POTENTIATION OF INDIRECTLY INDUCED MUSCLE TWITCHES
BY ORGANIC CALCIUM ANTAGONISTS IN PHRENIC
NERVE-DIAPHRAGM MUSCLE PREPARATIONS OF MICE

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Abstract—Organic calcium antagonists (l- and d-verapamil, D600, and
diltiazem) were examined for their effects on muscle twitches of isolated
phrenic nerve-diaphragm muscle preparations of mice. The calcium
antagonists (1×10^{-6} to 7.5×10^{-5} M) increased the amplitude of muscle
twitches induced by nerve stimuli with short durations (0.04 to 0.4 msec)
of rectangular pulses. However, these agents were poorly effective on
twitches induced by nerve stimuli with longer durations over 0.6 msec or by
direct shocks. The potentiative effect was reversible, reproducible and
dependent on their concentrations. Diltiazem was the most effective
among the four agents tested. The twitch increase produced by all of the
agents was demonstrated at concentrations of external Ca^{2+} above 0.6 mM.
At Ca^{2+} concentrations below 0.5 mM, the tension of the indirectly induced
muscle twitch was partially inhibited in the presence of these agents.
Caffeine, theophylline, isoproterenol or hypertonic potassium ions increased
the tension of indirectly induced muscle twitches. The potentiative effect
of the organic calcium antagonists, however, was discriminated from those
induced by the other agents under some conditions. From these results,
it is suggested that the organic calcium antagonists alter the reactivity of
the preparation to nerve shock. The potentiative effect of the agents on
indirectly induced muscle twitch may include an increase in the number
of fibers contracting per nerve impulse through increasing transmitter
release from the nerve terminal, but not an increase in contractility of an
individual muscle fiber.

The organic and inorganic calcium
antagonists affect cell function by altering
the transmembrane calcium flux (1).
Verapamil has been reported to inhibit
directly induced muscle twitches in rat
skeletal muscle with an initial increment in
their tensions, but not due to calcium
antagonistic action (2). In addition, verapamil
has been shown to inhibit neuromuscular
transmission in mammalian skeletal muscle
at a higher concentration (1×10^{-4} M) (3).
At concentrations ranging from 1×10^{-6} to
7.5×10^{-5} M, however, verapamil has been
reported to potentiate the tension of indirectly
induced muscle twitch in an isolated mouse
phrenic nerve-diaphragm preparation (4).
It has been noticed that verapamil (5) and
D600 (6) increase spontaneous transmitter
release in frog neuromuscular junction.

The present experiments have been
carried out to verify the effects of some organic calcium antagonists on muscle twitches induced directly or indirectly under some conditions including the alteration of external Ca\(^{2+}\) concentration and the presence of d-tubocurarine in a mouse phrenic nerve-diaphragm preparation.

**MATERIALS AND METHODS**

Adult male mice of the dd\(\text{y}\) strain weighing 20 to 25 g were used. Mice were killed by stunning and exsanguination. The phrenic nerve-diaphragm preparation (PND) was isolated by a conventional method (7). 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): Na, 152; K, 5; Ca, 2; Mg, 1; Cl, 146; HCO\(_3\), 15; HPO\(_4\), 1; and 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 also during the experiment, and kept at pH 7.4 and 37°C. Muscle twitches were induced by alternate electrical shocks (0.1 Hz) of nerve and muscle using an electronic stimulator SEN-7103 (Nihon Kohden Kohgyo). The motor nerve or muscle was stimulated with pulses of 0.01 to 8 msec duration and a supramaximal voltage. The muscle twitches were recorded isometrically by a force displacement transducer (Nihon Kohden Kohgyo).

**Drugs used:** d- and l-verapamil (donated by Eisai), diltiazem (donated by Tanabe), D600 (donated by Knoll), d-tubocurarine and caffeine (Wako Pure Chemicals), theophylline (Tokyo Kasei), isoproterenol (Kantoh Kagaku), propranolol (Sumitomo Chemicals).

**RESULTS**

In the normal Krebs-Ringer solution, all of the organic calcium antagonists used in this experiment increased the amplitude of indirectly induced muscle twitches (IT) elicited by pulses with 0.05 to 0.07 msec duration and a supramaximal voltage, but hardly affected directly induced muscle twitches (DT). Figure 1 shows the increase in the tension of IT induced by 5×10\(^{-5}\) M l-verapamil. All of the agents produced such a potentiative effect at concentrations ranging from 0.1 to 7.5×10\(^{-5}\) M. This effect was reversible, reproducible and dependent on their concentrations (Fig. 2). The potentiative effect gradually appeared and reached a plateau approximately 10 to 15 min after the application of the agent. Restoration from the increase by washing 4 or 5 times with drug-free media required approximately 30 min. Diltiazem was the most effective among the four agents tested. The potentiative effect of the agents lasted for 1 hr at least in the presence of the agents (5×10\(^{-5}\) M). On the other hand, when the concentration was over 1×10\(^{-4}\) M for all the agents, the amplitude of IT was inhibited with a short period (approximately 5 min) of increment by these agents.

Amplitude of IT induced by pulses with supramaximal voltage increased correspondingly with an increase in duration of the pulse (Fig. 3). The tension of IT induced by pulses of 0.02 to 0.4 msec duration and a supramaximal voltage increased correspondingly with an increase in duration of the pulse (Fig. 3).
duration and a supramaximal voltage was constant and the duration-response curve for IT formed a steady state. When the duration was over 0.5 msec, the amplitude of IT further increased and reached almost maximal level by 8 msec duration. At the first steady state, the amplitude of IT significantly (P<0.05) increased in the presence of 5 x 10^{-5} M I-verapamil. However, the agent did not increase the tension of IT induced by pulses of longer durations and a supramaximal voltage. A duration-response curve for DT was also obtained. The amplitude of DT induced by pulses of supramaximal voltage increased with the increase in the duration (0.01 to 8 msec) of the pulse. I-Verapamil (5 x 10^{-5} M) did not increase the amplitude of DT induced by pulses with all ranges of the duration.

The amplitude of IT was inhibited to some degree by 0.75 to 1.25 x 10^{-6} M d-tubocurarine (Fig. 4). In the presence of d-tubocurarine, the residual tension of IT reached a steady state for the first 10 to 15 min after the treatment. Verapamil (5 x 10^{-5} M) further reduced the residual tension of IT at the steady state level in the...
presence of d-tubocurarine (1.25x10^{-6} M) (Fig. 4). D600 (5x10^{-5} M) or diltiazem (5x10^{-5} M) also reduced the residual tension of IT in the presence of d-tubocurarine (1.25x10^{-6} M). On the other hand, caffeine (4x10^{-4} M), theophylline (4x10^{-4} M), isoproterenol (1x10^{-6} M) or hypertonically added 5 mM potassium chloride potentiatively affected the residual tension of IT in the presence of d-tubocurarine (1.25x10^{-6} M). The potentiative effect of verapamil, D600 or diltiazem was not inhibited at all by 1x10^{-5} M propranolol which completely inhibited the potentiative effect of 1x10^{-6} M isoproterenol on IT.

Ca^{2+} removal from the medium blocked IT, but not DT. Three min after blocking IT in Ca^{2+}-free solution, Ca^{2+} concentration was raised by a step wise manner from 0.1 to 4.0 mM at the 3 min interval, then the tension of IT recovered depending on the Ca^{2+} level (Fig. 5). At Ca^{2+} concentrations below 0.5 mM, 5x10^{-5} M l-verapamil slightly inhibited the amplitude of IT. However, at Ca^{2+} concentrations above 0.6 mM, the agent increased the amplitude of IT. Thus the potentiative effect of l-verapamil was demonstrated at Ca^{2+} concentrations above 0.6 mM. Similar results were obtained in the other experiments using d-verapamil, D600 or diltiazem. The potentiative effect of caffeine, theophylline or isoproterenol on IT was observed in all ranges of Ca^{2+} concentrations tested. On the other hand, the amplitude of DT correspondingly increased with a decrease in external Ca^{2+} concentration. In Ca^{2+}-free solution, however, the amplitude of DT was slightly reduced to approximately 80% of the amplitude of DT in the presence of 2.0 mM Ca^{2+}. In the presence of 0.2 to 0.5 mM Ca^{2+}, the amplitude of DT was slightly inhibited to approximately 80 to 90% by 5x10^{-5} M l-verapamil. However, the inhibitory effect of l-verapamil on DT was not antagonized by a rise in Ca^{2+} concentration.

**DISCUSSION**

Organic calcium antagonists used in this experiment increased the tension of IT induced by pulses of relatively short durations (0.04 to 0.4 msec) and a supramaximal voltage, but were poorly effective on IT produced by pulses of longer durations and a supramaximal voltage on DT. None of the organic calcium antagonists used increased the maximal twitch response, so it is unlikely that these agents increased the contractility of an individual muscle fiber. Thus the tension increase of IT by the organic calcium antagonists can probably be interpreted as an increase in the number of muscle fibers contracting per nerve stimulus.

In the case of an increase in the number
of fibers contracting at the nerve impulse, there must be some nerve-muscle units which fail to transmit in response to the stimulation. It is assumed that nerve-muscle units are not all active when stimulated, i.e. some units fail to transmit. On this assumption, the increase in the number of active nerve-muscle units is thought to be derived from an alteration of the reactivity of the preparation which may include an increase in transmitter release, a decrease in catabolism of transmitter, an increase in the sensitivity of end-plate to transmitter, and/or a lowering a mechanical threshold level of the plasma membrane of muscle fiber.

Spontaneous release of transmitter from the motor nerve terminal is reported to be increased by verapamil in the mammalian neuromuscular junction (3) and frog neuromuscular junction (5, 6). In addition, verapamil has been noted to increase the amplitude of evoked end-plate potential in the frog neuromuscular junction (5). In mammalian neuromuscular preparations, verapamil has been reported to increase the twitch potentiation induced by paired nerve shock, and the possibility of a presynaptic action was suggested (4). These suggest that the organic calcium antagonists may increase transmitter release which may serve to increase the number of fibers contracting at the nerve impulse. On the other hand, the present experiments indicated the organic calcium antagonists used in these experiments hardly affected the tension of DT. These suggest the possibility that the organic calcium antagonists increase the transmitter release, then may potentiate the tension of IT.

Despite the fact that the organic calcium antagonists used increased the tension of IT, they did not antagonize the inhibitory effect of d-tubocurarine. This may explain why these agents might have two contrary actions: first, they potentiate transmitter release at the nerve impulse and second, they inhibit the contraction induced by acetylcholine (8). In the ordinary state, they may cause the potentiative effect before the inhibitory one. In the presence of a small amount of d-tubocurarine, the non-competitive inhibitory effect of verapamil on the contraction induced by acetylcholine (8) may become dominant cooperatively with the inhibitory effect of d-tubocurarine on the end-plate.

The tension increase of IT by the organic calcium antagonists used was dependent on external Ca++ concentration above 0.6 mM. All of these agents reduced the tension of IT at Ca++ concentrations below 0.5 mM. The tension of DT, however, was not reduced by the lowering of Ca++ concentration below 0.5 mM. These suggest that at Ca++ concentrations below 0.5 mM, these agents cause a calcium antagonistic action on the nerve terminal and imply a possibility that the calcium antagonistic effect of the agents may be masked by their potentiative effect on IT at Ca++ concentrations above 0.6 mM. The inhibitory effect of verapamil and D600
on potassium-induced $^{45}\text{Ca}$ uptake by synaptosomes of rat brain are known to be weak (12). Thus the inhibitory effect of the organic calcium antagonists on the transmembrane Ca$^{2+}$ flux at the nerve terminal would not be demonstrated as an inhibitory effect on IT in the presence of a physiological level of Ca$^{2+}$ (1.8 to 2.5 mM).

Neuromuscular transmission is potentiated by several agents, i.e., caffeine, theophylline, catecholamines and potassium ions. The mechanisms of agents are suggested as follows: caffeine releases the axonal bound Ca$^{2+}$ into nerve terminals (13), theophylline increases the axonal level of cyclic AMP by the inhibition of phosphodiesterase (14), catecholamines accelerate the synthesis of cyclic AMP by activation of adenylate cyclase (15), and potassium ions depolarize the presynaptic nerve terminals (16). The organic calcium antagonists used were different in action from any of the other agents. It seems that the tension increase of IT by the organic calcium antagonists used do not take part in any of these modes of action.

From these information, it is suggested that the organic calcium antagonists used in this experiment potentiate the reactivity of the neuromuscular preparation to indirect shock, and the potentiation may include an increase in the number of fibers contracting in response to the nerve impulse through a possible increase in transmitter release, but not an increase in the contractility of an individual muscle fiber.

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