Blocking Effects of Blended Paeoniflorin or Its Related Compounds with Glycyrrhizin on Neuromuscular Junctions in Frog and Mouse

Masayasu KIMURA, Ikuko KIMURA, Kazuyoshi TAKAHASHI, Masashi MUROI, Masao YOSHIZAKI*, Matao KANAOKA** and Isao KITAGAWA***

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, *Institute of Herbary and **Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan
***Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Osaka University, Suita 565, Japan

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Abstract—The combined effects of paeoniflorin (PF), a main component of paeony roots, and glycyrrhizin (GLR), a main component of licorice roots, were investigated on isolated sciatic nerve-sartorius muscle preparations in frogs, or on isolated or in situ phrenic nerve-diaphragm muscle preparations in mice, intending to explain the effects of "Shakuyaku-Kanzô-Tô", composed of both these Chinese drugs, on clinical neuropathy. PF and GLR used together blocked indirectly stimulated twitchings at concentrations which when used alone induced no blocking effects. PF and GLR at a combining ratio of 1:2 (weight concentrations) corresponding to the amounts contained in "Shakuyaku-Kanzô-Tô", was more potent than when they were used at the ratio of 1:1 or 2:1. The synergistic effects induced by GLR were also confirmed for the other components, paeoniflorigenone or oxypaeoniflorin, which are contained in paeony roots, and for succinylcholine. The blocking effect of d-tubocurarine were not increased by GLR. Concludedly, PF and GLR were found to cause the pharmacological blend effect. The two combined compounds were mainly therapeutic components in "Shakuyaku-Kanzô-Tô".

The blended therapeutic, "Shakuyaku-Kanzô-Tô", which has been used as one of Kampô-Hozai by Chinese and Japanese in traditional medicine, consists of paeony roots and licorice roots in an even dose. It has been known to clinically improve neuropathy. There is no pharmacological evidence that the main principles of both roots, paeoniflorin (PF) and glycyrrhizin (GLR), have relaxing effects on skeletal muscles, although Takagi and Harada (1) have investigated several effects of PF and GLR on several tissues. Fortunately, our groups (2) have just found a new principle in paeony roots that we have named paeoniflorigenone (PFG). We found that PFG greatly blocked indirectly stimulated twitch responses (3) and reported preliminarily that the PFG-induced blocking effects were increased by GLR, although the effect was not potent. This finding has become a clue for investigating the blend effects of PF and GLR and now they are involved in the clinical improvement of neuropathy by "Shakuyaku-Kanzô-Tô". The experiments were designed to compare the combined effects of PF and GLR with those of GLR and PF-related compounds such as albiflorin, oxypaeoniflorin, benzoylpaeoniflorin (4) and benzoyloxy-paeoniflorin (5).

Materials and Methods
Sciatic nerve-sartorius muscle preparations isolated from the frog (Rana nigromaculata) weighing 17–34 g were suspended in 3 ml of a modified Ringer’s solution (111 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2 and 2.4 mM NaHCO3) being bubbled
with 95% \( O_2 \)+5% \( CO_2 \) at room temperature (22–26°C). For the twitch contraction of directly stimulated muscles, supramaximal square wave pulses of 2 msec duration were applied through one pair of electrodes placed directly on the muscle. The second pair of electrodes was applied to the nerve. The muscle was alternatively stimulated indirectly or directly by pulses of supramaximal intensity (duration: 2 msec and frequency: 0.2 Hz). The twitch contractions of muscles were measured with the classically isotonic lever and continuously recorded on an ink-writing drum.

The phrenic nerve-diaphragm muscle preparations isolated from mice (male ddY-strain, 29–46 g) were suspended in 5 ml of Krebs-Henseleit solution (118 mM \( NaCl \), 5.4 mM \( KCl \), 0.57 mM \( MgSO_4 \), 2.5 mM \( CaCl_2 \), 1.2 mM \( NaH_2PO_4 \), 11.1 mM glucose and 12–15.5 mM \( NaHCO_3 \), pH 7.3±0.5) being bubbled with 95% \( O_2 \)+5% \( CO_2 \) at 36–37°C. The phrenic nerve was placed over platinum electrodes and stimulated at a rate of 0.2 Hz by square wave pulses (supramaximally, 240–360 mV) of 1.0–2.5 msec duration. The twitch responses of the muscles were recorded with an isometric transducer (Nikon-Kohden, SB-1T-H) on a Bio-physiograph 110 system (San-ei). A resting force of 1 g was applied to each tissue.

ddY Male mice (7–8 weeks age) weighing 30–35 g were anaesthetized by intraperitoneal injection of urethane (1.7 g/kg). The mouse was transferred to a warm experimental table (36.5°C). A tracheal cannula was inserted and was connected to a Harvard rodent respirator (type 680) under the condition of 1.3 ml and 170 strokes/min. The drug at 0.1 ml/10 g weight was administered through the left femoral artery which was cannulated by a glass microtube (diameter of less than 0.1 mm) connected via a polyethylene catheter (ATOM, 1 mm) to a glass syringe. The left gastrocnemius muscle was freed from the adjacent muscles, leaving the vascular vessels intact. The sciatic nerve was separated and cut at a position as proximal as possible. All branches of the sciatic nerve, except the branch which innervates the gastrocnemius muscle, were cut. We always verified that there was no thrombosis in the femoral artery after the experiment. The sciatic nerve was stimulated under the same conditions as used in vitro.

The drugs used were d-tubocurarine Cl (d-TC, Nakarai), succinylcholine chloride (Suxamethonium, SuCh, Nakarai), neostigmine methylsulfate (Neo, Sigma), glycyrrhizin monoammonium salt (GLR, a kind gift from Dr. S. Yabuki, Minophagen Co.) and urethane (Nakarai). Paeoniflorigenone was a kind gift from Dr. M. Shimizu (Dept. of Pharmacognosy, the same University). PF

Table 1. The chemical structures of paeoniflorin-related compounds

| **PAEONIFLORIN** | \( R_1 \) | \( R_2 \) |
|-----------------|------|------|
| OXYPAEONIFLORIN | \( OH \) | \( H \) |
| BENZOYLPAEONIFLORIN | \( H \) | \( C=O \) |
| BENZOYLOXYPAEONIFLORIN | \( OH \) | \( C=O \) |

| **ALBIFLORIN** |
|----------------|
| \( OH \) | \( C=O \) |
| \( OH \) | \( C=O \) |

| **PAEONIFLORIGENONE** |
|-----------------------|
| \( CH_3O \) | \( COCH_3 \) |

| **PAEONOL** |
|-------------|
| \( CH_3O \) | \( COCH_3 \) |
and albiflorin (AF) were prepared according to the method reported by Yoshizaki et al. (6). Oxypaeoniflorin (O-PF), benzoylpaeoniflorin (B-PF), benzoyloxypaeoniflorin (B-O-PF) and paeonol (P) were prepared by the method reported by Kitagawa et al. (5). Glycyrrhetinic acid monoglucuronate were prepared by Dr. M. Kanaoka (unpublished methods). Fifty % ethanol extracts of licorice roots were prepared by the procedure reported by Namba et al. (7). The chemical structures of PF-related compounds are shown in Table 1.

**Results**

Concentration-dependent blocking effects caused by paeoniflorin and glycyrrhizin in “Shakuyaku-Kanzō-Tō”**: In frog sciatic nerve-sartorius muscle preparations, PF alone (600 μM, 289 μg/ml) or GLR alone (50 nM, 42 μg/ml) induced no blocking, but the combination of both drugs showed very marked blocking action (Fig. 1). When “Shakuyaku-Kanzō-Tō” was clinically used to improve neuropathy, the weight ratio of PF and GLR contained in the blended drugs is empirically estimated to be 1:2. As shown in Fig. 2, this weight concentration ratio (1:2) of PF and GLR was more potent in the blocking actions than those of 1:1 and 2:1, where % inhibition of twitch responses 60 min after applying these components were plotted for control responses for 1 min. When one PF was combined with 6-fold weight concentrations of 50% ethanol extracts (in which GLR was estimated to be contained in 30% yield), the blocking effect corresponded to that of a 1:2 ratio of PF and GLR, as shown in Fig. 2. In order to compare explicitly the extract-induced effect with that of the isolated compound, a weight concentration was plotted instead of a molar concen-

![Fig. 1. Blend effects of 600 μM paeoniflorin (PF) and 50 μM glycyrrhizin (GLR) on twitch responses of sartorius muscle evoked by alternate stimulation of sciatic nerves and muscles (0.2 Hz, 1 msec and supramaximal voltage) in Ringer’s solution bubbled with carbogen. Note that at the concentrations of PF or GLR alone which induced no effect, the combination of both revealed blocking effects which were not reversed by neostigmine (200 μM).](image-url)
tration. These results suggest that the blend effects are not attributable to any other component which is different from GLR in licorice roots, and that the effect of "Shakuyaku-Kanzō-Tō" may be caused by the blended PF and GLR.

Increase in blocking actions of indirectly stimulated twitching by the combination of glycyrrhizin and paeoniflorin-related compounds in vitro: In frog sciatic nerve-sartorius muscle preparations, 0.4 mM PF, oxy-

Fig. 2. The blend effects of various weight ratios (●: 1:2; ○: 2:1; and □: 1:1) of PF to GLR (straight line, n=2–5) and the effect of the 1:2 ratio of PF:GLR compared with that of the 1:6 ratio of PF plus 50% ethanol extracts of licorice roots (dotted lines, means±S.E., n=3–4). This extract was estimated to contain 30% of GLR. Note that the combination of one PF to two GLR induced the most potent effect and not less potent inhibition of twitch responses than the combination of PF with 6 vol. of the extract.

Fig. 3. The blend effects of 0.4 mM PF-related compounds alone or 0.05 mM GLR alone (open symbols) and 0.4 mM PF-related compounds plus 0.05 mM GLR (closed symbols) on isotonic twitch tones induced by indirect stimulation of sciatic nerve-sartorius muscle preparations of frogs (n=3).
were not reversed by 30 and 60 µg/ml neostigmine. These results suggest that the reaction mechanism was quite different from that of a competitive acetylcholine receptor blocker, d-tubocurarine, and it rather acted like a depolarizing blocker, succinylcholine (suxamethonium, SuCh).

FM 100 (300 µg/ml), another component in licorice roots, did not increase the PFG (300 µg/ml)-induced blocking action (data not shown).

The same blend effects in situ by the combination of glycyrrhizin and paeoniflorin:

Even when PF and GLR were intravenously administered at high concentrations (120 mg/kg), no blocking effects were observed (data not shown). Then the blocking effects by intraarterially administered SuCh were compared with those by intravenously applied

SuCh in Fig. 5, where % inhibition of twitch responses were plotted for control responses for 30 sec. The log dose-response curves of intraarterially administered SuCh was shifted to the left 10-fold more than those of intravenously administered SuCh. The 50% effective dose of the former was 31.5 µg/kg (25.8–38.5 µg/kg), and that for the latter was 217 µg/kg (183–256 µg/kg) (n=3–5).

When PF (8.5 mg/kg) and GLR (17 mg/kg) were intraarterially administered, the blend effects were clearly observed as shown in Fig. 6, where the concentration of PF alone demonstrated weak blocking effects and the concentration of GLR alone did not induce any blocking effects. The blend effects of PF and GLR were plotted against total concentrations of 18–36 mg/kg (26–54 µmolar/kg) in Fig. 7. GLR is a glycyrrhetinic acid diglucuronate. The chemically essential structure of GLR required to induce these combined effects was investigated with PF combined with glycyrretinic acid monogluconrate (GAG). The log dose-response curve of PF and GLR was compared with that of PF and GAG in Fig. 7. GAG alone (37 mg/kg, 57 µmolar/kg) induced no blocking (data not shown). These results demonstrated that the blocking effects of blended PF and GLR on neuromuscular junctions were not only confirmed in in vitro but also in situ, suggesting that the blend effect of GLR does not involve the reaction of GAG; one of the GLR metabolites.
A possible mechanism of GLR action coupled to the depolarization: The blend effects of representative neuromuscular blocking agents and GLR were investigated. In these experiments, the same ratios of weight concentrations of d-tubocurarine (d-TC) or SuCh and GLR were investigated. GLR (5 and 10 μg/ml) remarkably increased the blocking effects by SuCh (5 and 10 μg/ml) (Fig. 8). In contrast, the blocking actions by d-TC (3 and 5 μg/ml) was not increased by GLR (3 and 5 μg/ml) (data was not shown). These results indicated that GLR increased blocking effectively only in combination with depolarizing neuromuscular blocking agents, because the blocking action of PFG, one of the depolarizing agents (4), was also increased by GLR.

**Discussion**

"Shakuyaku-Kanzō-Tō" is the most simple blended therapeutic composed of two crude drugs, paeony roots and licorice roots, and it has been clinically used for neuropathy such as the improvement of muscle pain and the cure for a tremor in skeletal muscle (8). The purpose of our study was to investigate the mechanism of "Shakuyaku-Kanzō-Tō" on neuromuscular junctions, and thereby find an important and simple approach to the analysis of the complex principle in the blend effect. It is necessary to know the reason why paeony roots and licorice roots must be combined to improve neuropathy. First we investigated the neuromuscular blocking effects of the combination of PF (a main component of paeony roots) and GLR (a main component of licorice roots) in vitro and in situ. In a previous paper, we reported that PF induced no anti-cholinergic action in the slow contraction, whereas PFG produced weak anti-cholinergic actions. In the this
Fig. 8. Typical records of indirectly stimulated twitch responses in isolated phrenic nerve-diaphragm muscle preparations of mice in the presence of succinylcholine (SuCh) alone (upper, 5 µg/ml and lower left, 10 µg/ml), SuCh plus GLR (middle, 5 plus 5 µg/ml, and lower right, 10 plus 10 µg/ml). Note that GLR also increased the blocking action induced by SuCh alone. These blocking actions were hardly reversed by 2–4 µg/ml neostigmine.

paper, the remarkable combined effects by PF-related compounds plus GLR on fast contractions (twitching) were demonstrated.

Because the blend effect in “Kampo-Hōzai” has been conventionally exemplified in weight ratios of crude drugs, we used the weight concentration ratio of combined principles instead of a molar concentration. Thus, the blended weight ratio of PF and GLR in paeony roots is estimated to be 1:2. Effects of this combination ratio of both components was more potent than that with a ratio of 1:1 or 2:1. Fractions extracted with 50% ethanol from licorice roots are estimated to contain GLR in the yield of 30%. Because the combination ratio (1:1) of PF and GLR had almost the same potency as a 1:6 ratio of PF and EtOH extracts, any components in these extracts other than GLR are not considered to contribute to the blocking action. Therefore, the effectiveness of “Shakuyaku-Kanzō-To” can be explained by the combination of PF and GLR.

Paeony roots reportedly contain 2.2% PF, 0.22% AF, 0.05% PFG, 0.02% O-PF and 0.02% B-PF (6). Paeonol and B-O-PF were mainly contained in Moutan Cortex (5). In both mouse phrenic nerve-diaphragm muscle preparations in vitro and sciatic nerve-gastrocnemius muscle preparations in situ, the blocking action of PF was most potent of the PF-related compounds tested. The results suggested that PF seemed to play a main role in these actions induced by paeony roots.

As the effect of PF and GLR was more potent than that of PF and GAG, GAG, one of the metabolites of GLR (9), seemed not to play a direct role in these effects.

The blend effects with GLR are regarded to be restricted to depolarizing neuromuscular blockers, because GLR did not increase the blocking action of d-tubocurarine, but increased those of SuCh, PFG and PF. GLR may reveal the blend effect to a greater extent in a depolarizing blocked state in which there is a high permeability of potassium ion out of plasma membranes.

PFG alone and the combination of PF and GLR initiated the blocking action at longer
intervals after bath application. This mechanism is still uncertain.

In conclusion, PF and GLR were found to cause the pharmacological blend effect. The two combined compounds were the main therapeutic components in "Shakuyaku-Kanzō-Tō".

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