Depolarizing Neuromuscular Blocking Action Induced by Electropharmacological Coupling in the Combined Effect of Paeoniflorin and Glycyrrhizin

Masayasu KIMURA, Ikuko KIMURA and Hiroshi NOJIMA

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences,
Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan

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Abstract—Twitch tensions of indirectly stimulated diaphragm muscles of mice were blocked by a combination of paeoniflorin (PF) and glycyrrhizin (GLR). The mechanism of this effect was studied electropharmacologically. When twitch responses were completely prevented, miniature end-plate, end-plate, and muscle action potentials were still observed when PF and GLR were combined, suggesting that the mechanism is postsynaptic. Potential amplitudes induced by acetylcholine (ACh), which was injected iontophoretically, were inhibited by about 70% by PF (25 μ g/ml) plus GLR (75 μ g/ml), although neither agent alone caused an inhibition. The combined ratio (PF:GLR=1:3) by concentrations (g/ml) potentiated both the inhibition of ACh potential amplitudes and the depolarization of resting membrane potentials. These results indicate that the effect of combined PF and GLR is to depolarize the muscle membrane and to block ACh-receptor-linked processes. In chemically skinned (saponin-treated) muscles, the tension induced by 0.39 μ M of free calcium was inhibited by PF (300 μ g/ml), but it tended to be increased by GLR (300 μ g/ml). Caffeine-induced contractures in the skinned muscles was not influenced by PF, and they tended to be decreased with GLR treatment. Thus, in muscles with sustained depolarization, these combined compounds seem to block intracellular Ca^{2+} movement.

In earlier papers (1, 2), we reported a new compound, paeoniflorigenone, which blocks neuromuscular junctions in skeletal muscle. This effect was greatly increased by glycyrrhizin (GLR) (3). Furthermore, the combination of paeoniflorin (PF) with GLR blocked indirectly stimulated twitching. PF or GLR alone at the concentrations used had no such effects. The mechanism involved was similar to that of succinylcholine (SuCh) in that 1) during complete blockage of indirectly stimulated twitching, the diaphragm muscle membrane may be depolarized and 2) a potentiation effect was observed when GLR was used in combination with SuCh. There was no such effect when d-tubocurarine (d-TC), instead of SuCh, was used in combination with GLR. Here, we investigate whether the effect of the combination of compounds is closely related to depolariza-

tion and whether the site of action of the combined agents is presynaptic or postsynaptic. We use electropharmacology to study the mechanism of this phenomenon.

Materials and Methods

Experiments were carried out with isolated left diaphragm muscles from male ddY mice (body weight 30–40 g, 8–12 weeks of age). The muscles were removed after the mice were decapitated and bled, pinned in a muscle bath, and perfused with Krebs-Henseleit solution (pH 7.2–7.4) being aerated with 95% O_2 and 5% CO_2 . The solution contained 136.9 mM NaCl, 5.0 mM KCl, 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 15.0 mM NaHCO_3 and 10 mM glucose. The phrenic nerve was placed over platinum electrodes in an acrylic pool. The preparations were kept at 35–36°C.

Intracellular recording: Standard glass electrodes filled with 3 M KCl and with tip resistance of 20–40 M Ω were used for the intracellular recording of membrane potentials, which were measured using a micro-electrode amplifier (Nihon Kohden Co., MEZ 8101). Only those cells having miniature end-plate potentials (m.e.p.p.) were used for this study. M.e.p.p. were recorded on a Visilight (San-ei, Model 5M21). Acetylcholine (ACh) chloride was applied iontophoretically near the muscle-cell surface through an electrode filled with 1 M ACh, using a microiontophoresis unit (Diamedical, DPI-25) and a V-I conversion unit (DPI-25 T).

Saponin treatment: Diaphragm muscle fibers, 0.5 mm wide, were treated for 30 min with 0.1 mg/ml saponin in the contracting solution containing 60 mM K₂SO₄, 4 mM MgSO₄, 20 mM tris, 20 mM maleic acid, 4 mM ATP, and 2 mM EGTA. Then they were washed out for 15 min and contracted by calcium ions in the contracting solution with or without the compounds listed below. The free Ca²⁺ concentration in a mixture of 300 μ M CaCl₂ added to the above solution was calculated to be 0.39 μ M, using the dissociation constants of 2.3×10^{-6} M for (Ca²⁺) (EGTA)/(Ca-EGTA) (4), 3.17×10^{-5} M for (Ca²⁺) (ATP⁴⁻)/(CaATP²⁻), and 1.1×10^{-5} M for (Mg²⁺) (ATP⁴⁻)/(MgATP²⁻) and assuming that (ATP³⁻)=1.59 (ATP⁴⁻). After washing out the calcium solution with the contracting solution with or without the compounds below, contracture was immediately brought about using 20 mM caffeine with or without those compounds added. These procedures were essentially those of Endo et al. (5).

Compounds: SuCh $\cdot$ 2 H₂O (Nakarai), ACh (Dai-ichi Seiyaku) and GLR monoammonium salt (a kind gift of Dr. S. Yabuki, the Minophagen Co.) were used. PF was generously provided, by Prof. O. Tanaka, Department of Pharmacognosy, Hiroshima University.

Results

Relationship between iontophoretic ACh pulses and ACh potentials when PF and GLR are combined: The amplitude of ACh potentials were measured with pulses of 0.1,

0.15, 0.2, 0.3 and 0.4 nC applied iontophoretically (Fig. 1). The histogram shows the numbers of ACh potentials observed at each amplitude. Amplitudes in this logarithmic scale of ACh pulses were distributed normally. Normal distribution curves were shifted by a log pulse of ACh to the right, and did not by a pulse of 0.4 nC, indicating this is the submaximum pulse.

Since ACh potential amplitudes had a normal distribution, mean amplitudes were plotted against a logarithmic scale of the ACh pulse (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 nC) with 50 μ g/ml PF, 150 μ g/ml GLR, or 25 μ g/ml PF with 75 μ g/ml GLR (Fig. 2). Neither of the compounds alone at the concentration used blocked indirectly-stimulated twitching, but the combination of the agents blocked it completely (data not shown). ACh potentials with either compound alone did not differ from the control without it. The combination markedly suppressed these ACh potentials.

The ACh and the resting membrane potentials were plotted against the log of total concentrations (g/ml) of these substances, alone or in combination, in Fig. 3. The ratio PF:GLR was 1:3. ACh potentials were obtained using a constant maximum pulse of 3.2 nC. The results show that with these compounds together, the depolarizing and

Fig. 1. Histogram of ACh potential amplitudes (abscissa) of mouse diaphragm muscles. The ordinate shows the number of observations of potentials for each amplitude. ACh was ejected in pulses of 0.1 (○), 0.15 (△), 0.2 (●), 0.3 (▲) and 0.4 (□) nC (10 msec) iontophoretic application using a glass microelectrode filled with 1 M ACh.

postjunctional blocking action depended on concentration and that the blocking was much greater than for the compounds alone, so there was potentiation.

Effects on miniature end-plate and end-plate potentials (e.p.p.): The effect of the combination of 150 $\mu\text{g/ml}$ PF and 150 $\mu\text{g/ml}$ GLR on m.e.p.p. was compared with that of SuCh (10 $\mu\text{g/ml}$, Fig. 4). Measurements were made either 1 or 20 min after starting the treatment with either SuCh or combined compounds. The combination did block

indirectly-stimulated twitching, as we previously reported (3), but did not stop the m.e.p.p. However, SuCh completely suppressed m.e.p.p. In this respect the effect of the combination of PF and GLR is different from the effect of SuCh. The e.p.p. was recorded after PF and GLR treatment for 20 min had completely suppressed indirectly-stimulated twitchings (Fig. 5). In addition, this combination slightly decreased the

Fig. 2. Blocking by PF and GLR of ACh potentials in mouse diaphragm muscles. Mean amplitudes $\pm$ S.E.M. ($n=13$ to 15) of the potentials are plotted against 0.1, 0.2, 0.4, 0.8 and 3.2 nC (10–64 msec and 10–50 nA) with PF (50 $\mu\text{g/ml}$, $\blacktriangle$), GLR (150 $\mu\text{g/ml}$, $\bullet$), PF (25 $\mu\text{g/ml}$) + GLR (75 $\mu\text{g/ml}$) ($\blacksquare$), and the control ($\circ$).

Fig. 3. The relationship between ACh potentials (solid line, closed symbol, $n=10$ to 34) and resting membrane potentials (dotted line, open symbols, $n=8$ to 50) plotted against log concentrations (g/ml) of PF alone (triangle), GLR alone (circle), and the combination of PF and GLR (1:3) (square). Shading indicates the mean amplitude of the ACh potentials (upper, $n=9$ to 15) and the mean resting membrane potential (lower, $n=11$ to 40), with $\pm$ S.E.M.

Fig. 4. M.e.p.p. 1 min after SuCh (10 $\mu\text{g/ml}$, middle trace) or 20 min after PF+GLR (both 150 $\mu\text{g/ml}$, lower trace) treatment was started on isolated mouse diaphragm muscles.

Fig. 5. Effect of PF with GLR (both 150 $\mu\text{g/ml}$) on e.p.p. Mouse phrenic nerves were stimulated by 1 Hz pulses of supramaximal intensity, 1 msec in duration. E.p.p. was recorded after application of 20 min of PF+GLR treatment, when indirectly-stimulated twitching was completely depressed. Control e.p.p. is the e.p.p. after washing out (W).

Table 1. Inhibition by PF or GLR of free Ca^{2+} or caffeine contraction in saponin-treated diaphragm muscle of mice

	n	free Ca^{2+} (0.39 μM)	n	Caffeine (20 mM)
Control	26	$18.1 \pm 2.7\%^a$	26	$16.4 \pm 1.9\%$
PF (300 $\mu\text{g/ml}$)	6	$3.6 \pm 2.3^*$	6	13.1 ± 3.1
GLR (300 $\mu\text{g/ml}$)	4	26.3 ± 5.2^b	5	10.9 ± 4.8^b

^aContraction is shown as % of the contraction by 152 mM K^+ in untreated muscle, $\pm$ S.E.M. ^bNot significantly different from the control value ($P=0.05$). n: Numbers of observations. *: $P<0.05$.

frequency of e.p.p. These results showed that the combined compounds had little or no presynaptic action, which was therefore not part of the mechanism of the blocking action of the twitch response.

The direct effect of both compounds in activating muscles was examined by eliciting action potentials from a muscle fiber with a second electrode (5 msec, 45 nA). Treatment with the combination of PF and GLR (both 150 $\mu\text{g/ml}$) for 60 min did not affect the amplitude of the action potential during complete blocking of indirectly-stimulated twitching.

Effects on excitation-contraction coupling:

The mechanism of the combined compounds were further studied using skinned muscle fibers. When 0.5 mm wide muscle fibers were treated with 0.1 mg/ml saponin, various drugs can permeate through the membrane and can then affect excitation-contraction coupling. The free Ca^{2+} (0.39 μM) contraction was decreased at 15 min after the application of PF (300 $\mu\text{g/ml}$), but the contraction caused by caffeine (20 mM) was

not (Table 1). GLR (300 $\mu\text{g/ml}$) tended to increase the contraction caused by free Ca^{2+} , and it tended to reduce caffeine contracture.

Discussion

We previously (3) reported that GLR increased blocking by SuCh of indirectly-stimulated twitching, but it did not change that of d-TC. Since SuCh is a depolarizing neuromuscular blocker, this effect of GLR may be closely linked to depolarizing effects in muscle membranes. GLR also potentiated inhibition of indirectly-stimulated twitching by paeoniflorigenone (2), a depolarizing blocker, and PF. Here, the combination of GLR and PF depolarized resting membrane potentials more than either compound alone. This combination also potentiated the inhibition of ACh potentials. GLR+PF also revealed the potentiation effect. Either compound alone induced the depolarization of resting membrane potentials and the inhibition of ACh potentials only at high concentrations. Whether the combination of GLR and paeoniflorigenone had such effects could not

be studied, because the combination strongly depolarized resting membrane potentials.

The mechanism of the combined compounds was mainly postsynaptic, because m.e.p.p. and e.p.p. in the presence of the concentration of PF and GLR that caused neuromuscular blocking did not cease. Since amplitudes of muscle action potentials did not change during suppression of indirectly-stimulated twitching, the combination does not act like tetrodotoxin.

In chemically skinned muscles, GLR tended to increase free Ca^{2+} contraction and to inhibit caffeine-induced contraction. These findings indicate that GLR does not inhibit contractile protein, and they suggest that Ca^{2+} -induced Ca^{2+} release (5, 6) may be increased by GLR. Then GLR would tend to decrease caffeine contracture because of the resultant lesser load of Ca^{2+} in the sarcoplasmic reticulum. Since PF inhibited free Ca^{2+} contraction but did not affect caffeine contraction, it does not affect contractile protein or Ca^{2+} release from the sarcoplasmic reticulum.

The mechanism of the combined compounds is not that of dantrolene which inhibits contraction by reducing Ca^{2+} release from the sarcoplasmic reticulum (7), and weakly inhibits caffeine- and Ca^{2+} -induced Ca^{2+} release (8, 9).

We conclude that the main effect of these combined compounds at the neuromuscular junction is to depolarize the muscle membrane long-term and to block some part of the processes in which ACh receptors work. That the effect of the combination was greater than that of the compounds alone is possibly related to electropharmacological coupling connecting GLR with PF. Thus, in muscles with sustained depolarization, the combined compounds seem to block intra-

cellular Ca^{2+} movement.

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