated groups, a significant decrease in pD_2 was observed as compared with pD_2 of JM-1232(-) in the control group (Fig. 6). The relaxation by JM-1232(-) with PK11195 was converged (80-100%). Thus JM-1232(-) might be antagonized by competitive inhibition of PK11195 (Fig. 6). Meanwhile, a significant decrease in pD_2 was observed with diphenhydramine in the 3×10^{-5} and 10^{-4} M pretreated groups (Fig. 7). IC_{50} of PK11195 and that of diphenhydramine to JM-1232(-) (10^{-4} M) were $3.2 \pm 0.9 (\times 10^{-5})$ M and

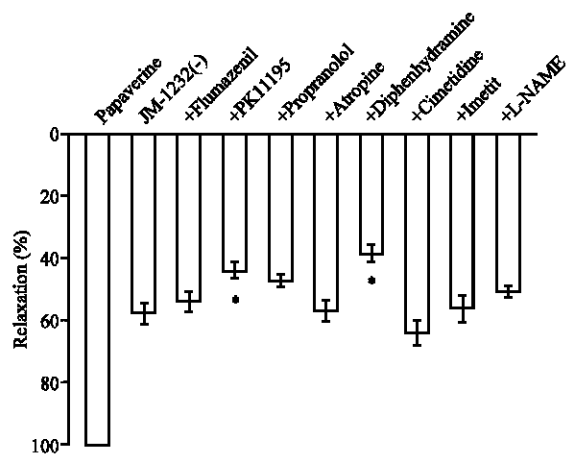


Fig. 5: JM-1232(-)-induced vasorelaxation in norepinephrine (10^{-7} M)-pretreated rat carotid arteries with and without antagonists. The relaxant effect of JM-1232(-) alone was significantly decreased ($*p < 0.05$) in the presence of PK11195 and diphenhydramine. However, the effect of JM-1232(-) in the presence of other drugs was not significantly different from that obtained with JM-1232(-) alone. The results were given as the percentages of the maximal relaxation for papaverine (10^{-4} M). Significant differences were determined by ANOVA followed by Tukey's test. Each bar represents Mean \pm SE (n = 6-10)

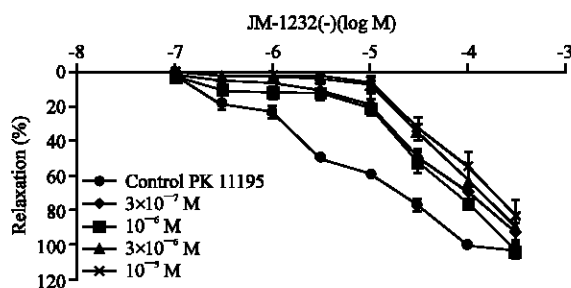


Fig. 6: Relaxation of norepinephrine (10^{-7} M)-pretreated rat carotid arteries by JM-1232(-) in the absence and in the presence of PK11195 (3×10^{-7} - 10^{-5} M). PK11195 above 3×10^{-7} M inhibited the relaxation responses to JM-1232(-) alone (control). The results were given as the percentages of the maximal relaxation for JM-1232 (10^{-4} M) before addition of the drugs. Each point represents Mean \pm SE (n = 6)

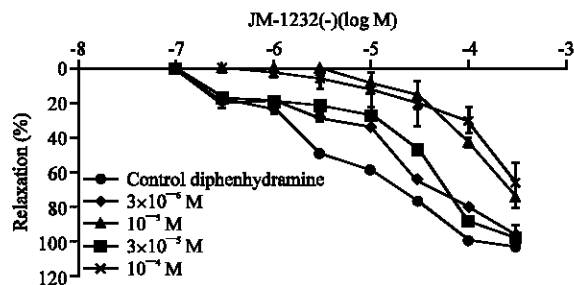


Fig. 7: Relaxation of norepinephrine (10^{-7} M)-pretreated rat carotid artery by JM-1232(-) in the absence and in the presence of diphenhydramine (3×10^{-6} - 10^{-4} M). Diphenhydramine above 3×10^{-5} M inhibited the relaxation responses to JM-1232(-) alone (control). The results were given as the percentages of the maximal relaxation for JM-1232 (10^{-4} M) before addition of the drugs. Each point represents Mean \pm SE (n = 6)

$5.6 \pm 1.7 (\times 10^{-5})$ M, respectively. The relaxation by JM-1232(-) with diphenhydramine was not converged (65-100%). Thus, JM-1232(-) might not be antagonized by competitive inhibition of diphenhydramine (Fig. 7).

DISCUSSION

It has been reported that many agents that affect BZ receptors has vessel relaxation which is caused not only by central depressant but also by direct interactions with peripheral blood vessels in high doses (Ishii *et al.*, 1983; Galindo *et al.*, 2001). Our concentration-contractile response curves for JM-1232(-) shows the maximum relaxation response (E_{max}) to be $57.8 \pm 2.4\%$ (about 10^{-4} M). Thus it follows that JM-1232(-) shows the maximum response at 10^{-4} M (Fig. 1). JM-1232(-) in high concentration (about 3×10^{-5} M) also indicated non-specific inhibition effects on artery contraction by all three of NE, KCl and $CaCl_2$ (Fig. 2-4). On the other hand, Fig. 1 might indicate the two types of the concentration-contractile response curve for JM-1232(-) (E_{max} was approximately 30% (around 3×10^{-6} - 10^{-5} M) and 60% (around 10^{-4} - 3×10^{-4} M), respectively). The concentration-contractile response curve represents the receptor response to the substrate (Ishii *et al.*, 1983; French *et al.*, 1989; Galindo *et al.*, 2001; Veenman and Gavish, 2006), but it might be suggested that the two types of concentration-contractile response curve for JM-1232(-) show that JM-1232(-) react two different receptors in high concentrations.

Artery relaxation response suppression effects were then examined using the following ligands: flumazenil, PK11195, propranolol, atropine, diphenhydramine, cimetidine, imetit and L-NAME. Suppression of relaxation response by JM-1232(-) with PK11195 and diphenhydramine was observed, whereas no suppression was observed with the others (Fig. 5). PK11195 significantly reduced pD_2 of JM-1232(-) from 3×10^{-7} M (Fig. 6). Diphenhydramine significantly reduced pD_2 of JM-1232(-) from 3×10^{-5} M or above (Fig. 7). It has been reported that diazepam acts on not only central BZ receptor but also peripheral BZ receptor (Ishii *et al.*, 1983; French *et al.*, 1989; Galindo *et al.*, 2001). These results suggested that JM-1232(-) might be antagonized by PK11195 specifically and other ligands non-specifically.

These results suggest that only a high concentration of JM-1232(-) (10^{-4} M) reacts for artery relaxation directly (EC_{50} : about 10^{-5} M). Thus on the concentration less than 10^{-6} M JM-1232 (-) might induce a vessel relaxation indirectly and on the concentration more than 10^{-6} M JM-1232(-) might induce a vessel relaxation directly through peripheral BZ receptors. JM-1232(-) might not cause an extensive hypotension on general dosage in rats.

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