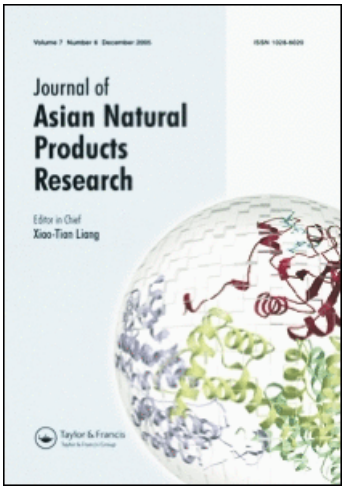

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Blood-Pressure Lowering, Positive Chronotropy and Inotropy by the *Veratrum* Alkaloids Germidine and Germerine but Negative Chronotropy by Veratridine in Mice

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To cite this Article Kimura, Ikuko , Islam, Md. Amirul , Honda, Ritsu , Nojima, Hiroshi , Tezuka, Yasuhiro and Zhao, Weijie(2000) 'Blood-Pressure Lowering, Positive Chronotropy and Inotropy by the *Veratrum* Alkaloids Germidine and Germerine but Negative Chronotropy by Veratridine in Mice', Journal of Asian Natural Products Research, 2: 2, 133 — 144
To link to this Article: DOI: 10.1080/10286020008039902
URL: http://dx.doi.org/10.1080/10286020008039902

JANPR, Vol. 2, pp. 133–144
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BLOOD-PRESSURE LOWERING, POSITIVE CHRONOTROPY AND INOTROPY BY THE *VERATRUM* ALKALOIDS GERMIDINE AND GERMERINE BUT NEGATIVE CHRONOTROPY BY VERATRIDINE IN MICE

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(Received 28 June 1999; In final form 25 August 1999)

Germidine and germerine, the *Veratrum* alkaloids lowered blood pressure accompanied with positive chronotropy and inotropy in mice. Germerine was more potent than germidine in both blood-pressure lowering and positive inotropy, whereas veratridine produced negative chronotropy and positive inotropy. An acyl group (an acetyl or a 2-methylbutyroyl group) at 3-*O*-R^1 position and a 2-methylbutyroyl group at 15-*O*-R^2 position in germine were important to produce the positive inotropy and chronotropy. The presence of a veratroyl group at 3-*O*-R^1 position and a free hydroxyl group at 15-*O*-R^2 position may be essential to produce the negative chronotropy by veratridine. The positive inotropy by germidine and veratridine may be due to TTX-resistant Na^+ channel activation.

Keywords: Germidine; Germerine; Blood-pressure lowering; Positive inotropy; Positive chronotropy

INTRODUCTION

The *Veratrum* (*V.*) herb (Liliaceae) lowers blood pressure (BP) and decreases the heart rate [1]. The potency of the crude alkaloids derived from roots and

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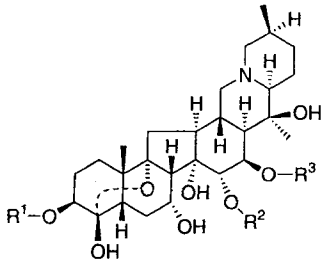
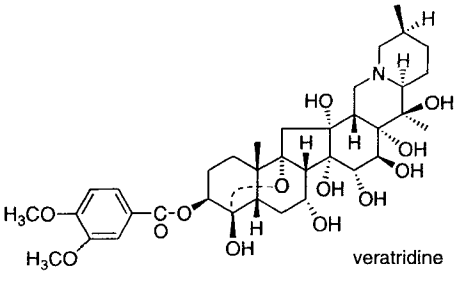
rhizomes of *Veratrum* plants fell in the order of *V. viride* (produced in America) >*V. grandiflorum* [2] >*V. album* var. *oxysepalum* (the same plant as *V. oxysepalum*) [3] >*V. stamineum* (produced in Japan) in dog [4,5]. The Chinese crude drug "Li-lu" is prepared from dried roots and rhizomes of several *V.* species: *V. nigrum* var. *ussurience*, *V. maackii*, *V. nigrum*, *V. album*, *V. patulum* and *V. schindleri* and is traditionally used for treating some kinds of vascular disease [6]. Several known and new steroidal alkaloids, germidine, germerine, 15-*O*-(2-methylbutyroyl)-germine, verussurinine [7], (+)-verussurine [8], neogermbudine and jervine [9] have been isolated and identified as constituents of *V. nigrum* var. *ussurience* (Fig. 1). Germidine and germerine also are contained in the crude alkaloids from *V. viride* [10,11], *V. oxysepalum* [12] and *V. nigrum* [13,14]. Veratridine, a steroidal alkaloid constituent derived from *V. album* activates Na^+ channel by blocking the inactivation, by prolonging the opening of the channel and the action potential, and then by augumenting the contractions to cardiac stimulation. The alkaloid alters the gating and permeability properties of Na^+ current [15]. We investigated cardiovascular effects of alkaloid fractions from *V. nigrum* var. *ussurience* and from *V. maackii*, and its derived compounds, germidine and germerine, and further compared the reaction

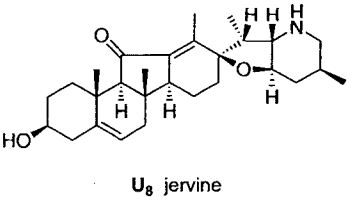


		R^1	R^2	R^3
U_7	germidine	Ac	MB	H
U_9	germerine	HMB	MB	H
U_{10}	15-*O*-(2-methyl-butyroyl)germine	H	MB	H
U_{11}	verussurinine	H	H	MB
U_{12}	neogermbudine	HMB	MB	H

Ac = Acetyl, MB = 2-Methylbutyroyl, HMB = 2-Hydroxy-2-methylbutyroyl



veratridine



U_8 jervine

FIGURE 1 Chemical structures of steroidal alkaloids derived from *V. nigrum* var. *ussuriense* and veratridine.

mode with that of veratridine *in vivo* and *in vitro* level of mice, and elucidated the structure–activity relationship among the steroidal alkaloids.

RESULTS AND DISCUSSION

Blood-pressure lowering and pulse rate increase by alkaloid fractions from V. nigrum var. *ussurience and V. maachii, and its constituents, germidine and germerine* The effects on pulse rate (PR) and BP by *Veratrum* alkaloids were compared. The changes in PR and BP by alkaloid fractions and by its derived compounds were calculated as a percentage of the basal values of PR and BP. Extract of *V. nigrum* var. *ussurience* (100 μg/kg, i.p.) produced a rapid increase in PR 2 min after injection (Fig. 2), and then recovered to the PR level by the saline control which was shown in Fig. 3. The extract gradually decreased BP. The effect by the alkaloid fractions from *V. nigrum* var. *ussurience* was prolonged for 60 min. The maximal hypotension was

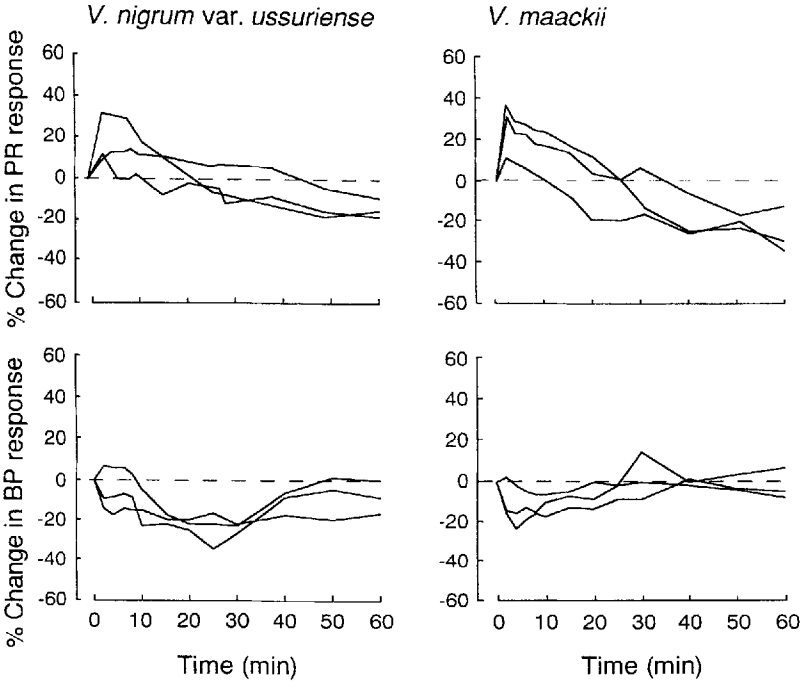


FIGURE 2 Time-dependent increase in PR response (upper panel) and BP lowering (lower panel) by alkaloid fractions of *V. nigrum* var. *ussuriense* (left) and *V. maackii* (right) (100 μg/kg, i.p.) in conscious mice. The PR and BP responses were estimated as percentage changes in the values of PR and BP before the administration of crude drugs, respectively. Values represent the response of each mouse.

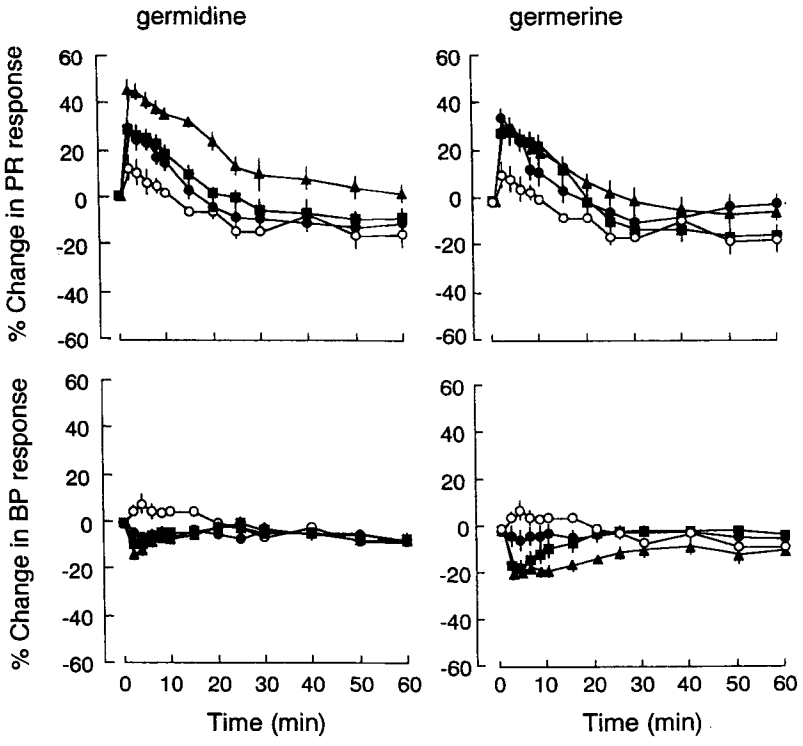


FIGURE 3 Time-dependent increase in PR response (upper panel) and BP lowering (lower panel) by germidine (left) and germerine (right) in conscious mice. Germidine (●, 10; ■, 30; ▲, 100 μg/kg), germerine (●, 10; ■, 30; ▲, 100 μg/kg) and saline (○) were administered i.p. into conscious mice. The PR and BP responses were estimated as in Fig. 2. All values represent mean ± SEM ($n = 7$).

obtained within 30 min. Extract of *V. maackii* (100 μg/kg, i.p.) also produced a rapid increase in PR to the same extent as that produced by *V. nigrum* var. *ussurience*. However, it weakly and transiently decreased BP. The alkaloid fractions from *V. patulum* and *V. schindleri* produced no change in BP responses, but increased PR (data not shown).

Germidine and germerine (10, 30 and 100 μg/kg, i.p.) produced a rapid increase in PR to a level higher than that of the saline control 2 min after injection. The basal values of PR 455 ± 8.3 and 488 ± 11.5 per min were changed to 657 ± 24.4 and 646 ± 26.5 per min by treating with germidine and germerine at 100 μg/kg, respectively ($n = 7$). The responses except at 100 μg/kg were then recovered within 20 min (Fig. 3). The extent of tachycardia by germidine was greater than that by germerine. Germidine and germerine at the same doses as above decreased BP. The basal values of BP 117 ± 3.2 and

118 ± 2.6 mmHg were changed to 101 ± 3.4 and to 96 ± 4.2 mmHg by treating with germidine and germerine at 100 μg/kg, respectively ($n = 7$). The hypotension by germerine was to the greater extent and prolonged longer than that of germidine. Jervine, 15-*O*-(2-methylbutyroyl)germine, verussurinine and neogermbudine (10 μg/kg, i.p.) produced no change in BP responses but increased PR (data not shown).

Positive chronotropic effects of germidine and germerine but negative chronotropic effects of veratridine on right atria Whether tachycardia induced by germidine and germerine was reflected by the BP-lowering was investigated using isolated right atrial preparations. The spontaneous beating rate (frequency, Hz) was measured. Germidine (0.3, 1 and 3 μM) and germerine (0.25, 0.5 and 0.7 μM) induced a positive, but veratridine (0.3, 1 and 3 μM) induced a negative chronotropic effects in a concentration-dependent manner (Figs. 4A and 4C). The maximal responses to germidine and germerine

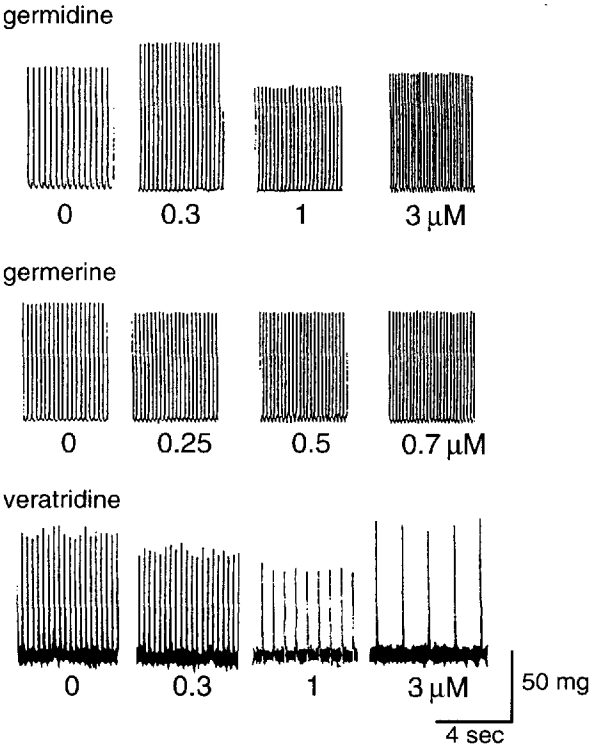


FIGURE 4A Typical traces showing the positive chronotropic effects by germidine (upper) and germerine (middle), and the negative chronotropic effects by veratridine (lower) in isolated right atria.

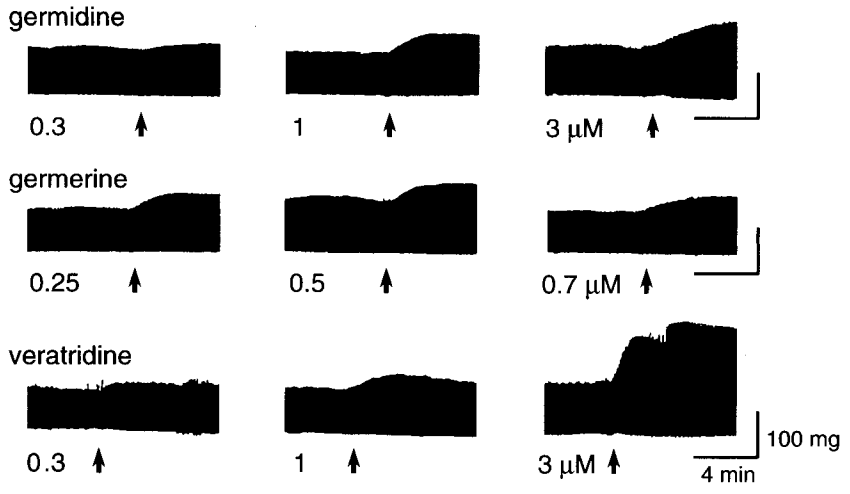


FIGURE 4B Typical traces showing the positive inotropic effects by germidine at 0.3, 1 and 3 μM (upper), germerine at 0.25, 0.5 and 0.7 μM (middle) and veratridine at 0.3, 1 and 3 μM (lower) in isolated left atria of mice. Each trace represents the maximum effects of the respective compound at the designated concentration.

were obtained 2 min whereas the responses to veratridine were observed 20–30 min after the application. These results suggest that tachycardia by germidine and germerine depends mainly on their positive chronotropy but not due to the reflex of BP lowering.

Positive inotropic effects of germidine, germerine, and veratridine on left atria Positive inotropy by three *Veratrum* alkaloids were compared using isolated left atrial preparations. Germidine (0.3, 1 and 3 μM), germerine (0.25, 0.5 and 0.7 μM) and veratridine (0.3, 1 and 3 μM) induced positive inotropic effects in a concentration-dependent manner (Figs. 4B and 4C). The maximal responses were obtained 4–5 min, after the application.

Propranolol-induced partial antagonism of the positive chronotropic and inotropic effect by germidine Whether the positive chronotropy and inotropy by germidine was related to β-adrenergic mechanisms were investigated. The time-dependent effects of the germidine (1 μM) was measured by its single application to the organ bath in the presence and in the absence of propranolol (0.3 and 1 μM), a β-adrenergic antagonist. Propranolol was administered 5–10 min prior to the application of germidine and was present throughout the experiments. The chronotropic and inotropic responses to germidine in the presence of propranolol was observed 10 and 6 min, respectively. The atria treated with 1 μM propranolol still showed triggered activity 6 min after germidine application. Propranolol at 3 μM which is a

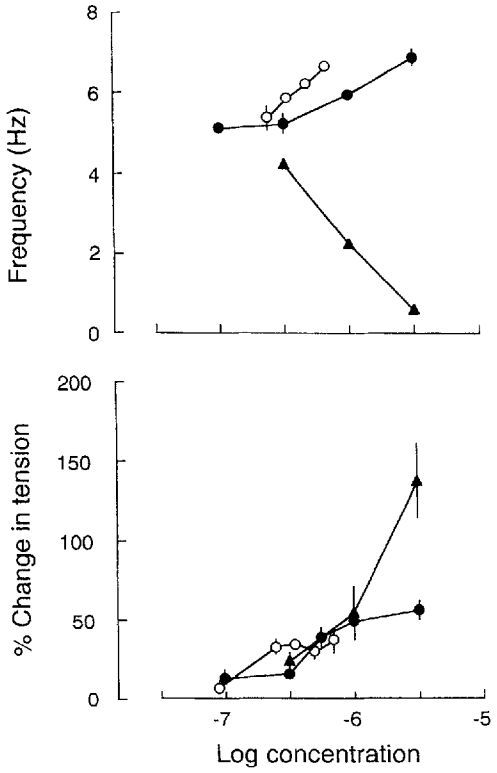


FIGURE 4C Log concentration–response curves of chronotropic (upper) and inotropic (lower) effects by germidine (●), germerine (○) and veratridine (▲) in isolated atrial preparations of mice. The spontaneous beating rate (frequency, Hz) and the percentage change in the values of contraction force before the administration of *Veraturum* alkaloids were measured in isolated right atria and in left atria electrically stimulated at 4 Hz, respectively. All values represent mean ± SEM ($n = 5$).

high concentration enough to block the responses induced by isoproterenol [16] partially blocked both the chronotropic and inotropic responses by germidine. These results suggest that the effects by germidine is not β-adrenergic.

Tetrodotoxin (TTX)-induced blockade of the negative chronotropic effect by veratridine but not the positive inotropic effect by veratridine and germidine The time-dependent effects of the veratridine (1 μM) was measured by its single application to the organ bath in the absence and presence of TTX (1 μM). TTX was administered 15 min prior to the application of veratridine and was present throughout the experiments. The responses to veratridine was observed for 30 min. TTX abolished the negative chronotropy by veratridine (Fig. 5) but not the positive inotropy by veratridine and germidine (1 μM) (data not shown).

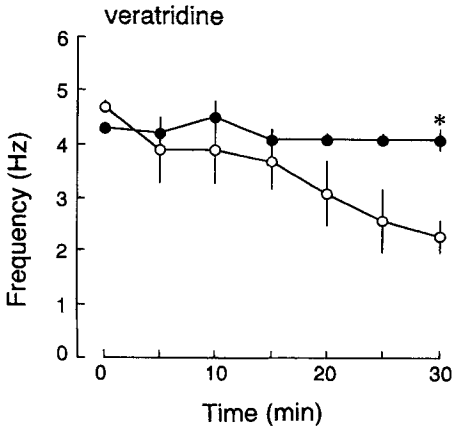


FIGURE 5 TTX-induced blockade of the negative chronotropic effect of veratridine in isolated right atria of mice. The time-dependent effects of the veratridine (1 μM) were measured by its single application to the organ bath in the presence (●) and absence (○) of TTX (1 μM). All values represent mean ± SEM ($n = 3$). $^{*}P < 0.05$: significant difference from data in the absence of TTX by unpaired Student's t-test.

No relaxation effect of germidine on noradrenaline-induced contraction in mouse mesenteric veins Whether the BP lowering by germidine was caused by the relaxation of blood vessels was investigated. Germidine (30 μM) was administered when noradrenaline (NA) (60 μM)-induced contraction was saturated, or 30 min before application of NA (30 μM). In either experiment, germidine did not affect the NA responses. These results suggest that BP lowering by germidine was not upset by the dilatating of blood vessels.

The structure–activity relationship in seven steroidal alkaloids used was as follows: an acyl group (an acetyl or a 2-methylbutyroyl group) at 3-*O*-R^1 position and a 2-methylbutyroyl group at 15-*O*-R^2 position in germine are important to produce the positive inotropy and chronotropy (Fig. 1). Germerine was more potent than germidine in positive inotropy and BP lowering. The presence of a more bulky acyl goup at 3-*O*-R^1 position may be important to induce the effects. The presence of a veratroyl group at 3-*O*-R^1 position and a free hydroxyl group at 15-*O*-R^2 position may be essential to produce the negative chronotropy by veratridine.

There is reflex bradycardia by increased vagal tone, by dilatation of resistance vessels due to reduction of sympathetic vasomotor tone, and by homeostatic feed back control in coordination with respiration. *Veratrum* alkaloids lower BP by eliciting a depressor reflex [17]. The alkaloids abolish the carotid sinus pressor response in dog [17]. The hypercontracture of rat myocyte by veratridine is explained by increases both in intracellular Na^+

and Ca^{2+} because it is not caused in the absence of extracellular Ca^{2+} [18]. There are TTX-sensitive and TTX-resistant sodium channels in rat dorsal root ganglion neurons [19]. TTX- and veratridine-sensitive voltage-gated Na^+ channel which Ca^{2+} permeates is reported in guinea pig ventricular myocytes [15]. The present results suggest the presence of TTX-resistant Na^+-channel activated by veratridine in mouse positive inotropy. The germidine-induced positive inotropy may be caused by the same mechanisms. The positive chronotropy produced by germidine and germerine in isolated atria seems to be inconsistent to their BP lowering effects. Furthermore, BP lowering caused by germidine and germerine may be related neither to positive inotropy nor to relaxation of vascular vessels.

In conclusion, germidine and germerine lower BP accompanied with tachycardia in mice, and positive chronotropy and inotropy in isolated atrial preparations. Germerine was more potent than germidine in BP lowering and positive inotropy, whereas veratridine produced negative chronotropy. The positive inotropy by germidine and veratridine may be due to TTX-resistant Na^+ channel activation.

EXPERIMENTAL SECTION

Plant Material

Roots and rhizomes of *V. nigrum* var. *ussuriense*, *V. maackii*, *V. patulum*, and *V. schindleri* were collected at Qianshan in Liaoning Province, People's Republic of China, in 1985 and identified by Dr. Guo Yun-Zhen at Shenyang Pharmaceutical University. A voucher specimen is deposited at the Research Institute for Medical and Pharmaceutical Science, Dalian.

Extraction and Isolation

Dried roots and rhizomes of *V. nigrum* var. *ussuriense*, *V. maackii*, *V. patulum*, and *V. schindleri* were cut into small pieces and extracted with EtOH at room temperature, respectively. The EtOH solutions were concentrated *in vacuo*. Each residue was dissolved in 5% aqueous tartaric acid solution and insoluble material was removed by filtration. After the filtrate was defatted with ether, a part of the filtrate was basified (pH > 10) by addition of 20% aqueous Na_2CO_3 solution and extracted with $CHCl_3$ to give an alkaloid fraction, which showed a positive reaction with Dragendorff reagent. On the other hand, other part of the filtrate was used for the isolation of the alkaloids, germidine, germerine, 15-*O*-(2-methylbutyroyl)germine,

verussurinine [7], neogermbudine, and jervine [9]. The procedures were reported previously [7].

In Vivo Recordings of the Pulse Rate and Blood Pressure Responses

Male ddY mice weighing 30–42.9 g (7–9 weeks of age) were used. Mice were purchased from Japan Shizuoka Laboratory Center (Shizuoka, Japan) and maintained under constant temperature (23 ± 1°C). They were fed the usual laboratory diet (CA-1, Clea Japan, Inc.) and took tap water freely with lights on from 8 a.m. to 6 p.m. PR and systolic BP were determined simultaneously with a tail artery-cuff using a photoelectric sensor plethysmograph (PS-200, Riken-Kaihatsu, Tokyo, Japan) at a constant temperature (37 ± 1°C), while animals were fully conscious. The pulse detector of plethysmograph was attached to the tail proximal to the occluding cuff. The PR response was continuously measured by a tachometer which was triggered by the detector. The pressure inside the cuff was expressed as the systolic BP. After monitoring the PR and BP every 5 min for 30 min, 0.9% NaCl (saline) or *Veratrum* alkaloid solution was administered intraperitoneally (i.p.) and then the PR and BP were measured for a duration of 60 min. The changes in PR and BP were expressed as the % change from the basal level.

In Vitro Mesurements of the Rate and Force of Contraction in Atria

Mice were sacrificed by decapitation and exanguination under light anesthesia with ether. The heart was rapidly removed and placed in Krebs–Henseleit solution. The solution was composed of 118.4 mM NaCl, 4.69 mM KCl, 2.0 mM $CaCl_2$, 1.16 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$ and 5.0 mM glucose and gassed with 95% O_2–5% CO_2 (pH = 7.4) at 30 ± 1°C. Right and left atria were separated from the ventricles and other tissues. The atrial preparations were connected to an isometric transducer (model UL-2GR, Minebea, Nagano) by silk thread, and were suspended under resting tension of 1 mN in an organ bath containing 2.5 mL Krebs–Henseleit solution. The right and left atrial preparations were equilibrated for 1 h. The spontaneous beating rate (frequency, Hz) was measured in isolated right atria. The left atria were electrically stimulated using two bipolar electrode at 4 Hz (3-ms duration, 1.5 times the threshold potential). The contraction force (tension) was measured in the isolated left atria stimulated electrically at 4 Hz. The stimulus pulse was generated by an electronic stimulator (SEN-3201; Nihon-Kohden, Tokyo) and applied through an

isolator (SS-201J, Nihon-Kohden). The rate and the force of contraction were recorded on a pen-writing oscillograph (type 5108, Nihon-Kohden). The time-dependent effects of the *Veratrum* alkaloids were measured by the single application at each concentration to the organ bath.

In Vitro Measurements of Isometric Tensions in Mouse Mesenteric Veins

Isolated mesenteric veins of mice were used for isometric tension recording. The longitudinal muscle strips (10 mm length, 0.3 mm diameter) were suspended in an organ chamber filled with Krebs–Henseleit solution (5 mL, 37°C) under continuous gassing with a mixture of 95% O_2 + 5% CO_2. A resting tension of 95 mg was applied and changes in isometric contraction were recorded by a pen-recorder as above.

Drugs

Alkaloid fractions of three *Veratrum* plants, and the constituents, germidine, germerine, 15-*O*-(2-methylbutyroyl)-germine, verussurinine, neogermbudine, jervine, and veratridine (Sigma, St. Louis, MO, USA), propranolol hydrochloride (Nacalai Tesque, Kyoto, Japan), (±)-noradrenaline and tetrodotoxin (Sankyo, Tokyo, Japan) were used. Alkaloid fractions and *Veratrum* alkaloids were dissolved in 100 μL of 0.1 N HCl, and then diluted with distilled water. Propranolol, noradrenaline and tetrodotoxin were dissolved in distilled water.

Statistics

Data were expressed as the mean ± SEM. Significant differences were analyzed by the unpaired Student's *t*-test.

Acknowledgments

We are grateful to Mr. M. Mizumoto and Ms. M. Takada for their skillful technical assistance, and to emeritus Profs. Drs. T. Kikuchi and M. Kimura for their suggestive encouragement.

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