Proctolin induces rhythmic contractions and spikes in *Limulus* heart muscle

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WATSON, WINSOR H., III, AND TOSHINORI HOSHI. *Proctolin induces rhythmic contractions and spikes in Limulus heart muscle.* Am. J. Physiol. 249 (Regulatory Integrative Comp. Physiol. 18): R490–R495, 1985.—The *Limulus* heart is neurogenic. If the cardiac ganglion is removed, all spontaneous contractions of the heart are abolished. Application of the pentapeptide proctolin (>1 μM) causes the deganglionated heart muscle to beat with a frequency and amplitude slightly greater than those of a normal heart with an intact cardiac ganglion. At a proctolin concentration of 1 μM, rhythmic beating requires 2–10 min to develop, and up to 1 h of continuous washing is required to reverse the effect. A contracture often precedes the rhythmic contractions. Proctolin-induced rhythmicity occurs in the presence of tetrodotoxin (TTX) and in Na^+-free saline. These effects of proctolin are not mediated by residual portions of the cardiac ganglion. Contractions are inhibited by Ca^{2+}-free EGTA saline, $CoCl_2$, $MnCl_2$, and $CdCl_2$. Proctolin causes no significant long-term changes in the myocardial resting potential or apparent input resistance. However, proctolin causes rhythmic 10- to 20-mV spikes that precede each contraction of the myocardium. Production of these spikes appears to be the mechanism by which proctolin causes rhythmic contractions in normally quiescent deganglionated myocardium of *Limulus*.

neurogenic heart; deganglionated heart; myogenicity; intracellular recording; peptide

THE HEART OF THE HORSESHOE CRAB *Limulus polyphemus* is neurogenic (6). Pacemaker neurons within the cardiac ganglion drive the follower cells, or motor neurons, that innervate the cardiac muscle (1, 2, 17, 23, 29). The follower neurons produce summating, compound, excitatory junctional potentials (EJP) in the nonspiking muscle fibers. Physical or pharmacological removal of the cardiac ganglion abolishes the EJPs and terminates the cardiac rhythm (1, 2, 6, 17, 21, 23, 25).

A deganglionated heart (without cardiac ganglion) can be induced to beat in several ways. Perfusion with Ca^{2+}-free saline elicits rhythmic contractions (7, 8). This rhythmicity is the result of tetrodotoxin (TTX)-insensitive Na^+-dependent action potentials (18, 26).

Excessive stretch applied to a deganglionated heart also induces rhythmic contractions (10, 11). Each contraction appears to be accompanied by action potentials in the muscle fibers, but definitive proof is lacking (16, 18). The magnitude of the heart contractions is so large that intracellular recordings are extremely difficult to obtain. Moreover, when the heart is immobilized to facilitate intracellular recordings, stretch no longer elicits rhythmic contractions. Thus freedom of the muscle fibers to move seems necessary for stretch-induced rhythmicity.

The pentapeptide proctolin can also elicit rhythmic contractions in a deganglionated *Limulus* heart when applied at concentrations of >0.1 μM (14, 30). Proctolin or a close analog is present in the *Limulus* nervous system and appears to be involved in enhancing the strength of heart contractions (4). Proctolin is also present in other arthropods and affects their muscle activity. For example, proctolin causes contracture of lobster opener muscle (27), induces rhythmic contractions of locust extensor tibiae muscle (20, 24), and produces both contracture and rhythmic contractions of the cockroach hindgut (5, 9, 28).

This paper details proctolin-induced rhythmic contractions in the deganglionated *Limulus* heart. The results show that proctolin induces the rhythmic contractions through direct actions on the cardiac muscle fibers. The underlying mechanism appears to be the initiation of spike-like potentials.

METHODS

Animals. The horseshoe crabs (*L. polyphemus*) were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and were maintained in a recirculating seawater system filled with natural seawater (15–18°C). Both male and female specimens, carapace widths between 13 and 20 cm, were used.

Tissue preparation. Hearts were removed and prepared for recording as previously described (3). When mechanical activity alone was monitored, intact hearts were pinned out in a 5-ml Plexiglas chamber through which various solutions were perfused at an average rate of 20 ml/min. Several different methods were utilized to record intracellular electrical activity. *1)* Intact hearts were pinned out as described above, and a small (~1 cm × 0.5 cm) piece of nylon mesh was secured over a region of the heart. The secured section of myocardium could not move excessively; thus stable intracellular recordings could be obtained for 30 min while tension was being monitored simultaneously in an adjacent region. The major drawback of this technique was that it was impossible to record electrical activity from the same muscle

fibers from which tension measurements were obtained. 2) Deganglionated hearts were turned inside out to expose the luminal side of the myocardium. The muscle fibers lining the lumen were more easily impaled with microelectrodes, and the inside out preparations responded to proctolin as well as intact hearts. Recordings were made with floating microelectrodes and without mesh. Thus we could electrically record from the same fibers from which tension was being measured. However, proctolin-induced contractions were so large that even floating microelectrodes were dislodged within a few seconds. 3) A single sucrose gap chamber was used to record the electrical activity of a small piece of heart muscle. The chamber was constructed as previously described by Lloyd (19). Briefly, two sections (5 ml each) of the chamber were separated by a gap, through which sucrose was perfused. One section of the chamber contained isotonic KCl and the other normal saline. Small rings (cross section of heart, 1 × 0.5 cm) of cardiac muscle were pulled through the chamber. One end of the muscle was attached to a force transducer. This arrangement allowed simultaneous measurement of tension and membrane potential, although the electrical activity of single muscle fiber could not be monitored. This technique proved to be most successful, and it provided most data on intracellular electrical activity.

Instrumentation. Tension was recorded from the lateral margin of the hearts, in the region between the 3rd and 5th ostia, with a Grass FTO3 force transducer. Intracellular recordings from muscle fibers were made with 10–30 MΩ microelectrodes filled with 3 M KCl. Floating microelectrodes were constructed as described by Lang (18). Outputs of the force transducer and electrometer were displayed on an oscilloscope and a Grass polygraph or a Gould pen recorder. Occasionally experiments were tape recorded (Tanberg UM model 115) for subsequent analysis.

Solutions. Two normal Ringer solutions were used: *1)* natural seawater obtained from Portsmouth Harbor, New Hampshire, and 2) *Limulus* saline [(in mM) 445 NaCl, 12 KCl, 10 $CaCl_2$, 46 $MgCl_2$, 10 tris(hydroxymethyl)aminomethane-maleate buffer, pH 7.4]. Na^+-free solutions were made by substituting choline Cl for NaCl. Two types of Ca^{2+}-free solutions were used: *1)* Ca^{2+}-free EGTA (2 mM) solutions and 2) isotonic (600 mM) NaCl solution without any EGTA. Verapamil was made up fresh as a 10 mM stock. Sucrose gap experiments were performed with isotonic KCl (0.54 M) and sucrose (0.72 M) made up in deionized water. All drugs were obtained from Sigma, except that TTX was purchased from Calbiochem-Behring (La Jolla, CA 92037) and that verapamil was a gift from Knoll Pharmaceutical (Whippany, NJ 07981).

RESULTS

General characteristics of proctolin-induced contractions and contracture. Bath application of proctolin (>0.1 μM) caused rhythmic contractions in deganglionated *Limulus* hearts and an increase in the strength of contractions in normal hearts (Fig. 1). The magnitude and frequency of these contractions in deganglionated hearts were greater than those recorded from the same hearts, with cardiac ganglia attached, in normal seawater containing no proctolin. The rhythmic contractions typically started 4.3 ± 2.7 (SD) min (n = 12) after addition of 1 μM proctolin, and they reached maximal amplitude 11.2 ± 5.2 (n = 12) after addition of the peptide (1 μM). The effects of proctolin greatly outlasted the application period. A 30-s treatment with a 1-ml dose of 1 μM proctolin led to contractions that continued for up to 30 min, and after 30 min exposure, more than 2 h of continuous perfusion (20 ml/min) with normal seawater were required to eliminate the contractions.

Proctolin-induced rhythmic contractions were often preceded by slow contractures (Figs. 1, 2). These proctolin-induced contractures could be separated from the rhythmic contractions also induced by proctolin by repeatedly exposing the deganglionated hearts to the peptide. Such exposures eventually blocked the contractions but not the contracture (middle trace, Fig. 2). The time course of the development of these contractures paralleled the increase in contractility of normal hearts that

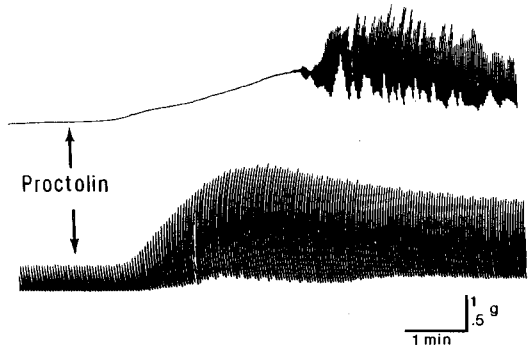

FIG. 1. Proctolin induces rhythmic contractions in deganglionated hearts and enhances contractility in normal hearts. Comparison of effects of proctolin (1 μM) on a deganglionated (*top*) vs. normal (*bottom*) *Limulus* heart. Proctolin application caused deganglionated heart to develop a slow contracture followed by rhythmic contractions. Normal hearts responded to proctolin with a large increase in strength of contractions. Note that force calibration bar for deganglionated heart trace (*top*) is 1 g and that for normal heart trace is 0.5 g.

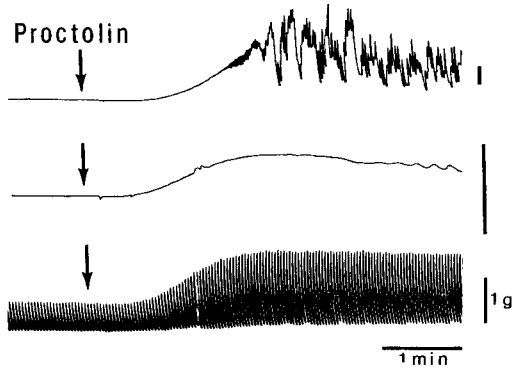

FIG. 2. Proctolin also induces contractures. Proctolin, at a concentration of 1 μM, usually produces a contracture followed by rhythmic contractions (*top*). If a small dose (0.5 ml of 1 μM squirted directly into chamber) is applied repeatedly, it occasionally produces a pure contracture without subsequent contractions (*middle*). *Bottom*, response of a normal heart in same chamber. Note similarity in time course of contracture (*middle*) and increase in contractility (*bottom*).

had been exposed to proctolin in the same recording chamber (bottom trace, Fig. 2). The proctolin-induced rhythmic contractions took about the same amount of time to develop as the maximal increases in contractility produced in normal hearts by proctolin. (Figs. 1, 2).

Response of normal hearts to proctolin in Na^+-free saline. The contractions of normal hearts, with intact cardiac ganglia, were blocked readily by perfusion of a Na^+-free saline solution (Fig. 3*A*). The voltage-dependent Na^+ channel blocker TTX also blocks conduction of impulses from the ganglion to the heart muscle and thus inhibits the normal heart contractions (21, 25, 30). Application of proctolin to the nonbeating normal hearts in Na^+-free solution initiated rhythmic contractions preceded by contractures (Fig. 3*B*) similar to those observed in deganglionated hearts after application of peptide. In Na^+-free solution, synaptic transmission between the follower motor nerve terminals and cardiac cells was also blocked (Fig. 4). Glutamate is the putative neuromuscular transmitter in *Limulus*, and the response of cardiac muscle fibers to glutamate is Na^+-dependent (unpublished results).

In conclusion, Na^+-free solutions inhibit contractions of the normal heart by blocking axonal conduction and neuromuscular transmission, functionally deganglionating the hearts. In contrast, the response to proctolin is unaffected by the absence of Na^+, so the effect of the peptide under these conditions is probably due to a direct action on the muscle fibers.

Ca^{2+} sensitivity of proctolin-induced contractions. Proctolin-induced rhythmic contractions were inhibited by inorganic divalent ions that block voltage-dependent Ca^{2+} channels (in mM): 30 $CoCl_2$, 5 $MnCl_2$, 1 $CdCl_2$, and 10 $NiCl_2$. In most experiments the blocking effects were reversible (Fig. 5). An organic Ca^{2+} channel blocker verapamil (0.1 mM) did not block the contractions.

When deganglionated hearts were perfused with a solution containing no added Ca^{2+} and Ca^{2+} chelator EGTA (2 mM), application of proctolin did not produce contractions (Fig. 6). When the preparation was then washed with normal saline, a burst of rhythmic contractions was observed, even though proctolin was not present in the bath (Fig. 6).

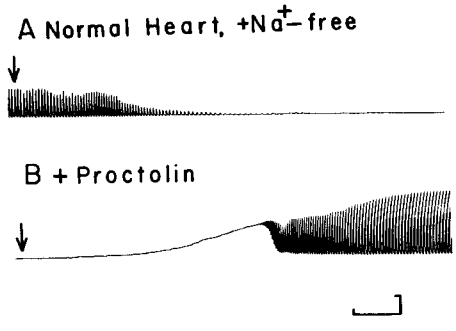

FIG. 3. External Na^+ is not required for proctolin-induced contractions. Tension recording was made from a normal heart with cardiac ganglia. *A*: at *arrow*, bath solution was changed from normal Ringer solution to Na^+-free solution. This treatment eventually eliminated contractions. *B*: at *arrow*, proctolin (1 μM) was added in Na^+-free solution. This produced a contracture followed by rhythmic contractions. Calibration: 1 min, 1 g.

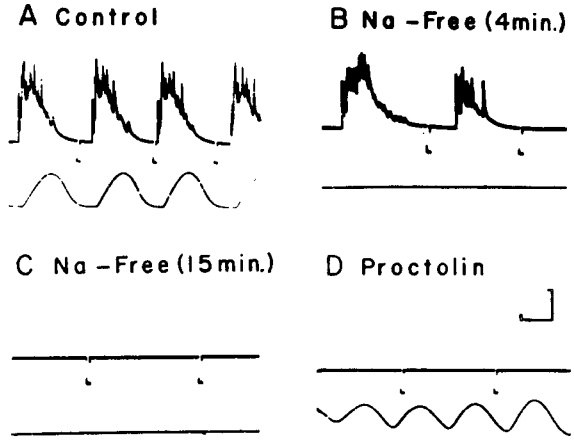

FIG. 4. Na^+-free saline blocks neuromuscular transmission but not proctolin-induced contractions. *A*: control records showing compound excitatory junctional potentials (EJP) (*top*) with periodic resistance check (0.5 nA) and rhythmic contractions of heart (*bottom*). *B*: perfusion with Na^+-free saline reduced amplitude of EJPs and contractions after 4 min, and after 15 min in Na^+-free saline EJPs were abolished, apparent input resistance was unchanged, and there were no contractions (*C*). *D*: addition of proctolin (1 μM) induced rhythmic contractions, but there were no changes in the membrane potential or apparent input resistance of impaled fiber. Note intracellular recordings were made from an immobilized region of heart, whereas tension was measured in an adjacent region. Calibrations: 10 mV, 2 s.

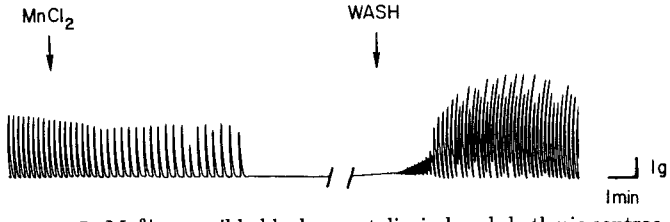

FIG. 5. Mn^{2+} reversibly blocks proctolin-induced rhythmic contractions. Rhythmic contraction of a deganglionated heart was elicited by exposing it to 1 μM proctolin. Addition of $MnCl_2$ (5 mM) at *left arrow* slowly blocked contractions despite continuous presence of proctolin. On washing with normal saline solution (*right arrow*), rhythmic contractions reappeared. A 6-min section of record has been removed.

Proctolin-induced spikes. Intracellular recordings were obtained from muscle fibers while the mechanical activity was simultaneously being monitored with a force transducer. The following four methods of intracellular recordings were tried: *1*) conventional microelectrodes, *2*) conventional microelectrodes poked through a nylon mesh used to immobilize a small portion of the heart, *3*) floating microelectrodes, and *4*) single sucrose gap. Conventional microelectrodes were dislodged or broken by the force of the proctolin-induced contractions. With the nylon mesh method, no consistent changes in membrane potential or apparent input resistance were recorded from the immobilized fibers, although rhythmic contractions elicited by proctolin could be monitored in adjacent nonimmobilized muscle fibers. The only changes observed were very small (<1 mV) fluctuations in membrane potential. The same results were obtained in normal ganglionated hearts that were pharmacologically deganglionated by perfusion with Na^+-free solution. Fibers were impaled with electrodes and normal EJPs recorded; then the heart was perfused with Na^+-free

saline to eliminate EJPs and contractions, and proctolin was finally added. Despite strong contractions in adjacent muscle fibers, those under mesh displayed no obvious changes in membrane potential or apparent input resistance (Fig. 4).

When the sucrose gap method was used to obtain intracellular recordings from deganglionated heart muscle, rhythmic spikes were observed after application of proctolin (Fig. 7). The average resting potential obtained in 13 experiments using 0.54 M KCl was −32.4 ± 9.0 mV. This was more depolarized than values recorded with microelectrodes from preparations treated the same way (−50 ± 2.6 mV, $n = 20$). The differences in magnitude and variability were most probably caused by shunting of current across the gap and by the concentration of KCl (0.54 M) employed. Application of 1 μM proctolin produced little change in resting potential (0.82 ± 2.2 mV, $n = 23$) during the contracture phase of the response. After the contracture phase, small slow fluctuations in membrane potential appeared and were accompanied by slow waves of contraction and relaxation (Fig. 7*A*). These small fluctuations in membrane potential were comparable in size and time course to those recorded using microelectrodes with nylon mesh. Gradually the slow waves developed into small spike-like potentials (Fig. 7*B*). Finally, much larger (10–20 mV) rhythmic spikes, preceding each contraction, were observed (Fig. 7*C*). Proctolin-induced spikes were, on average, 10–20 mV and 500–800 ms long. The size of the muscle contractions was usually proportional to the spike amplitude. Episodes of rhythmic activity observed in the sucrose gap chamber, such as the one shown in Fig. 7, were brief despite the continuous presence of proctolin. This behavior probably resulted from the use of small pieces of muscle because small rings of heart muscle, pinned out in a dish containing 1 μM proctolin, did not beat for more than a few minutes either.

The spikes recorded from deganglionated heart muscle of *Limulus* in response to proctolin were similar to those recorded from the same preparation in response to isotonic NaCl (Fig. 8). Rhythmicity elicited by isotonic NaCl also started with slow waves and small fluctuations in membrane potential. Eventually, large spikes were evident, and their durations and amplitudes were com-

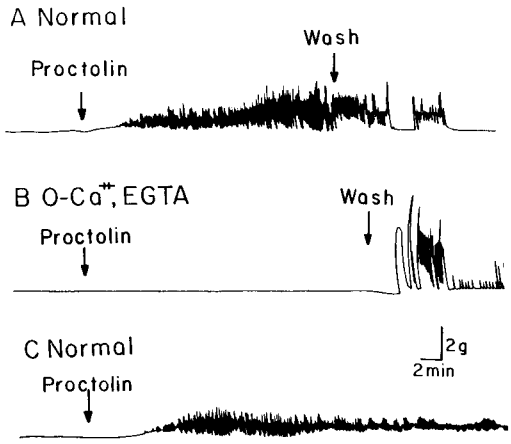

FIG. 6. Proctolin cannot induce rhythmic contractions when external Ca^{2+} concentration is lowered by EGTA. *A*: addition of proctolin (1 μM) in normal saline to a deganglionated heart caused rhythmic contractions. Perfusion with proctolin-free normal saline gradually terminated contractions. *B*: 1 h later the same heart was perfused with Ca^{2+}-free EGTA solution for 15 min, and proctolin (1 μM) was then added to bath at *left arrow*. During next 30 min in Ca^{2+}-free EGTA solution with proctolin, there was no indication of either a contracture or rhythmic contractions. When heart was washed with normal saline without proctolin (at *right arrow*), a burst of rhythmic contractions appeared for a short time. *C*: after another 1 h of wash with normal saline without proctolin, the same heart was exposed to proctolin (1 μM) again to ensure that results obtained in *B* were not caused by a permanent deleterious change in heart's responsiveness to proctolin.

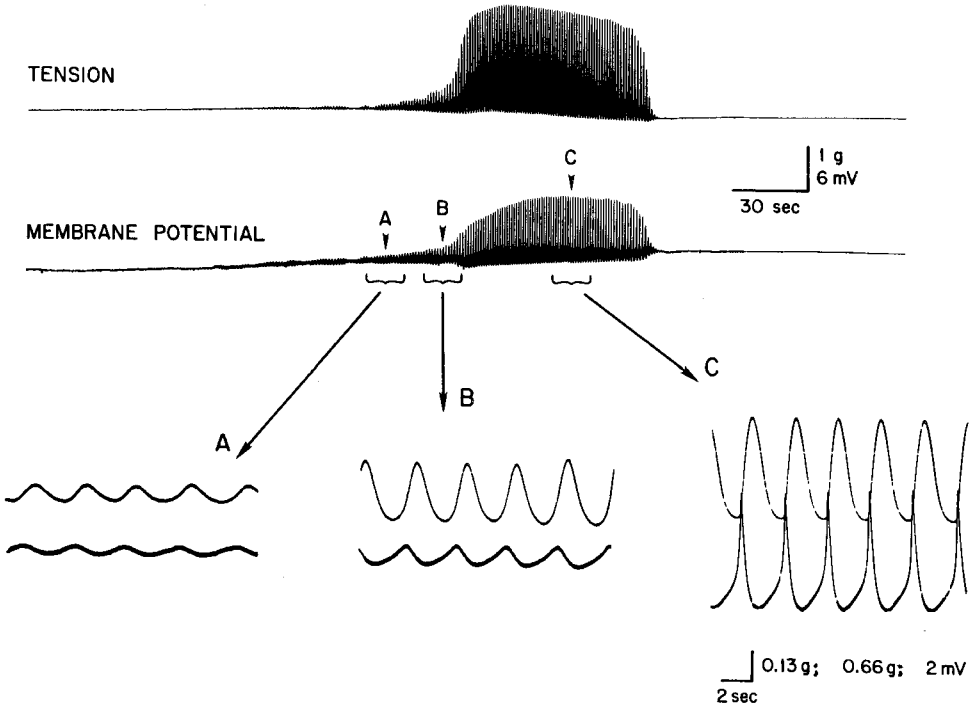

FIG. 7. Proctolin induces rhythmic spikes that accompany contractions. Simultaneous recordings of tension and electrical activity were obtained during proctolin-induced contractions, using sucrose gap technique. A small ring of heart muscle was placed in a single sucrose gap chamber so that both tension and membrane potential changes could be monitored at the same time. Application of proctolin (1 μM) just before this section of record caused slow waves to develop (*A*), then small spike-like potentials (*B*), and finally large spikes (*C*). All changes in membrane potential were associated with cardiac muscle contractions. Records shown were obtained at times indicated by *arrows* on top traces. Tension: *A* and *B*, 0.13 g; *C*, 0.66 g.

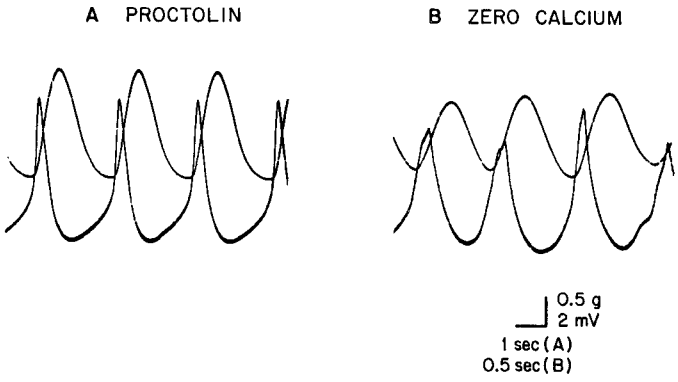

FIG. 8. Comparison of proctolin- and isotonic NaCl-induced spikes and contractions in deganglionated hearts. In both *A* and *B*, top traces are force recordings from muscle fibers, and bottom traces are transmembrane potential. In *A*, 1 μM proctolin was applied to induce the rhythmic contractions and spikes. After proctolin treatment the same heart was perfused with isotonic NaCl to induce rhythmicity shown in *B*. Note difference in time calibration in *A* and *B*.

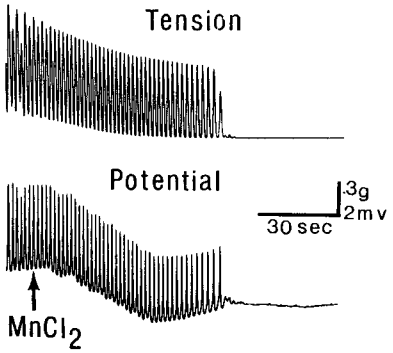

FIG. 9. $MnCl_2$ blocked proctolin-induced rhythmic contractions and spikes. Proctolin was perfused over a ring of muscle in a single sucrose gap chamber before record shown. Rhythmic contractions and spikes appeared, and then application of 20 mM $MnCl_2$ blocked contractions and spikes after 1 min in most cases. Before complete blockage, Mn^{2+} treatment slowed rate of contractions and spikes. Small hyperpolarization after Mn^{2+} treatment is probably an artifact, since it was observed only in some experiments.

parable with those of proctolin-elicited spikes (Fig. 8). However, the rate of isotonic NaCl-induced contractions was typically twice as great as the proctolin-induced ones, even in the same preparations.

The inorganic Ca^{2+} channel blockers that inhibited the proctolin-induced contractions (see Fig. 5) also blocked the rhythmic spikes recorded with the sucrose gap technique. $CoCl_2$, $MnCl_2$, and $NiCl_2$ reduced the rate and amplitude of proctolin-induced spikes and gradually eliminated the rhythmic activity altogether (Fig. 9).

DISCUSSION

Proctolin-induced rhythmic contractions in deganglionated *Limulus* heart appear to be caused by a direct action of the peptide on the myocardium, rather than on remnants of the cardiac ganglion. Proctolin-induced contractions do not require Na^+, as shown in Figs. 3 and 4. Nerve impulses from the cardiac ganglion to the muscle fibers should have been blocked by TTX or Na^+-free Ringer solutions (30), assuming that the Ca^{2+}-dependent action potentials shown for some molluscan axons (13) do not occur in *Limulus*. Regardless of the ionic dependency of the axonal nerve impulse, EJPs in the cardiac muscle fibers are Na^+ dependent (see Fig. 4). In the absence of external Na^+, neurotransmitters released onto the cardiac muscle fibers cannot directly generate the rhythmic depolarizations necessary to produce contractions. Furthermore, rhythmic EJPs were not observed from deganglionated hearts treated with proctolin during the intracellular recordings.

Proctolin-induced rhythmic contractions of *Limulus* deganglionated hearts are accompanied by rhythmic spikes in the cardiac muscle fibers. Each spike (10–20 mV) precedes a contraction, and amplitudes of the spike and contraction are positively correlated. Furthermore, treatments that inhibit the contractions, such as Mn^{2+}, also inhibit the spikes. The pharmacological properties of the proctolin-induced spikes, judging from the divalent blocker sensitivity, are similar to those of the voltage-dependent Ca^{2+} channels in other preparations. Therefore the proximate effect of proctolin may be to elicit Ca^{2+}-dependent myocardial spikes that in turn generate the rhythmic contractions.

Rhythmic contractions of deganglionated *Limulus* hearts produced by isotonic NaCl or by stretch are also accompanied by rhythmic myocardial spikes (18, 25). These spikes are insensitive to TTX (18) and are blocked by Ca^{2+} (18), Mn^{2+}, Cd^{2+}, and Ni^{2+}. Na^+, Ba^{2+}, and Sr^{2+} can serve as charge carriers (26). These ion selectivity properties, including Na^+ permeability (12), suggest that voltage-dependent Ca^{2+} channels are involved in stretch-, isotonic NaCl-, and proctolin-induced contractions.

In *Limulus* myocardium, proctolin produces two effects: *1*) rhythmic contractions accompanied by spikes and *2*) contractures that are not associated with changes in either membrane potential or apparent input resistance (Fig. 4). Both phenomena are dependent on external Ca^{2+}. The lobster opener muscle (27) also gives similar responses to proctolin, and the effects are also Ca^{2+} dependent. Thus proctolin probably acts through a similar mechanism to generate contractions of the muscle fibers in both preparations.

In most muscles studied, proctolin either induces spikes [*Lucilia* larvae (15), lobster opener muscle (27)], initiates contracture in the absence of membrane depolarization [lobster (27)], or enhances the ability of existing potential changes to promote contraction [locust extensor tibiae muscle (24), cockroach hindgut (9, 23)]. In *Limulus*, proctolin shows all three actions, depending on the concentration. When the normal heart is exposed to low concentrations of proctolin (0.1–100 nM), there is a substantial increase in contractility (4). The precise mechanism responsible for this effect is unknown. As described in this paper, higher proctolin concentrations lead to contracture without depolarization and then rhythmic contractions accompanied by spikes. Although these latter effects may not occur in intact animals, the same underlying mechanisms may be responsible for proctolin's enhancement of contractility at lower concentrations.

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