EFFECTS OF VERAPAMIL ON TWITCH POTENTIATION INDUCED BY INDIRECT CONDITIONING STIMULATION IN MOUSE PHRENIC NERVE-DIAPHRAGM MUSCLE PREPARATION

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Verapamil is known to inhibit calcium entry into squid giant axon (1). Calcium movement is a prerequisite event in neuromuscular transmission and muscle contraction. When the presynaptic nerve is stimulated by conditioning shocks with short intervals, two phenomena, facilitation (2) and post-tetanic potentiation (3), are observed. Both these phenomena are considered to be produced by an increased transmitter release which is associated with an intracellular accumulation of calcium during the conditioning shocks (4). In mouse phrenic nerve-diaphragm muscle preparation, we observed an increase in twitch amplitude following the application of conditioning pulse on the nerve. These phenomena seemed to be dependent on extracellular calcium. In the present experiments, the effects of verapamil (donated by Eisai) on the twitch potentiation were examined.

Adult male mice of ddy strain weighing 20 to 25 g at the age of 30 to 45 days were used. Mice were stunned, exsanguinated and the phrenic nerve-diaphragm muscle isolated by a conventional method (5). The preparation was set up under 0.5 g tension in a 20 ml organ bath containing Krebs-Ringer solution with the following composition (in mM/l): Na, 152; K, 5; Ca, 2; Mg, 1; Cl, 146; HCO_3 , 15; HPO_4 , 1; glucose, 11. The solution was gassed with a mixture of 5% CO_2 in O_2 for

at least 30 min prior to use and during the experiment, and kept at pH 7.2 to 7.5 and 37°C . In some experiments, 12.3 mM sodium bicarbonate in the above solution was replaced with equimolar sodium chloride to decrease the pH of the solution to 6.4. The nerve or muscle was stimulated by electrical shocks using an electronic stimulator (SEN-7103, Nihon Kohden Kohgyo). The muscle twitches were recorded isometrically by force displacement transducer (Nihon Kohden Kohgyo).

In the first experiments, the nerve or muscle was stimulated by alternate shocks (0.1 Hz). The motor nerve was stimulated with 0.05 msec duration and supramaximal voltage. The muscle was stimulated with 8 msec duration and voltages which were selected to produce a twitch tension at a similar level to the one induced by the indirect stimuli. In normal Krebs-Ringer solution, 5×10^{-5} M verapamil increased the amplitude of indirectly induced muscle twitches (IT) but not that of directly induced ones (DT). When the stimulus-voltage was gradually increased, the amplitude of DT increased gradually and reached a plateau level which was 2 to 3 fold larger than that of IT in the normal Krebs-Ringer solution. The DT induced by the higher voltage was not increased in the amplitude by verapamil (5×10^{-5} M). Verapamil in concentrations

from 1×10^{-8} to 1×10^{-6} M had little effect on the amplitudes of both IT and DT. Lowering the pH of the solution to 6.4 slightly decreased the amplitudes of both IT and DT. Verapamil (5×10^{-5} M) reduced the tension of both IT and DT in the low pH solution.

In the next experiments, the phrenic nerve was stimulated with a paired rectangular square pulses (0.1 Hz). The interval of the paired pulses was 10 to 1000 msec and each pulse had a 0.05 msec duration and supra-maximal voltage. The paired pulses were applied every 10 sec. When the interval of the paired pulse was shortened to less than 25 msec, the twitch induced by the second pulse was always larger than that induced by the first pulse. The increase in the twitch amplitude corresponded with the shortening of the interval of the paired stimuli. Similar

results were obtained in the solution with pH 6.4. Verapamil (5×10^{-5} M) further increased the twitch potentiation produced by the paired stimulation in the solution with pH 7.5 but not in the pH 6.4 solution. These results are summarized in Fig. 1.

A paired stimulation applied directly to muscle is known to produce a twitch summation (6). The diaphragm muscle of mouse was stimulated with paired pulses. The interval of the paired pulse was 10 to 50 msec and each pulse had a 5 msec duration and supramaximal voltage. The paired pulses were applied every 10 sec. A twitch summation in mouse diaphragm muscle was observed as an increase in the tension of the second twitch in the presence of 1×10^{-5} M d-tubocurarine in both normal pH and low pH solutions when the interval of the paired

Fig. 1. Effect of verapamil (5×10^{-5} M) on twitch potentiation (upper) induced by paired indirect stimulation and on twitch summation (lower) induced by paired direct stimulation in mouse phrenic nerve-diaphragm muscle preparation. pH of the bathing solution was either 7.5 or 6.4. Percentile twitch tension shows the relative tension of the second twitch to the first one. $\times$; $p < 0.05$. $\times \times$; $p < 0.01$. Vertical bar indicates the S.E. of the mean of 8 experiments. ○; control. ●; verapamil (5×10^{-5} M).

Fig. 2. Effect of verapamil (5×10^{-5} M) on post-tetanic twitch potentiation in mouse phrenic nerve-diaphragm preparation. The upper two traces are two control responses, 30 min intervals. Middle trace shows the effect of verapamil on indirectly induced muscle twitches at 0.3 Hz. In the lower two traces, Ver-I is obtained 15 min after the application of verapamil and Ver-II, 30 min later. T; tetanic stimulation at 30 Hz, for 10 sec.

shocks was between 10 and 25 msec. Verapamil (5×10^{-5} M) inhibited the twitch summation phenomenon at both pH ranges (Fig. 1).

Figure 2 shows the effects of 5×10^{-5} M verapamil on the post-tetanic twitch potentiation. The phrenic nerve was stimulated by rectangular pulses with 0.3 Hz frequency, 0.05 msec duration and supramaximal voltage. Parameter of tetanic stimulation of the nerve was 30 Hz for 10 sec with the same duration and voltages as the pre- or post-tetanic stimulation. The potentiation in twitch amplitude induced by the conditioning shocks lasted 30 to 40 sec. Similar potentiation was observed following the second conditioning stimulation applied after 15 min or longer

(upper two traces in Fig. 2). The potentiation was followed by a decrease in twitch amplitude lasting 4 to 5 min. Verapamil (5×10^{-5} M) increased the twitch response induced indirectly at 0.3 Hz and the effect reached a plateau level after 10 to 15 min (middle trace in Fig. 2). Tetanic stimulation (30 Hz for 10 sec) was applied to the phrenic nerve 15 or 30 min after the treatment with verapamil. Magnitude of the tetanic contraction, amplitudes of the post-tetanic twitches and the duration of the potentiation of twitch tension were significantly reduced in the presence of verapamil. Verapamil, however, shortened the duration of the decrease in twitch tension following the potentiation. When the muscle was stimulated with tetanic shocks (8 msec duration, supramaximal voltage, 30 Hz) for 10 sec, post-tetanic twitch potentiation was observed. This potentiation was partially inhibited by verapamil (5×10^{-5} M). Verapamil in the concentrations lower than 1×10^{-6} M had little effect on the twitch potentiation.

The twitch response to nerve stimulation and to paired indirect stimulation were potentiated but those to direct stimulation, to paired direct stimulation and to post-tetanic stimulation of the nerve were not potentiated by verapamil (5×10^{-5} M). Low concentrations (1×10^{-8} to 1×10^{-6} M) of verapamil had no effect on the twitch responses to various stimulations.

In the first experiments, it was shown that a high concentration (5×10^{-5} M) of verapamil potentiated the twitch tension induced by indirect shocks but not by direct shocks, suggesting that the site of action of verapamil is not the skeletal muscle. Since it has been reported that verapamil reduced the sensitivity of frog muscle to acetylcholine (7) and also reduced the amplitude of miniature end-plate potentials in rat neuromuscular junction (8), the sensitivity of end-plate to transmitter may not be involved in the potentiation of reactivity

induced by verapamil. It is, therefore, possible that verapamil somehow changes nerve function to potentiate the twitch response. Such a possibility is supported by the second experiments showing that verapamil potentiated the reactivity of the preparation in response to paired indirect stimulation but not to paired direct stimulation. According to the residual calcium hypothesis of neuromuscular facilitation (4), residual but available calcium ions in the nerve terminal remaining after the first nerve impulse are utilized to increase the amount of transmitter released during the second impulse. The potentiation by verapamil on the facilitation could be due to the increase in the residual calcium in the nerve terminals. Such an effect of verapamil was seen only in the solution with pH 7.5 but not in the solution with pH 6.4. Verapamil has a pKa of 6.6 (9) and is in almost basal form (RH^+ : $R=0.112 : 0.888$) at pH 7.5. It is, therefore, possible that the potentiation effects of verapamil are dependent on the amount of the basal form.

The post-tetanic potentiation has been suggested to be a presynaptic event, due to an increase in the amount of transmitter release per each nerve stimulus (10). It has also been suggested that excess calcium ion is stored in the nerve terminal during the tetanic stimulation and this stored calcium is utilized to increase the amount of transmitter release following the post-tetanic stimulation (3). In the present experiments, verapamil inhibited the post-tetanic twitch potentiation as well as the tetanic contraction. This effect may be due to the inhibitory effect of verapamil on transmembrane calcium influx (1). The different effects of verapamil on the twitch responses to the paired stimulation and post-tetanic stimulation on the nerve might be attributable to the suggested difference (11) between the mechanisms to

increase the release of transmitter in paired stimulation and post-tetanic stimulation on the nerve.

From the results, the potentiative effect of verapamil on twitch potentiation induced by paired indirect shocks could be due to the increase in the amount of the residual calcium in the nerve terminals and the effect possibly depends on its basal form.

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