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Stimulatory effect of paeoniflorin on the release of noradrenaline from ileal synaptosomes of guinea-pig in-vitro

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Abstract

The effect of paeoniflorin (an active principle of Paeoniae Radix, commonly used in traditional Chinese medicine) on the release of noradrenaline (norepineprhine) from nerve terminals was investigated using guinea-pig isolated ileal synaptosomes. Release was determined as the amount of noradrenaline, quantified by high-performance liquid chromatography–electrochemical detection, from samples incubated with paeoniflorin or vehicle. Paeoniflorin stimulated the release of noradrenaline in a concentration-dependent manner without an effect on the level of lactate dehydrogenase in the bathing medium. Tetrodotoxin abolished the action of paeoniflorin at concentrations sufficient to block sodium channels. The depolarizing effect of paeoniflorin on the membrane potential was also illustrated by a concentration-dependent increase in the fluorescence of bisoxonol. Moreover, the effect of paeoniflorin on bisoxonol fluorescence in ileal synaptosomes seems more potent than that of 4-aminopyridine. That paeoniflorin causes influx of calcium ions via the depolarization of nerve terminals could be considered. The noradrenaline-releasing action of paeoniflorin was abolished by removal of calcium chloride from the bathing medium. This action of paeoniflorin was also attenuated by Rp-cAMP at concentrations sufficient to inhibit the action of cyclic AMP. Therefore, paeoniflorin could induce a calcium-dependent and cyclic-AMP-related release of noradrenaline from sympathetic nerve terminals of guinea-pig ileum. Guanethidine inhibited the noradrenaline-releasing action of paeoniflorin in a concentration-dependent manner. The effect of paeoniflorin on the increase of bisoxonol fluorescence was not modified by atropine. Release of noradrenaline by paeoniflorin from noradrenergic nerve terminals was characterized. These findings suggest that paeoniflorin can stimulate tetrodotoxin-sensitive depolarization of membranes to result in a calcium-dependent and cyclic-AMP-related release of noradrenaline from noradrenergic nerve terminals.

Introduction

Paeoniae Radix is one of the common herbs used in traditional Chinese medicine for treatment of abdominal pain and syndromes involving stiffness of abdominal muscles (Hattori et al 1985). Extracts of Paeoniae Radix produce endothelium-dependent vasodilatation in rat aorta (Goto et al 1996) and inhibition of Na^+,K^+-ATPase (Satoh et al 1997). Paeoniflorin (Figure 1) was purified from Paeoniae Radix as an active principle (Yoshizaki et al 1977) and has been reported to have hypoglycaemic (Hsu et al 1997), cognition-enhancing (Watanabe 1997) and anticonvulsant actions (Abdel-Hafez et al 1998). Also, an inhibitory effect of

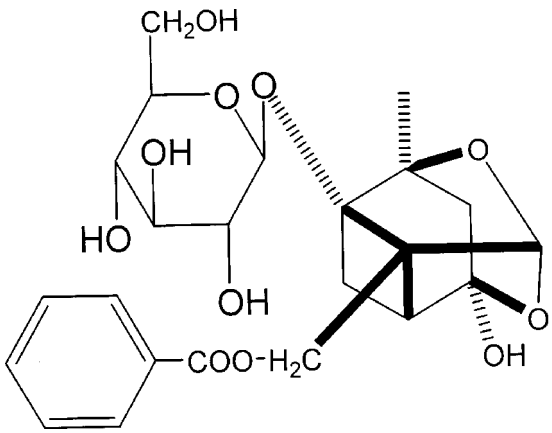

Figure 1 Chemical structure of paeoniflorin.

paeoniflorin on intracellular Ca^{2+} mobilization was observed in the nerve-stimulated skeletal muscle of mice (Dezaki et al 1995). In addition to steroid-like action (Tamaya et al 1986), paeoniflorin has been suggested to attenuate memory deficits following cholinergic dysfunction via noradrenergic mechanisms (Ohta et al 1993). Also, paeoniflorin has the ability to reduce gastrointestinal functions (Takagi & Harada 1969). Both actions of paeoniflorin could be due to an increase of noradrenaline (norepinephrine) release. However, no reports mention this view and the effect of paeoniflorin on the noradrenergic neurotransmission in the gut remains obscure. Thus, we examined the effect of paeoniflorin, purified from Paeoniae Radix as in our previous report (Hsu et al 1997), on noradrenaline release in isolated synaptosomal preparations from the myenteric plexus of guinea-pig ileum. Synaptosomal preparations, the isolated fraction consisting of pinched-off nerve terminals, are useful for analysing the release of neurotransmitter (Nicholls 1993).

Materials and Methods

Preparation of ileal synaptosomes

Guinea-pigs of either sex, weighing 360–430 g, were killed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The ileal segment midway between stomach and ileo-caecal junction (20–25 cm) was dissected from the mesentery and then removed. A crude synaptosomal fraction was prepared according to our previously reported method (Cheng et al 1997). In brief, the minced tissues of the longitudinal muscle strips (Paton & Zar 1968) were homogenized in 0.32 M sucrose-phosphate buffer at 5 mL (g of wet tissue)$^{-1}$. After centrifugation twice at 1000 *g* for 10 min, the two supernatants were pooled and centrifuged at 17000 *g* for 20 min to pellet. Synaptosomes were obtained from a discontinuous sucrose–metrizamide gradient by centrifugation at 20000 *g* for 20 min as described previously (Briggs & Cooper 1981). All procedures were carried out at 4°C.

Experimental protocols

Portions of the synaptosomal preparation (about 1.8 mg protein) were washed and suspended in 1 mL of oxygenated Tyrode's solution with 10 mM xylamine and 0.1 mM pyrogallol. Release was initiated by incubating the preparations with paeoniflorin at the desired concentration in a continuous shaking water bath (65 strokes per min) at 37 ± 1°C for 30 min, the time required to induce maximal response in preliminary experiments. Paeoniflorin was extracted from the root of *Paeoniae lactiflora* (purity $> 98.6\%$) with the same physical properties (including water solubility) as described previously (Hsu et al 1997). Reactions were then terminated by chilling the tubes in an ice bath. Following centrifugation of the tubes at 5000 *g* for 10 min, supernatants were collected for determination of noradrenaline concentrations. Release was calculated as the amount of noradrenaline from samples incubated with paeoniflorin minus the parallel blank treated with the same volume of vehicle (control). Treatment with tetrodotoxin (RBI, Natick, MA), Rp-cAMP (RBI, Natick, MA) or guanethidine (Sigma, St Louis, MO) was started 30 min before incubation with paeoniflorin. Incubation in the absence of calcium chloride was carried out in calcium-free Tyrode's solution with 1 mM ethylene diamine tetraacetic acid (EDTA).

Measurement of released noradrenaline

The concentration of noradrenaline in the supernatants was estimated using HPLC with an electrochemical detector (BAS 200) according to our previous study (Cheng et al 1997). Samples spiked with 20 ng of dihydroxybenzylamine (Sigma, St Louis, MO), the internal standard, were adsorbed onto activated alumina by continuous shaking for 30 min. The alumina was then washed three times with 1 mL of distilled water. The catechols were eluted by 0.1 M perchloric acid by shaking for 10 min. Then, they were lyophilized and

dissolved in 0.03 mL of 0.1 M perchloric acid for injection into the HPLC through an autoinjector. All values were corrected for recovery (78–82%) and expressed as pmol noradrenaline per mg of synaptosomal protein determined as previously reported (Lowry et al 1951). Also, incubation of standard noradrenaline with the compounds tested in this study showed that they did not interfere with the measurement of noradrenaline.

Determination of lactate dehydrogenase (LDH) activity

Activity of lactate dehydrogenase (LDH), the enzyme located in cytosol, was determined using a commercial kit (Besteck Biotech., USA). As described previously (Mercer 1978), the obtained supernatant (0.02 mL) was mixed with 1 mL of working solution containing 0.35 mM NADH and 0.63 mM sodium pyruvate in 0.1 M phosphate buffer (pH 7.6). After incubation at 37°C for 1 min, kinetic measurement at 340 nm for 2 min was carried out in duplicate using an ultraviolet spectrophotometer (Hitachi U-3210, Tokyo, Japan).

Determination of synaptosomal membrane potential variations

Following previously reported methods for monitoring changes in membrane potential (Lakos et al 1990; Regazzi et al 1990), the lipophylic anion bisoxonol was added at 300 nM into the quartz cuvette of the spectrofluorimeter containing 2 mL of the pre-warmed medium. One minute later, synaptosomal protein (0.3–0.5 mg) was pipetted into the cuvette. Like 4-aminopyridine (Sigma, St Louis, MO), paeoniflorin at the desired concentration was then added into the cuvette during the stable state of fluorescence recorded in the spectrophotometer; an excitation and emission wavelength of 485 and 515 nm was used, respectively (Lakos et al 1990). Treatment with atropine (Sigma, St Louis, MO) or acetylcholine (Sigma, St Louis, MO) was started 30 min before incubation with paeoniflorin. Similar to a previous report (Tagialatela et al 1990), bisoxonol (Molecular Probes, Inc., Eugene, OR) fluorescence intensity variations were not converted into absolute membrane potential values using the valinomycin null-point method (Rink et al 1980). Data of intensity variation were expressed as the arbitrary unit of F/Fo where F is the peak of intensity increase by paeoniflorin or 4-aminopyridine and Fo is the basal fluorescence, as previously described (Rodriguez-Pascual et al 1995). In preliminary experiments, paeoniflorin or 4-aminopyridine at the concentrations tested had no effect on the bisoxonol fluorescence in the absence of synaptosomal protein.

Statistical analysis

Values of mean ± s.e.m. for each group were obtained from number (n) of samples. Number (n) of experiments was the number of separate studies from different synaptosomal preparations. Statistical analysis of the differences between two mean values was assessed using Student's *t*-test; a P value of $\leqslant 0.05$ was considered significant. Where two or more of the obtained means were compared with one control mean, analysis for significance ($P < 0.05$) was carried out using one-way analysis of variance in conjunction with Dunnett's post-hoc test.

Results

Effect of paeoniflorin on the release of noradrenaline from ileal synaptosomes

Incubation of guinea-pig ileal synaptosomes with paeoniflorin induced an increase in noradrenaline release in a time-related manner; the release of nor-

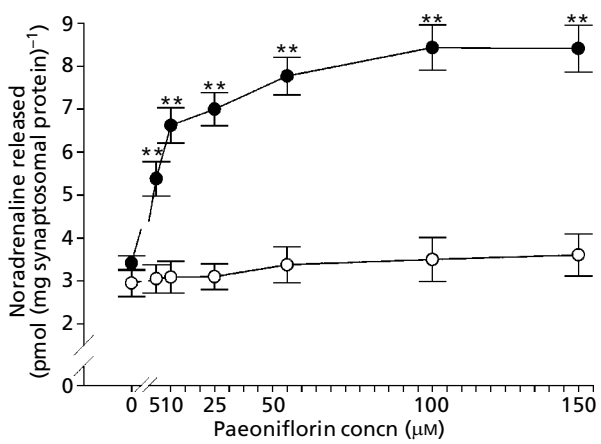

Figure 2 Concentration–response effect of paeoniflorin on noradrenaline release from isolated ileal synpatosomes of guinea-pig. The noradrenaline release was measured in samples incubated for 30 min with the indicated concentration; 0 indicates the spontaneous release of noradrenaline. Each point represents the mean ± s.e.m. of 8 separate experiments in normal (●) or in calcium-free (○) medium. $^{**}P < 0.01$ vs corresponding value in the absence (0) of paeoniflorin via unpaired comparison.

adrenaline reached a plateau within 25 min of treatment in 6 preliminary experiments (data not shown). In all subsequent experiments, synaptosomes were incubated with paeoniflorin for 30 min. The release of noradrenaline was increased in a concentration-dependent manner, over the range 5–50 μM, by paeoniflorin (Figure 2). The stimulatory effect of paeoniflorin was not further increased even at 150 μM, the supramaximal concentration. To produce marked release of noradrenaline, 50 μM of paeoniflorin was used in subsequent experiments.

Effect of paeoniflorin on lactate dehydrogenase (LDH) activity in ileal synaptosomes

Lactate dehydrogenase (LDH) activity was not modified in synaptosomes incubated with paeoniflorin. The activity of LDH in samples treated with paeoniflorin at 50 μM, the maximal concentration tested, was 0.10 ± 0.01 IU mL^{-1} (n = 8) which was not different ($P > 0.05$) from the vehicle-treated control (0.11 ± 0.01 IU mL^{-1}; n = 8).

Effect of guanethidine on the action of paeoniflorin

Guanethidine significantly inhibited the paeoniflorin-induced release of noradrenaline from guinea-pig ileal synaptosomes at a concentration of 1.5 μM (Table 1). A higher concentration of guanethidine (3 μM) reduced the paeoniflorin-induced noradrenaline release to 4.7 ± 0.4 pmol (mg synaptosomal protein)$^{-1}$, which was not different ($P > 0.05$) from the vehicle-treated control (4.0 ± 0.8 pmol (mg synaptosomal protein)$^{-1}$). Also, guanethidine at 3 μM attenuated the spontaneous secretion of noradrenaline mildly without statistical significance ($P > 0.05$) when compared with the vehicle-treated control (Table 1).

Effect of paeoniflorin on membrane potential

The release of noradrenaline stimulated by paeoniflorin was markedly reduced in synaptosomes pretreated (30 min) with tetrodotoxin ; a concentration-related attenuation of paeoniflorin-induced noradrenaline release was observed (Table 2). At the highest concentration (2 μM), tetrodotoxin abolished the paeoniflorin-induced release of noradrenaline (4.0 ± 0.7 pmol (mg synaptosomal protein)$^{-1}$) to a level not different ($P > 0.05$) from the vehicle-treated control (4.1 ± 0.7 pmol (mg synaptosomal protein)$^{-1}$). However, the spontaneous secretion of noradrenaline was not significantly influenced by tetrodotoxin (Table 2). When synaptosomal preparations were exposed to paeoniflorin, a concentration-dependent depolarization, monitored by the increase of bisoxonol fluorescence, was obtained (Figure 3). Depolarization of the membrane potential by paeoniflorin was observed at a concentration of 10 μM and reached a maximum at 50 μM. However, depolarization of the membrane potential by paeoniflorin was not further increased even at the supramaximal concentration of

Table 1 Effect of guanethidine on paeoniflorin-induced release of noradrenaline from isolated ileal synaptosomes of guinea-pigs.

	Noradrenaline released (pmol (mg synaptosomal protein)$^{-1}$)	
	Spontaneous	Paeoniflorin (50 μM)
Vehicle	4.0 ± 0.8 (n = 8)	7.4 ± 0.5 (n = 8)
Guanethidine (μM)		
0.1	3.9 ± 0.6 (n = 6)	7.0 ± 0.6 (n = 8)
1	3.8 ± 0.4 (n = 6)	6.0 ± 0.5 (n = 6)
1.5	3.7 ± 0.3 (n = 7)	5.1 ± 0.4* (n = 6)
3	3.5 ± 0.5 (n = 8)	4.7 ± 0.3* (n = 8)

All values shown are means$\pm$s.e.m. from 6–8 separate experiments. Incubation with paeoniflorin was for 30 min while guanethidine was added 30 min before paeoniflorin. Vehicle solutions were without addition of drug. *$P < 0.05$ vs vehicle-treated control, respectively, via unpaired comparison with Dunnett's post-hoc test.

Table 2 Effect of tetrodotoxin on paeoniflorin-induced release of noradrenaline from isolated ileal synaptosomes of guinea-pigs.

	Noradrenaline released (pmol (mg synaptosomal protein)$^{-1}$)	
	Spontaneous	Paeoniflorin (50 μM)
Vehicle	4.1 ± 0.7 (n = 8)	7.5 ± 0.4 (n = 8)
Tetrodotoxin (μM)		
0.1	4.0 ± 0.6 (n = 8)	7.0 ± 0.6 (n = 8)
0.5	3.9 ± 0.7 (n = 7)	5.7 ± 0.7* (n = 8)
1	3.8 ± 0.5 (n = 6)	4.7 ± 0.8* (n = 8)
2	3.8 ± 0.4 (n = 7)	4.0 ± 0.7* (n = 7)

All values shown are means$\pm$s.e.m. from 6–8 separate experiments. Incubation with paeoniflorin was for 30 min while tetrodotoxin was added 30 min before paeoniflorin. Vehicle solutions were without addition of drug. *$P < 0.05$ vs vehicle-treated control, respectively, via unpaired comparison with Dunnett's post-hoc test.

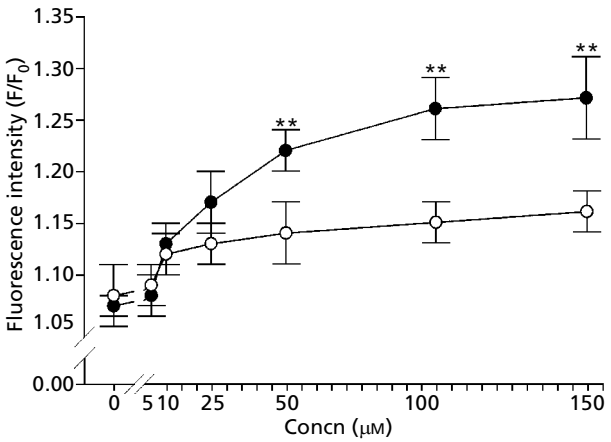

Figure 3 Effect of paeoniflorin (●) or 4-aminopyridine (○) on membrane potential in ileal synaptosomes of guinea-pig monitored by bisoxonol dye. Values are the arbitrary unit of F/Fo where F represents the peak intensity induced by drugs and Fo the basal fluorescence. Each point shows the mean±s.e.m. of 6 separate determinations. $^{**}P < 0.01$ vs vehicle-treated control (0) via unpaired comparison. Vehicle solutions were without addition of drugs.

Table 3 Effect of atropine on membrane potential induced by paeoniflorin in isolated ileal synaptosomes of guinea-pigs monitored with bisoxonol dye.

	Fluorescence intensity (F/Fo)
Vehicle	1.09±0.01 (n = 7)**
Paeoniflorin (50 μM)	
+vehicle	1.27±0.03 (n = 6)
+acetylcholine (1 μM)	1.16±0.05 (n = 7)**
+atropine (1 μM)	
+vehicle	1.25±0.03 (n = 8)
+acetylcholine (1 μM)	1.26±0.02 (n = 8)

All values shown are means±s.e.m. of arbitrary unit of F/Fo from 6–8 separate experiments. F represents the peak intensity induced by paeoniflorin and Fo the basal fluorescence. Vehicle solutions were without addition of drug. $^{**}P < 0.01$ vs paeoniflorin (50 μM)-treated group via unpaired comparison with Dunnett's post-hoc test.

150 μM. No marked change in basal fluorescence was observed during incubation with distilled water at the same volume under similar conditions. The concentration-dependent increase of bisoxonol fluorescence was also observed in ileal synaptosomes incubated with 4-aminopyridine (Figure 3), but the depolarization of the membrane potential of ileal synaptosomes was markedly lower than that produced by the same concentration (50 μM) of paeoniflorin.

Moreover, the effect of paeoniflorin (50 μM) on the increase of bisoxonol fluorescence was not modified by atropine at a concentration sufficient to block muscarinic receptors. As shown in Table 3, the value of F/Fo (1.25±0.02; n = 7) was not different ($P > 0.05$) from that in the absence of 1 μM atropine (1.27±0.03; n = 6). Incubation with acetylcholine (1 μM) reduced the depolarization produced by paeoniflorin markedly ($P < 0.05$); the value of F/Fo became 1.16±0.05 (n = 7), and this inhibition was reversed by 1 μM atropine (1.25±0.03; n = 7).

Role of calcium ions and cyclic AMP in the action of paeoniflorin

In the absence of calcium ions (calcium chloride-free medium), both the spontaneous noradrenaline release from guinea-pig ileal synaptosomes and the stimulatory action of paeoniflorin were markedly lowered as compared with that in the presence of calcium ions (Figure 2). Also, incubation with Rp-cAMP attenuated the paeoniflorin-induced release of noradrenaline over the concentration range 1–100 nM (Figure 4). The spontaneous secretion of noradrenaline was also lowered by Rp-cAMP at the highest concentration of 100 nM (3.4±0.7 pmol (mg synaptosomal protein)$^{-1}$, n = 8). However, it was not statistically different ($P > 0.05$)

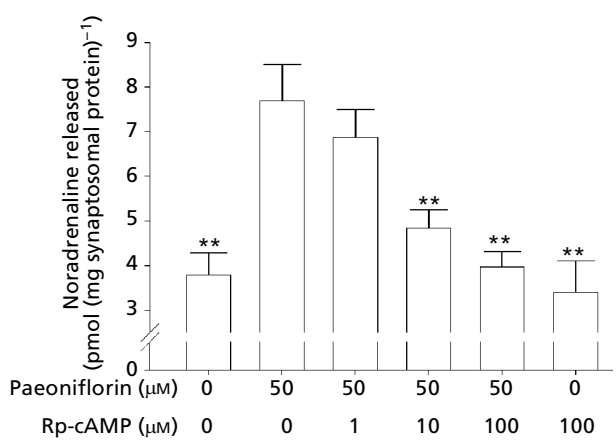

Figure 4 Effect of Rp-cAMP, the antagonist of cyclic AMP, on paeoniflorin-induced release of noradrenaline from ileal synaptosomes of guinea-pig. The noradrenaline release was measured in samples incubated for 30 min with paeoniflorin at 50 μM while Rp-cAMP at the indicated concentration was added 30 min before paeoniflorin; 0 indicates the spontaneous release of noradrenaline. Each point represents the mean±s.e.m. of 6 separate experiments. $^{**}P < 0.01$ vs the value of paeoniflorin at 50 μM in the absence of Rp-cAMP via unpaired comparison.

from the vehicle-treated control (3.9±0.5 pmol (mg synaptosomal protein)$^{-1}$, n = 8).

Discussion

In this study, we found that paeoniflorin stimulates the secretion of noradrenaline from noradrenergic terminals of guinea-pig ileum in a concentration-dependent manner over the range 5–50 μM. At these concentrations, paeoniflorin did not influence the level of LDH, one of the cytosolic enzymes, suggesting its action is not due to damage of the cell membrane.

Release of noradrenaline by paeoniflorin from noradrenergic nerve terminals was characterized in two ways. One was blockade with guanethidine at concentrations sufficient to block noradrenergic nerve terminals (Cheng et al 2000). Guanethidine has been documented as a specific blocker of noradrenergic nerve terminals (Starke 1972). Another was that atropine failed to modify the action of paeoniflorin (Table 3) at a concentration sufficient to block muscarinic receptors which may be stimulated by acetylcholine release from the cholinergic nerve terminals. Therefore, specific action of paeoniflorin on the noradrenergic nerve terminals can be considered.

The increase in noradrenaline release produced by paeoniflorin was abolished by the removal of calcium chloride from the bathing medium, indicating dependence on calcium ions. Moreover, Rp-cAMP, an inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinase A (Wang et al 1991), suppressed the noradrenaline-releasing action of paeoniflorin (Figure 4) within a concentration range effective at blocking the action of cyclic AMP (Murthy et al 1995). It has been documented that cyclic AMP is involved in the release of neurotransmitter (Ouedraogo et al 1994). The results we obtained are consistent with this and show that paeoniflorin induced a calcium-dependent and cyclic-AMP-related release of noradrenaline from noradrenergic terminals of guinea-pig ileum.

It has been established that influx of calcium ions is related to the depolarization of nerve terminals (Tagialatela et al 1990). Thus, we used bisoxonol, the fluorescencent dye, to investigate the effect of paeoniflorin on the membrane potential of isolated synaptosomes. This dye is a lipophilic anion that freely permeates the cell, its distribution across the cell membrane being dependent upon membrane potential (Lakos et al 1990). When the membrane potential is depolarized, as described in cerebral synaptosomes of the rat (Tagialatela et al 1990), it allows more of this negatively charged dye to enter the cells, leading to an increase of fluorescence. The significant increase in bisoxonol fluorescence in ileal synaptosomes brought about by paeoniflorin (Figure 3) demonstrates the involvement of membrane depolarization. As a positive control, we employed 4-aminopyridine that can stimulate the release of neurotransmitters from nerve endings (Thesleff 1980). We observed that paeoniflorin-induced increase in bisoxonol fluorescence in ileal synaptosomes was more marked than that induced by 4-aminopyridine (Figure 3). In general, 4-aminopyridine is introduced as a K^+ channel blocker (Tapia et al 1985; Sugimori et al 1987). The marked difference from 4-aminopyridine ruled out the K^+ channel blocker-like action of paeoniflorin. Moreover, as shown in Table 2, paeoniflorin-stimulated noradrenaline release was attenuated by tetrodotoxin at concentrations sufficient to inhibit the depolarization-evoked release of various neurotransmitters from guinea-pig myenteric plexus (Katsoulis et al 1992). Tetrodotoxin is introduced as a specific blocker of sodium channels (Narahashi 1974) and the tetrodotoxin-sensitive release of neurotransmitter is usually due to an excitation of nervous cells (Ritchie & Rogart 1977). The release of noradrenaline from guinea-pig myenteric plexus by paeoniflorin via a tetrodotoxin-sensitive depolarization can thus be considered. Taken together, therefore, it is indicated that paeoniflorin causes influx of calcium ions via the depolarization of nerve terminals. Otherwise, reduction of calcium influx by paeoniflorin has been observed in isolated atria (Tsai et al 1997) and aorta (Tsai et al 1999). The difference in calcium influx between nerve and muscle can be used to explain this discrepancy.

The interaction of paeoniflorin with neurons is still not clearly investigated, except for the protective effect on neuron damage in the hippocampus induced by the cobalt epilepsy model (Tsuda et al 1997). Improvement of impaired learning by paeoniflorin has been mentioned (Ohta et al 1993). CNS effects of paeoniflorin (Cheng et al 1999) or paeony root extract (Tsuda et al 1997) have been documented. In a rat hippocampal slice, paeoniflorin reversed muscarinic M1 receptor antagonist-induced suppression of long-term potentiation (Tabata et al 2000). This view is consistent with the cognition-enhancing action of paeoniflorin (Watanabe 1997). However, there is no information indicating the effect of paeoniflorin on the nervous activity of peripheral tissues. Also, the reason why paeoniflorin affects noradrenaline release but not acetylcholine release is still unknown. Thus, the detailed mechanisms for paeoniflorin-induced depolarization of noradrenergic terminals remain to be clarified. Nevertheless, our findings are available to

explain the decrease of gastrointestinal functions produced by paeoniflorin (Takagi & Harada 1969). Decrease in intestinal tone by paeoniflorin due to noradrenaline release would be useful in its clinical application.

In conclusion, our results suggest that paeoniflorin can stimulate noradrenaline secretion from noradrenergic nerve terminals of guinea-pig ileum via a tetrodotoxin-sensitive depolarization of the membrane.

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