Picrotoxin Potentiates Contraction while Inhibiting Ca Current but Increasing Birefringence Signal in Frog Skeletal Muscle Fibers

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Summary: In single frog skeletal muscle fibers, picrotoxin (5 mM) potentiated the voltage-dependent component of contractions in response to 2-s depolarizing pulses while greatly inhibiting the simultaneously recorded tubular Ca current in a normal-Ca, Na- and Cl-deficient solution, provided the contractions were generated at long time intervals (2 min). In normal Ringer’s solution, picrotoxin reversibly increased the amplitude of the early large birefringence signal and the amplitude and duration of the simultaneously recorded twitch tension, suggesting that the drug may increase, directly or indirectly, the release of Ca from the SR. [Japanese Journal of Physiology, 46, 99–104, 1996]

Key words: picrotoxin, Ca current, birefringence.

It has been shown in frog skeletal muscle fibers that picrotoxin, by acting on the sarcotubular system, affects excitation–contraction (E–C) coupling [1–11]. Takeda [7] and Potreau and Raymond [12] have shown that picrotoxin reversibly inhibits the slow Na current in the transverse tubular (T) membrane produced under appropriate experimental conditions, which, as shown by Almers and McCleskey [13], flows through the Ca channels, when, as shown by Almers et al. [14], external Ca is reduced below 1 μM. Potreau and Raymond [12] have also shown that picrotoxin reversibly inhibits the part of the contraction associated with the sodium-induced calcium release mechanism, while apparently not affecting the potential-dependent component of contraction, which is present in normal physiological solution. This inhibition of contraction with picrotoxin appeared to be inconsistent with its previously known potentiating effects [1–4, 6]. To clarify this problem, the effects of picrotoxin on the Ca channels and on contraction were examined under experimental conditions similar to those of Ildefonse et al. [15] who have shown that, when current carrier of the Ca channels is confined to the Ca at normal external concentration, contraction is composed of a rapid voltage-dependent component and a component related to the inward Ca current. The results of the present experiments show that picrotoxin potentiates voltage-dependent contraction even when it inhibits the inward Ca current, provided that contractions are generated at long enough time intervals (2 min), although it depresses succeeding contractions at shorter stimulation intervals.

The early large birefringence signal (second component in [16]) is considered to reflect the process linking excitation to contraction in the sarcoplasmic reticulum (SR) and to release Ca in frog skeletal muscle fibers [17–21], and is correlated with the myoplasmic free Ca transient [22, 23]. The effect of picrotoxin on the birefringence signal in single frog skeletal muscle fibers was examined as an approach to clarify the action of picrotoxin in the SR. The results show that the amplitude of the birefringence signal increases reversibly together with the twitch tension in the presence of picrotoxin. Preliminary accounts of the pre-
Methods and Materials

Ca current and tension experiments. The equipment used was the same as that used before [15] and described in detail previously [26]. Single twitch fibers freshly dissected from the semitendinosus muscle of the frog *Rana esculenta* were used. The frogs were killed by decapitation. Isolated single fiber was immersed in cold solution (5°C) used for electrophysiological recordings for about 15 min before mounting in a double mannitol-gap voltage clamp apparatus. The solution used had the following composition: 1.8 mM CaCl₂; 122 mM TEA-CH₃SO₃H; 5 mM glucose; 3 mM 4-aminopyridine; 10 mM HEPES-KOH (pH 7.4). The solution contained a normal concentration of Ca, but most Cl was substituted with impermeable CH₃SO₃H and most K channels were blocked with TEA and 4-aminopyridine. Isotonic mannitol (236 mM) was used in the isolating compartments. The muscle fiber in the solution was generally depolarized. A holding potential (estimated to be ca. −90 mV) adjusted to have a contractile threshold at −50 mV was applied to maintain normal resting potential. The currents and tensions in response to voltage-clamped 2 s depolarizing pulses, which were given usually at relatively long time intervals (up to at least 2 min), were recorded simultaneously. Picrotoxin at 5 mM (ca. 3 mg/ml) was used because it is about the solubility limit and we expected quick effects of the drug. Experiments were performed at about 17°C.

Birefringence and tension experiments. The equipment used for the experiments was the same as that used before [21] and originally described in detail previously [17]. Single fibers were isolated from the semitendinosus muscle of *Rana temporaria*. The Ringer's solution used contained (in mM): NaCl, 115; KCl, 2.5; CaCl₂, 1.8; HEPES, 10. The tendons were tied with 50-μm silver wire to loops made from 100-μm silver wire. A muscle fiber was suspended vertically in a chamber between a stainless-steel hook at the lower end and a Plexiglas hook attached to the transducer at the upper end. The fiber was stretched to 130% slack length, yielding a sarcomere length of approximately 2.7 μm. The fiber was flanked by two platinum electrodes for field stimulation. A piezoelectric transducer was used to record twitch tension. To record the birefringence signals, the chamber containing the muscle fiber was placed between crossed polarizers and illuminated with a tungsten halogen light source. The light intensity transmitted by the second polarizer was measured with a photodiode. Changes in light intensity, indicative of changes in muscle fiber birefringence are given relative to the resting light intensity. The outputs of the force transducer and the photodiode were amplified, digitized and the data were stored on a hard disk. When 2-Hz stimulation was used, data of 8 sweeps were signal-averaged. Data were printed out by X–Y plotter. Picrotoxin at 5 mM was added to the solution to examine its effect. Experiments were performed at room temperature (20–23°C).

Results and Discussion

Ca current and tension experiments. The amplitude and time course of inward Ca current and of tension recorded simultaneously in the solution (e.g. Fig. 1A, B) were rather similar to those in the previous study [15]. During a 2-s depolarizing pulse, the amplitude of tension gradually decreased after a peak, but the tension was retained until the end of the pulse. After adding 5 mM picrotoxin to the solution, the inward Ca current was inhibited almost completely with time. In all the records examined, the amplitude of simultaneously recorded tension in response to first depolarizing pulse (e.g. to −40 mV) applied after adding picrotoxin was larger than that of control tension produced by the same depolarizing pulse be-
fore adding picrotoxin. However, when stimulated at intervals of 1 min or less, the amplitude of tension in response to successive depolarizing pulses became smaller than the control value in all of the several fibers examined.

Figure 1 shows typical current and tension records of the same fiber before and after adding picrotoxin, and after the removal of picrotoxin when the depolarizing pulses were applied at intervals of at least 2 min. At 6 min after adding picrotoxin (Fig. 1C), the peak inward Ca current in response to a depolarizing pulse to $-40$ mV was reduced to 26%, but the outward current was increased to 1.4 times and peak tension was increased to 2.5 times the values of control records (Fig. 1A). At 8 min after adding picrotoxin (Fig. 1D), the peak inward Ca current in response to a depolarizing pulse to $-20$ mV was reduced to 31%, but the outward current was increased to 1.28 times and peak tension was increased to 2.86 times the values of control records (Fig. 1B). Picrotoxin was washed out immediately after the records in Fig. 1D were taken. At 8 min after removal of picrotoxin (Fig. 1E), the peak inward Ca current and peak tension in response to a depolarizing pulse to $-40$ mV were almost identical, but the outward current was increased to 1.28 times the value of control records (Fig. 1A). These results indicate that, in the presence of normal external Ca (1.8 mM) and by stimulating at long enough time intervals (2 min or more), picrotoxin potentiates contraction while it is inhibiting the inward Ca current, and that these effects are reversible, if the exposure time to picrotoxin is short (ca. 8 min in this fiber). The depolarization dependency of the tension in the absence (Fig. 1A, B) and in the presence (Fig. 1C, D) of picrotoxin indicates that they represent potential-de-

Figure 2. Simultaneous records of twitch tensions (upper traces) and early large birefringence signals (lower traces with early large fast change, upward deflexion represents positive change in transmitted light) before (dotted traces) and 30 s after adding 5 mM (ca. 3 mg/ml) picrotoxin (solid traces). The dotted traces represent an average of 8 sweeps at 2-Hz stimulation and solid traces are single sweep records. The same scales are applied to the two tension and the birefringence records, respectively.
the optical signal could be reversed by prolonged soaking in normal Ringer’s solution. In all three other fibers examined, including those stimulated with trains-of-eight pulses at 2 Hz also after adding picrotoxin, similar results were obtained.

Figure 3 shows typical records of other fiber stimulated at 2-min intervals, since, as already described, picrotoxin showed stable potentiation of contraction at the intervals. Although some irregularities are noticed in the traces of the records, the amplitude of the birefringence signal after adding picrotoxin increased to 2.7 times normal in the 2nd (ca. 5 min after adding picrotoxin) and to 3.5 times normal in the 3rd record (ca. 7 min), respectively. The amplitude of tension increased to about 1.5 times normal, and the duration of tension at half amplitude increased to about 2.1 times normal (from 104 to 220 ms) in these three records after adding picrotoxin. In all three fibers stimulated at 2-min intervals, the amplitude of the birefringence signal increased by a factor of 1.5–3.5, the amplitude of tension by a factor of 1.5–1.9, and the duration of tension at half amplitude by a factor of 2.0–2.1, when measured between 5 and 7 min after adding picrotoxin.

**Discussion.** The sodium-induced calcium release mechanism shown by Potreau and Raymond [12] reveals a mechanism for the Na current to induce a large contraction, which is inhibited with picrotoxin, when the extracellular ionic composition is modified. These authors have also shown the potential-dependent component of contraction, but not the effect of picrotoxin on this component. A sodium-induced calcium release mechanism has also been shown by Caillé et al. [27] and is discussed as sodium-modulated calcium release by Allard and Rouger [28].

The present results of the Ca current and tension experiments show that picrotoxin greatly inhibits the Ca current through the Ca channels in the T membrane when, in the presence of normal external Ca, Ca is the only current carrier of the channels. It has been shown by Potreau and Raymond [29], Ildefonse et al. [15] and by Blaineau et al. [30] that, during a long depolarizing pulse comparable to that in the present experiments, the Ca current through the Ca channels in the T membrane induces a Ca current-dependent part of contraction, which should be mostly abolished with picrotoxin in the present experimental conditions. In the present Ca current and tension experiments, potentiation of contraction with picrotoxin is free from its effects on action potential [1–3]. It has been shown that the inward rectifier due to K conductance in the T membrane is virtually completely abolished with picrotoxin [5, 10]. Moreover, most K channels were blocked with TEA and 4-aminopyridine in the present experiments. Therefore, the results of the present Ca current and tension experiments show, under the experimental conditions used: that (i) picrotoxin potentiates contraction mainly via voltage-dependent processes of transmission from the T membrane to the SR, mostly unaffected from the current events in the T membrane [31]; and that (ii) the effect of the drug responsible for the potentiation is expected to be detected in the SR.

In the present birefringence and tension experiments, in which twitch contraction lasted at most for less than about 300 ms even when the twitch duration

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**Fig. 3.** Simultaneous records of early large birefringence signals (upper traces) and twitch tensions (lower traces) before (4 sweeps with almost identical time course) and after adding 5 mM picrotoxin (3 sweeps with numbers in the order of recording). The fiber was stimulated at 2-min intervals and all sweeps were superimposed. Same scales are applied to all tensions and the birefringence records, respectively.
increased in the presence of picrotoxin, stimulating intervals as long as 2 min may not be required to examine the effects of picrotoxin. However, the great inhibition with picrotoxin of the Ca current in the T membrane and the resultant Ca current-dependent contraction, and also virtually complete abolition with picrotoxin of the inward rectifier due to K conductance in the T membrane, should occur in the present experimental conditions. Therefore, the potentiation of twitch contraction and the increase in the early large birefringence signal observed should be achieved mainly by transmission via voltage-dependent processes from the T membrane to the SR. As suggested [17–19, 21] and discussed in detail [20], the early large birefringence signal is considered to reflect the process in the SR and to release Ca. Baylor et al. [22, 23] have shown, using either Arsenazo III, Antipyrilazo III or Dichlororhodopsinazo III to measure free Ca, that the time course of the early large birefringence signal is remarkably similar to those of the Ca transient signals through time to peak, or except at late times in the falling phase, before serious movement artifacts occur. Baylor et al. [23] also show that the time course of these signals is distinct from that of the concentration of Ca-regulatory sites on troponin which are complexed with Ca, which argues against the suggestion that the birefringence signal may reflect a conformational change in the thin filament as a consequence of Ca binding to troponin. The significant increase with picrotoxin of the birefringence signal in the present results indicates that picrotoxin actually modifies, directly or indirectly, a parameter of the event in the SR. These results, together with those of Baylor et al. [22, 23], suggest that picrotoxin increases the release of Ca from the SR. In this connection, it has been shown that picrotoxin increases about ten times the efficiency of the voltage and duration product above the rheobase mechanical threshold needed for short depolarizing pulses, which are comparable in duration to the action potential, to reach the mechanical threshold [11]. Possible ways of discriminating between hypotheses in E–C coupling are provided by examining the effects of changes in ionic composition of the extracellular bathing fluid, and by using drugs to block specific transmembrane ion fluxes, as stated in Milehi et al. [32], and the use of picrotoxin as a blocker of the T membrane current is an example. The present results suggest that picrotoxin might modify the voltage-dependent processes of transmission from the T membrane to the SR by acting on the dihydropyridine receptor [31], and/or the drug might affect directly the processes to release Ca from the SR. The Ca current and tension study was done by V. J., K. T., and O. R. at Université Claude Bernard, and the birefringence and tension study by H. O. and K. T. at University of Bern. K. T.'s visits to Villeurbanne and Bern were supported by an overseas visiting research grant from the Ministry of Education, Science, Sports and Culture of Japan.

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