VERAPAMIL-INDUCED TRANSMITTER RELEASE IN RAT DIAPHRAGM MUSCLE

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Abstract—Verapamil was examined for its effect on the frequency of miniature end-plate potential (m.e.p.p.) in rat diaphragm muscles. Verapamil (5x10^-5 M) raised the m.e.p.p. frequency. This effect was reversible, reproducible, and concentration dependent. The rise in the frequency was maintained in the presence of external Ca++ but was transient in the absence of external Ca++. Lowering the temperature to 20°C slightly decreased the average frequency of m.e.p.p. in the normal medium. The effect of verapamil was also present at a low temperature but was delayed in its onset. The resting membrane potential of the muscle fiber was not affected by the agent. These results suggest the possibility that verapamil increases the transmitter release from motor nerve terminals, and the effect is possibly due to a release of Ca++ for its initiation, but is dependent on external Ca++ for its maintenance.

It is known that verapamil inhibits Ca++ movements across the cell membrane not only in cardiac muscles (1), but in nerve fibers (2, 3). As the evoked transmitter release from motor nerve terminal is dependent on external Ca++ (4, 5), it can be anticipated that verapamil and other calcium antagonists inhibit the transmitter release. On the other hand, verapamil (6) and its methoxy derivative D600 (7) have been reported to increase the spontaneous transmitter release in frog neuromuscular junction. However, its dependency on external Ca++ is still unknown.

The present experiments have been carried out to elucidate the effect of verapamil on transmitter release from motor nerve terminals in rat diaphragm muscles in the presence or absence of external Ca++. MATERIALS AND METHODS

The left hemidiaphragm muscles were dissected from male Wistar rat weighing 200 to 250 g. The diaphragms were continuously bathed in Krebs-Ringer solution bubbled with 95% O2 and 5% CO2. The Krebs-Ringer solution had the following composition (in mM): Na, 152; K, 5; Ca, 2; Mg, 1; Cl, 146; HC03, 15; HPO4, 1; and glucose, 11. Ca++ removal from the medium was made by omission of calcium chloride, but no addition of EDTA or EGTA. The temp. of the bathing fluid was maintained at approx. 37°C and the pH was kept at approx. 7.3.

Intracellular recording of miniature end-plate potentials (m.e.p.p.) was made with glassmicro-electrodes filled with 3-M KCl solution by a conventional method (8). Potentials were fed through an oscilloscope (Nihon Kohden, VC-9). The m.e.p.p. was recorded on a cassette recorder (Nihon Kohden, RMG-5204) and partly on moving film and counted by a computer (Nihon Kohden, ATAC-350).
Verapamil HCl (donated by Eisai) was dissolved in distilled water to make a $2 \times 10^{-2}$ M stock solution. An appropriate amount of the stock solution was added to the test solution.

RESULTS

The frequency of m.e.p.p. was intracellularly recorded at the rat neuromuscular junction in the normal Krebs-Ringer solution (Fig. 1, left), and the effect of verapamil on the frequency at the same end-plate was examined (Fig. 1, right). The number of m.e.p.p. apparently increased 10 min after application of verapamil ($5 \times 10^{-5}$ M), showing a rise in the frequency. The giant m.e.p.p. was observed both in the control and after the application of verapamil. The number of m.e.p.p. was counted in each end-plate from a record for 5 min from 10 to 15 min after the application of verapamil. The frequency of m.e.p.p. ($F$) was expressed as the number per sec (nps) of m.e.p.p. Figure 2 shows a dose-response curve of verapamil’s effect on the $F$ value. The effect of verapamil on $F$ was examined with respect to concentration dependence at the end-plate showing a control value for $F$ of $2.2 \pm 0.22$ (mean±S.E., N=6) nps. Verapamil at $2.5 \times 10^{-5}$ and $5 \times 10^{-5}$ M increased the $F$ value depending on its concentration. The effect of verapamil in increasing the $F$ value was reversible and reproducible.

The effect of verapamil on the resting membrane potential of the muscle fiber was examined. The resting membrane potential was $88 \pm 2.3$ (mean±S.E., N=12) mV in normal Krebs-Ringer solution. Ten min or more after applications of verapamil in concentrations of $2.5 \times 10^{-5}$ and $5 \times 10^{-5}$ M, the resting membrane potentials of muscle fibers were $86 \pm 2.6$ (N=8) and $86 \pm 2.2$ (N=8) mV, respectively, showing that verapamil did not affect the resting membrane potential of muscle fibers.

Influence of Ca$^{2+}$ concentration on verapamil’s effect was examined, and the results are presented in Fig. 3. At a normal Ca$^{2+}$ level, the control value for $F$ was approx. 2.5 nps. This was gradually increased with time after the application of verapamil ($5 \times 10^{-5}$ M) and reached a plateau (approx. 6 nps) at 10 to 15 min after. This effect of verapamil continued for at least 40 min or more. Ca$^{2+}$ removal from the medium

![Fig. 1. Effect of verapamil ($5 \times 10^{-5}$ M) on m.e.p.p. frequency at the rat neuromuscular junction: Left, control; Right, 10 min after application of verapamil. The photographs of moving film are continuous 5.5 sec samples at the same end-plate. The higher frequency was observed after the application of verapamil.]

![Fig. 2. Effect of verapamil on average frequency of m.e.p.p. at the rat neuromuscular junction: Each point represents the mean of successive 1 sec counts during the 5 min observation period from 10 to 15 min after application of each concentration of verapamil. Vertical bar shows S.E. of the mean of 6 experiments in which the end-plates were selected for showing a control frequency of $2.2 \pm 0.22$ nps (mean±S.E., N=6).]
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**Fig. 3.** Effect of verapamil (5×10^{-5} M) on the average frequency of m.e.p.p. in the presence or absence of external Ca^{++} at the rat neuromuscular junction: Ca^{(+)}, 2.0 mM-Ca^{++}; Ca^{(-)}, 0 mM-Ca^{++}. Each point represents the mean of successive 1 sec counts (number per sec, nps) during the 5 min observation period. Vertical bar shows S.E. of the mean of 6 experiments.

**Fig. 4.** Effect of verapamil (5×10^{-5} M) on m.e.p.p. frequency at the rat neuromuscular junction under low temp. at 20°C: Each point represents successive 1 sec counts of m.e.p.p. (number per sec, nps) during the 5 min observation period in 3 individual end-plates from 3 muscles. Ver; verapamil.

decreased the value of F to approx. one third of the control F value. The F value in the absence of external Ca^{++} without addition of EDTA or EGTA was also increased by verapamil (5×10^{-5} M). This effect was transient. The difference between the F value 30 min after the application of verapamil and the control value in Ca^{++}-free solution was not significant (P>0.05). Verapamil was less active in the Ca^{++}-free medium than in the normal Krebs-Ringer solution.

The effect of verapamil on F was examined at a low temp. (20°C) and the results are presented in Fig. 4. Lowering the temp. of the medium slightly decreased the value for F in the normal Krebs-Ringer solution. The F value was also gradually increased by verapamil (5×10^{-5} M) with time after the application at the low temp. Though the stimulatory effect of verapamil on F reached a plateau level 10 min after the application at 37°C (Fig. 3, Ca^{(+)}), the onset of the effect was delayed at the low temp. and the effect was less potent than at 37°C.

**DISCUSSION**

It has been well known that Ca^{++} ions are important in the release of transmitter at the neuromuscular junction (9). The m.e.p.p. is produced by a spontaneous release of a minute amount of transmitter from motor nerve terminals (4). An elevation of intracellular Ca^{++} generally results in an increase in transmitter release (9). It has also been noticed that m.e.p.p. frequency is largely controlled by intracellular Ca^{++} at the presynaptic terminals (10, 11). It is known that an increase in Ca^{++} entry into nerve terminals brings about facilitation of neuromuscular transmission because of an increased transmitter release (12). Although verapamil is a typical calcium antagonist in nerve fibers (2, 3), it increased transmitter release at the rat neuromuscular junction in the present experiments. This observation was consistent with the findings in the frog neuromuscular junction (6, 7). Furthermore, the verapamil-induced transmitter release at the rat neuromuscular junction continued in the normal Krebs-Ringer solution, but was transient in Ca^{++}-free medium. Thus it appears likely that the initiation of the stimulatory effect of verapamil on transmitter release is dependent on the release of Ca^{++} from intracellular store(s), and sustained transmitter release is not maintained by only intracellularly stored Ca^{++}. Indeed, the
possible Ca\textsuperscript{++} releasing action of verapamil has also been noticed in frog sartorius muscle (13).

It has been suggested that Ca\textsuperscript{++} ions enter the nerve terminals through the opened voltage-gated channels, by translocation with an ionophore or by leaking through the resting membrane, and Ca\textsuperscript{++} always stimulates transmitter release (14). In the present experiments, the sustained effect of verapamil was dependent on the presence of external Ca\textsuperscript{++}, suggesting the possibility that the sustained effect is supported by an influx of Ca\textsuperscript{++} into nerve terminals at the rat neuromuscular junction. Since verapamil did not alter the resting membrane potential of the muscle fiber in the present experiments, it appears that an influx of Ca\textsuperscript{++} would not occur through the opened voltage-gated channels.

It has been reported that m.e.p.p. frequency at the frog neuromuscular junction is probably raised by elevating the cytoplasmic Ca\textsuperscript{++} level through reducing the activity of the Ca\textsuperscript{++} pump of the plasma membrane which is inhibited by lowering temp. (6, 15–17). Publicover and Duncan (6) noticed that the stimulatory effect of verapamil on transmitter release disappeared at the frog neuromuscular junction at low temp. They suggested that verapamil increases transmitter release through the inhibition of the membrane Ca\textsuperscript{++} pump. In the present experiments, however, the stimulatory effect of verapamil on the m.e.p.p. frequency at the rat neuromuscular junction was present at low temp, although delayed and less active. It seems unlikely that the stimulatory effect of verapamil is mainly due to the reduction of the activity of the Ca\textsuperscript{++} pump of the nerve terminal plasma membrane in this rat preparation.

From the results, it is suggested that verapamil increases spontaneous transmitter release from motor nerve terminals, and the effect is possibly mediated by releasing Ca\textsuperscript{++} into the axonal plasma from intracellularly stored Ca\textsuperscript{++} which is continuously supplied by external Ca\textsuperscript{++}.

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