Complementary Effects of Paeoniflorin and Glycyrrhizin on Intracellular Ca^{2+} Mobilization in the Nerve-Stimulated Skeletal Muscle of Mice

Katsuya Dezaki, Ikuko Kimura*, Kayoko Miyahara and Masayasu Kimura

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan

Received August 17, 1995 Accepted September 26, 1995

ABSTRACT—Effects of paeoniflorin (PF) and glycyrrhizin (GLR), contained in paeony and licorice roots, respectively, on contractile and non-contractile Ca^{2+} mobilization were examined by measuring the Ca^{2+} -aequorin luminescence (Ca^{2+} transients) of the nerve-stimulated skeletal muscle of mice in the presence of neostigmine (0.3 μM). PF (0.1–1 mM) prolonged the duration of non-contractile Ca^{2+} transients, which may induce the desensitization of nicotinic acetylcholine receptor, but did not affect contractile Ca^{2+} transients. GLR (0.3–1 mM) depressed contractile Ca^{2+} transients without affecting non-contractile transients. These results suggest that PF and GLR may have complementary effects on intracellular Ca^{2+} mobilization to block the neuromuscular transmission.

Keywords: Paeoniflorin and glycyrrhizin, Calcium, Neuromuscular junction

Paeoniflorin (PF) and glycyrrhizin (GLR) are the principal components of paeony and licorice roots, respectively. Paeony and licorice roots are prescribed in *Shakuyaku-Kanzo-To*, which has been used as one of the Kampo-Hozai to relieve muscular pain by traditional Chinese and Japanese medicine. In the previous study, we have reported that PF in combination with GLR inhibits both the nerve-stimulated twitch response and acetylcholine (ACh) potentials and has depolarizing effects (1, 2). Therefore, we predicted that these compounds may have similar effects to a depolarizing neuromuscular blocker like succinylcholine.

We have reported that non-contractile Ca^{2+} mobilization (not accompanied by muscle contraction) is generated at the neuromuscular junction by nerve stimulation in the presence of anticholinesterase agents (3). Except via the initial activation of the postjunctional nicotinic acetylcholine receptor (AChR), the mechanism of non-contractile Ca^{2+} mobilization is completely different from that of contractile Ca^{2+} mobilization (3–6), which is closely related to intracellular Ca^{2+} release from the sarcoplasmic reticulum. In the present study, we investigated the effect of PF or GLR on the contractile and non-contractile Ca^{2+} mobilization in the nerve-stimulated skeletal muscle of mice.

Male ddY mice (7–9-weeks-old, 28–42 g) were killed by decapitation. Segments of the phrenic nerve-diaphragm muscles were used to measure the Ca^{2+} -aequorin luminescence (Ca^{2+} transients). Modified Krebs' solution (122 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 15.5 mM NaHCO_3 and 11.5 mM glucose) was equilibrated with 95% O_2 and 5% CO_2 , maintained at 36°C and perfused through the chamber in which the muscle segments were fixed. Procedures for measurements of Ca^{2+} transients were as already described (7). Briefly, the aequorin solution (1 mg/ml) was pressure-injected at 5–6 atm for 2 sec through a micropipette into the myoplasm in the endplate region of a muscle. Usually, 40–50 fibers of a diaphragm segment were intracellularly loaded with aequorin. The phrenic nerve was then electrically stimulated (0.1 Hz, 0.4–0.8 V, 0.1-msec duration). Ca^{2+} transients were led to a photomultiplier tube (R464; Hamamatsu Photonics, Shizuoka) and measured by a photon counter (C767, Hamamatsu Photonics). The GLR monoammonium salt was a kind gift from Minophagen Co. (Tokyo). Either PF (0.1–1 mM) or GLR (0.3–3 mM) was bath-applied for 1 hr. PF or GLR alone had a negligible effect on the contractile Ca^{2+} transients (data not shown). After these treatments, the anticholinesterase neostigmine (0.3 μM) was added for 15 min. The 30 Ca^{2+} transient signals obtained 10–15 min after neostigmine application were averaged by a signal

*To whom correspondence should be addressed.

processor (7T07A; San-ei, Tokyo) in order to improve the signal-to-noise ratio. The peak amplitude of both contractile and non-contractile Ca^{2+} transients was expressed as a percentage of the contractile Ca^{2+} transients obtained during the 5 to 0 min before neostigmine was applied. The duration of non-contractile Ca^{2+} transients was determined at $1/e$ amplitude of the signal. Data are expressed as means $\pm$ S.E. One-way analysis of variance (ANOVA) and Scheffe multiple-comparison test were used to determine statistical significances at P levels of 0.05 or 0.01.

PF (0.1–1 mM) prolonged the duration of non-contractile Ca^{2+} transients in a concentration-dependent manner, but did not affect contractile Ca^{2+} transients (Figs. 1 and 2). We have reported that non-contractile Ca^{2+} is mobilized through the nicotinic AChR under the

Fig. 1. Typical traces of Ca^{2+} transients elicited by nerve stimulation with $0.3 \mu\text{M}$ neostigmine in the absence (upper) and presence of 1 mM paeoniflorin (PF; middle) or 1 mM glycyrrhizin (GLR; lower) in the mouse diaphragm muscle. These traces are the averaged records for 30 Ca^{2+} transient signals 10–15 min after the neostigmine application. The large, rapid increase in Ca^{2+} represents the contractile transients, and the slower prolonged increase represents the non-contractile transients. The ordinate calibration bar represents 100% amplitude of the contractile Ca^{2+} transients before application of neostigmine. Similar results were obtained in 8–9 separate experiments.

Fig. 2. Prolongation of the duration of non-contractile Ca^{2+} transients by PF in the nerve-stimulated diaphragm muscle of the mouse. The muscle was pretreated with PF for 1 hr, and then neostigmine ($0.3 \mu\text{M}$) was applied to it for 15 min. The 30 Ca^{2+} transient signals obtained 10–15 min after neostigmine application were averaged. Peak amplitude of contractile (A) and non-contractile Ca^{2+} transients (B, top) is expressed as percentages of contractile Ca^{2+} transients before the neostigmine application. The duration of non-contractile transients was determined at $1/e$ amplitude of the signal (B, bottom). The data are means $\pm$ S.E., $n=8-9$. * $P<0.05$, ** $P<0.01$; significantly different from the values of the control with neostigmine alone based on one-way ANOVA and the Scheffe multiple-comparison test.

desensitizable conditions prepared by cholinesterase inhibition and induced nicotinic AChR desensitization via protein kinase-C activation, depressing contractile Ca^{2+} mobilization in the mouse neuromuscular junction (8). Therefore, PF may enhance the non-contractile Ca^{2+} -induced nicotinic AChR desensitization and then may cause neuromuscular blockade indirectly.

GLR (0.3–1 mM) depressed contractile Ca^{2+} transients in the diaphragm muscles with neostigmine in a concentration-dependent manner (Figs. 1 and 3A). Nevertheless, GLR at the same concentration ranges affected neither the peak amplitude nor the duration of non-contractile Ca^{2+} transients (Fig. 3B). At the high concentration (3

Fig. 3. Inhibitory effect of GLR on contractile Ca^{2+} transients in the nerve-stimulated diaphragm muscle of the mouse. The muscle was pretreated with GLR for 1 hr, and then neostigmine ($0.3 \mu\text{M}$) was applied to it for 15 min. The 30 Ca^{2+} transient signals obtained 10–15 min after neostigmine application were averaged. Peak amplitude of contractile (A) and non-contractile Ca^{2+} transients (B, top) is expressed as percentages of contractile Ca^{2+} transients before the neostigmine application. The duration of non-contractile transients was determined at 1/e amplitude of the signal (B, bottom). The data are means $\pm$ S.E., $n=8-9$. * $P<0.05$; significantly different from the values of the control with neostigmine alone based on one-way ANOVA and the Scheffe multiple-comparison test.

mM), GLR depressed peak amplitudes of non-contractile Ca^{2+} transients in addition to contractile ones, but did not affect the duration of non-contractile Ca^{2+} transients (data not shown). These results indicate that GLR depressed contractile Ca^{2+} transients more predominantly than non-contractile Ca^{2+} transients. In our previous study, GLR reduced the caffeine-induced increase in Ca^{2+} transients in directly-stimulated diaphragm muscle of the mouse (9). GLR seems to depress a Ca^{2+} -releasing process related to the site of action of caffeine. In addition, GLR inhibits phospholipase A_2 activity in several tissues (10, 11). We previously reported that GLR inhibited phospholipase A_2 activity in skeletal muscle

myotubes (12). Arachidonic acid from membrane phospholipids produced by the action of phospholipase A_2 stimulates the rate of Ca^{2+} release from the sarcoplasmic reticulum (13, 14). Thus, the effect of GLR on the neuromuscular functions may be partly attributed to its inhibitory action on phospholipase A_2 .

PF in combination with GLR shows inhibitory effects on the nerve-stimulated twitch responses in frog and mouse muscles at concentrations that were without effects when applied separately (1). PF enhanced non-contractile Ca^{2+} mobilization, which may induce the desensitization of nicotinic AChR, at the neuromuscular junction. On the other hand, GLR depressed contractile Ca^{2+} mobilization. Therefore, the inhibition of twitch response by combining PF with GLR may be caused by the complementary effects of these compounds on the intracellular Ca^{2+} mobilization.

PF and GLR depolarize the skeletal muscle membranes, similar to the effects caused by depolarizing neuromuscular blockers like succinylcholine and decamethonium (2). However, the extent of depolarization (only a few mV) may be too small to explain the modulation of Ca^{2+} mobilization caused by these compounds. We have reported that succinylcholine depresses both contractile and non-contractile Ca^{2+} mobilization (15). In particular, succinylcholine inhibited both the peak amplitude and the duration of non-contractile Ca^{2+} mobilization simultaneously. Decamethonium depresses the peak amplitude of non-contractile Ca^{2+} mobilization more predominantly than contractile Ca^{2+} mobilization (15). Therefore, our present findings suggest that the effects of PF and GLR on the neuromuscular junction are different from those of depolarizing neuromuscular blockers. This suggestion is supported by our previous results that the combination of PF and GLR blocked the nerve-stimulated twitch response, but did not suppress the occurrence of the miniature endplate potentials at the mouse neuromuscular junction, whereas succinylcholine suppressed the occurrence of both of them by its presynaptic inhibitory action (2).

In conclusion, the present study indicates that PF and GLR have different effects on intracellular Ca^{2+} mobilization in the nerve-stimulated skeletal muscle. These results suggest that PF and GLR may have complementary effects on intracellular Ca^{2+} mobilization to block the neuromuscular transmission.

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