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Peptide Cotransmitter Potentiates Calcium Channel Activity in Crayfish Skeletal Muscle

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The activity of 2 types of Ca^{2+} channels (38 and 14 pS in 137 mM Ba^{2+}) in the plasma membrane of the crayfish tonic flexor muscle is modulated by the peptide proctolin. This peptide serves as a cotransmitter in 3 of the 5 excitatory tonic flexor motoneurons and greatly enhances tension after depolarization by the conventional neurotransmitter. Proctolin alone has no effect on these channels, but renders them capable of sustained activity following depolarization. After depolarization induces activity, 5×10^{-9} M proctolin increases the open probability of the larger channel up to 50-fold due to a marked decrease in the mean channel closed time. There is also at least a 4-fold increase in the percentage of patches with active channels for the large channel and a 2-fold increase for the small channel. Proctolin modulation appears to occur via an intracellular messenger, possibly cAMP. The peptide's effect on channel activity is dose dependent in a manner that parallels its effect on tension. These results indicate that the activation of these channels and the resulting influx of Ca^{2+} into the muscle fiber play a role in the potentiation of tension in this muscle.

We have been investigating the role of a peptide cotransmitter in the well-studied tonic flexor neuromuscular system in the crayfish. This system consists of 6 identified motoneurons (5 excitatory) and approximately 40 muscle fibers located in each hemisegment in the animal's abdomen, where it controls slow abdominal flexion (Kennedy and Takeda, 1965; Wine et al., 1974). We have shown that, in addition to the conventional neurotransmitter L-glutamate, 3 of the excitatory motoneurons in this system contain the peptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH). Proctolin is released from these motoneurons in response to neural stimulation. Selective depletion of the peptide from motoneuron terminals, leaving the levels of the conventional neurotransmitter unchanged, results in a reduction of as much as 50% of tension. Thus, endogenous proctolin appears to be an important contributor to the magnitude of tension generated in this muscle (Bishop et al., 1984, 1987).

Proctolin, by itself, does not induce tension in the muscle at any concentration. Rather, it acts directly on the muscle to amplify the depolarization-induced tension of the conventional neurotransmitter. It also lowers the threshold for excitation–contraction coupling. The threshold for the peptide tension effect is 5×10^{-11} M, and the maximum effect is reached with 5×10^{-9} M. Proctolin tension amplification occurs without observable depolarization of the muscle or changes in synaptic potentials (Bishop et al., 1987).

To understand further the significance of the peptide cotransmitter in this system, we are investigating the mechanisms by which proctolin modulates tension in this muscle. Because proctolin has no obvious effect on EPSPs and can amplify tension produced by direct depolarization of the muscle fiber via current injection, it presumably acts by binding to its own receptor on the muscle and not on the glutamate receptor. One possibility is that proctolin regulates the influx of extracellular Ca^{2+} into the muscle fiber in response to depolarization. We examined this possibility using the patch-clamp technique, and we found that proctolin potentiates the activity of 2 voltage-inducible Ca^{2+} channels in the plasma membrane of the muscle. This effect appears to require an intracellular messenger, possibly cAMP.

A preliminary presentation of these results appeared in a symposium chapter (Bishop et al., 1990).

Materials and Methods

Adult crayfish, *Procambarus clarkii,* of either sex were obtained from a local supplier (Niles Biological, Sacramento, CA) and maintained in laboratory tanks until use. The 12 medial fibers of the tonic flexor muscle were isolated from the third abdominal segment of the crayfish (Fig. 1) and treated with 0.05 to 0.15% collagenase IA for 20 min. Patch-clamp experiments were performed at 21°C between April and October of 1987 and 1988.

For most experiments, the inside-out patch configuration was used. In these experiments, muscles were bathed in 137 mM $BaCl_2$ [to enhance conductance of Ca^{2+} channels and block K^+ currents (Hille, 1984)], 1 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 10.1 mM Trizma (pH, 7.0). Pipets contained 205 mM Na-gluconate, 2.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 10.1 mM Trizma (pH, 7.0). Barium and chloride conductances were distinguished at 0 mV patch potential in terms of inward and outward currents. Calcium channel openings in the inside-out configuration (outward barium currents) are indicated as upward deflections in the figures. Seal resistances of excised patches were commonly greater than 50 GΩ. When proctolin was used, it was added to the bath at least 5 min prior to seal formation. Patches were excised at 0 mV clamp potential and then depolarized in 20- or 50-mV steps up to 100 mV or until Ca^{2+} channel activity was seen; patches were held for at least 30 sec at each depolarizing step and for a total of at least 5 min. Patches with stable seals were then hyperpolarized in 20–25-mV steps to at least −75 mV. Patches without channel activity were usually more stable than those with active Ca^{2+} and could thus be examined for longer periods, permitting several series of depolarizing and hyperpolarizing steps. In some experiments, the excised patch was transferred from the barium bath into one in which the barium was replaced with 137 mM $CaCl_2$, using the technique of Quartararo and Barry (1987). The barium or calcium concentration used in these experiments represents a 10-fold increase compared to normal physiological solution (van Harreveld, 1936); this increase was used to enhance single-channel currents.

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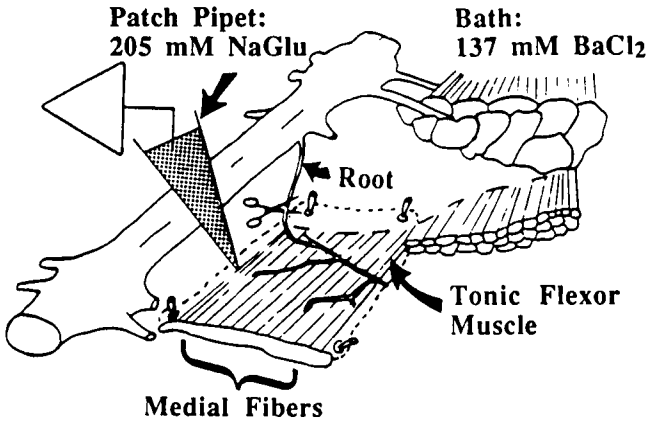

Figure 1. Crayfish tonic flexor muscle preparation used for single-channel patch-clamp recordings. Twelve medial muscle fibers were isolated from the third abdominal segment and treated with collagenase. Patch recordings were usually accomplished using the inside-out configuration and asymmetric solutions of 205 mM Na-gluconate in the pipet and 137 mM $BaCl_2$ in the bath. Patches were taken from random positions on the dorsal surface of the muscle fibers.

A few experiments were conducted using the cell-attached configuration. In these experiments, pipets contained the $BaCl_2$ solution described above, and the bath was normal physiological solution. In some of these experiments, a second pipet containing 5×10^{-9} M proctolin in physiological solution was lowered in to the bath *after seal formation.* The proctolin-containing solution was subsequently pressure ejected from this pipet onto the muscle fiber. Calcium channel openings (inward barium currents) from cell-attached experiments are indicated as downward deflections.

Current records were usually filtered at 500 Hz with an 8-pole Bessel filter, digitized at 400 μsec/point, and analyzed on an IBM PC/AT computer using the programs pCLAMP (Axon Instruments), version 4.0, and RS1 (BBN Software Products). Channel activity is expressed as the percentage of patches with active channels and as single-channel open probability, P_o [(mean open time/mean open time + mean closed time)/N, where N is the total number of active channels, defined as the maximum number of observed channel overlaps]. This interpretation of P_o results in an *underestimation* of the true current increase in the presence of proctolin, because multiple channels were usually present in proctolin-treated patches and were never seen in control patches.

Results

Two calcium and at least 3 chloride conductances are observed in patches of tonic flexor muscles

In excised patches in control barium bath, we identified 2 calcium conductances (38 and 14 pS in 137 mM Ba^{2+}) and 3 chloride conductances (25, 14, and 6 pS in 280 mM Cl^-) in the plasma membrane of the tonic flexor muscle (Fig. 2). The calcium channels usually required depolarization to 50 mV or greater to activate, while the chloride channels were generally active at 0 mV patch potential or became active with slight hyperpolarization (−25 mV or less). Once activated, the Ca^{2+} channels often remained active after repolarization, suggesting a slow time constant for closure. Large calcium channel openings were only rarely seen in the control bath, occurring in 17% (5/29) of depolarized patches. (In these experiments, patches were depolarized to 100 mV or until channel activity was seen; see Materials and Methods.) Small calcium channel openings were seen

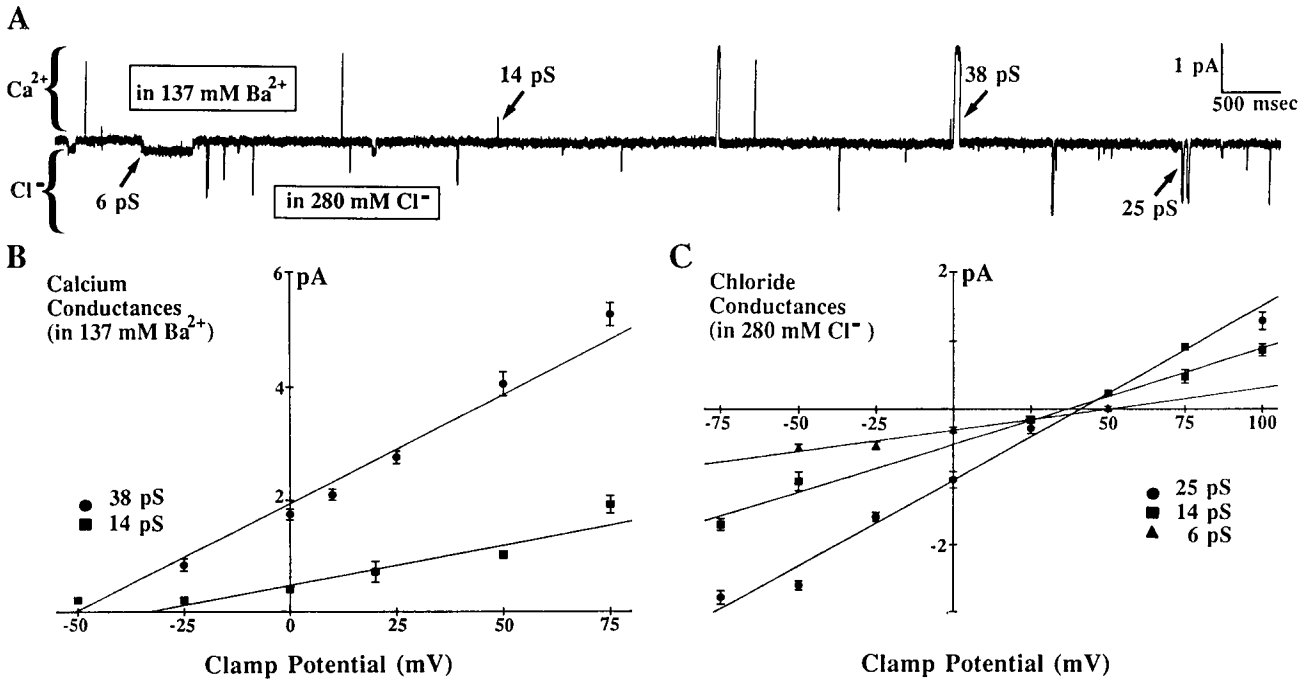

Figure 2. Two Ca^{2+} and at least 3 Cl^- conductances were observed in excised patches from tonic flexor muscles. *A,* Current record from inside-out patch at 0 mV clamp potential in control solution, showing 2 Ca^{2+} channels (outward currents) and 2 Cl^- channels (inward currents). The patch had been depolarized by 50 mV to activate the calcium channels; the Cl^- channels were already active upon excision. Once the Ca^{2+} channels were activated, they often persisted after repolarization, suggesting a slow time constant for closure. *B,* Current–voltage (*I–V*) relationships for the 2 Ca^{2+} channels observed in inside-out patches (n = 5 for each *point*; patches were previously depolarized to about 50 mV to activate channels). Channel conductances, as indicated by the slopes of the weighted least-squares fits, were 38 and 14 pS in 137 mM Ba^{2+}. (An exponential would provide a better fit of these points and less dissimilar reversal points for the 2 channels; linear fits, however, were used to more easily compare conductances of Ba^{2+} and Ca^{2+}; see Fig. 6.) No true reversal was ever detected in our experiments, indicating that the channels were selective against Na^+. *C, I–V* relationships for the 3 commonly observed Cl^- conductances in inside-out patches (n = 5 for each *point*). Channel conductances were 25, 14, and 6 pS in 280 mM Cl^-.

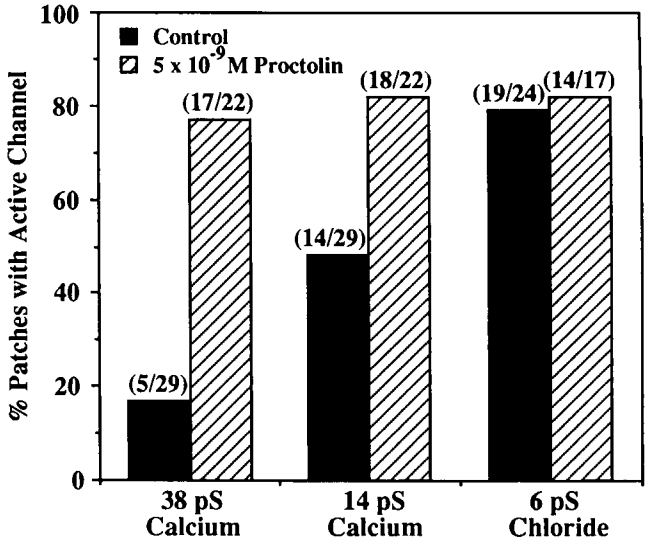

Figure 3. Proctolin selectively increased proportion of patches containing depolarization-induced Ca^{2+} channels. The *black bars* show the percent patches with active 38 pS Ca^{2+}, 14 pS Ca^{2+}, and 6 pS Cl^- channels in control solution; the *hatched bars* show the effect of 5×10^{-9} M proctolin (the fraction of total sample is given in *parentheses* above each bar). Excised patches were depolarized until the Ca^{2+} channels were seen or up to 100 mV, and hyperpolarized until the Cl^- channel was seen or to a maximum or −75 mV. The proportion of patches with active Ca^{2+} channels was significantly increased in proctolin, while the proportion of patches with active Cl^- channels was unchanged. Because 80% of patches had Cl^- channels in the control condition, this measure may have been insensitive to further increases. However, we also did not observe any increases in the number of Cl^- channels in a patch or P_o in proctolin (see Results). Control results were accumulated from muscles taken from 16 animals, while proctolin results were from 10 animals.

more frequently in patches in the control bath, but the channel was active in less than 50% (14/29) of the patches. The chloride channels, on the other hand, were active in most patches (patches in these cases were hyperpolarized −75 mV or until channel activity was seen). For example, the 6-pS channel was present in 79% (19/24) of the patches (Fig. 3).

Proctolin selectivity potentiates depolarization-induced activity of Ca^{2+} channels

Proctolin increased the probability of Ca^{2+} channel occurrence in depolarized patches. When 5×10^{-9} M proctolin was added to the bath at least 5 min before seal formation, the Ca^{2+} channel openings were still only rarely recorded in excised patches, clamped at 0 or negative clamp potentials. However, in depolarized patches (usually 50 mV or more), channel occurrence was significantly increased. Ca^{2+} channels occurred in about 80% of the depolarized patches (17/22 patches had large Ca^{2+} channels and 18/22 patches had small; Fig. 3). This represented more than a 4-fold increase in the number of patches with large Ca^{2+} channels compared to control and a nearly 2-fold increase in the number with small Ca^{2+} channels ($p < 0.025$ for both differences; χ^2 test). In contrast, the number of patches with 6-pS Cl^- channels did not significantly change in the presence of the peptide; this channel remained present in approximately 80% of the patches (14/17 patches; Fig. 3) after exposure to proctolin. Similar results were obtained when responses of patches from muscles from different animals were compared. (For example, 3/16 animals had patches with active large Ca^{2+} channels in the control bath, while 10/10 animals had patches displaying channel activity in proctolin.) Thus, individual variability does not seem to be a factor in these results.

Proctolin increased the activity of the large Ca^{2+} channel within a patch. Ca^{2+} channel activity within an individual patch was also selectively enhanced in the presence of the peptide, as shown in Figures 4–6. Figure 4 shows the activity of the large Ca^{2+} channel in the control bath (*A*), 5×10^{-9} M proctolin (*B*), and 10^{-8} M proctolin (*C*) at 50 mV clamp potential. (This clamp potential was threshold for channel activation in each of these 3 patches.) In the control bath (the record in Fig. 4*A* represents the *most active* channel record out of 29 depolarized patches), the channel displayed only brief and sporadic openings. This

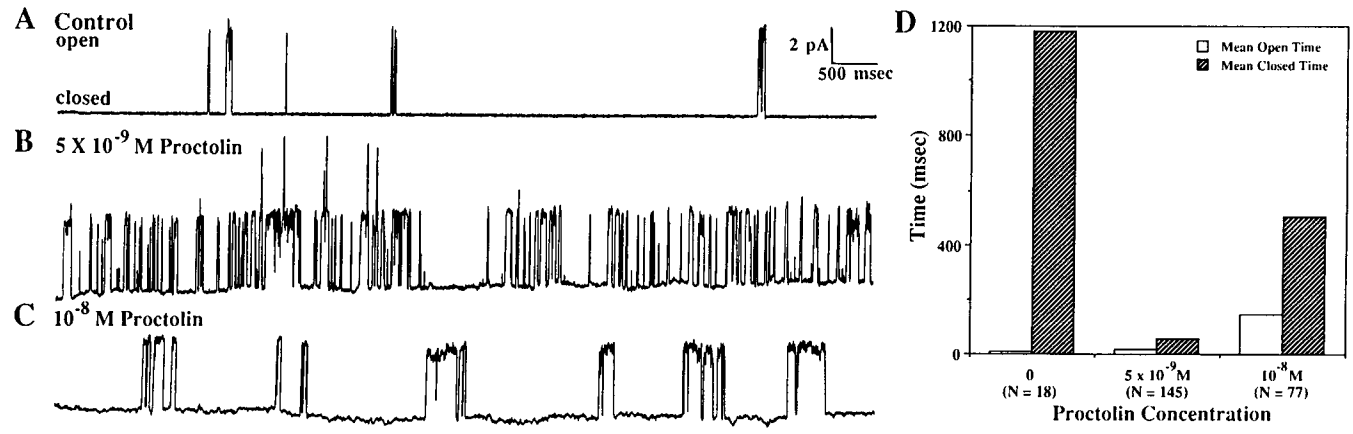

Figure 4. Proctolin increased open probability of 38 pS Ca^{2+} channel in patches. *A,* Current record from the *most active* inside-out patch in the control bath. Clamp potential for this and following records was 50 mV, which was also the threshold for channel activation. *B,* Current record from a *typically active* inside-out patch from a muscle preexposed to 5×10^{-9} M proctolin. *C,* Current record from the *most active* inside-out patch from a muscle preexposed to 10^{-8} M proctolin, which is known to be a level that causes desensitization (Bishop et al., 1987). *D,* Proctolin enhanced the open probability of the large Ca^{2+} channel mainly by decreasing the mean closed time. Mean open and closed times for the large Ca^{2+} channel in the 3 patch recordings are shown. The *numbers* in parentheses beneath each bar pair indicate the number of events analyzed. The small number indicated for the control condition underscores the rare occurrence of the channel in the absence of proctolin. Mean closed time was not as reduced in 10^{-8} M proctolin, consistent with other evidence that desensitization occurs at this peptide concentration.

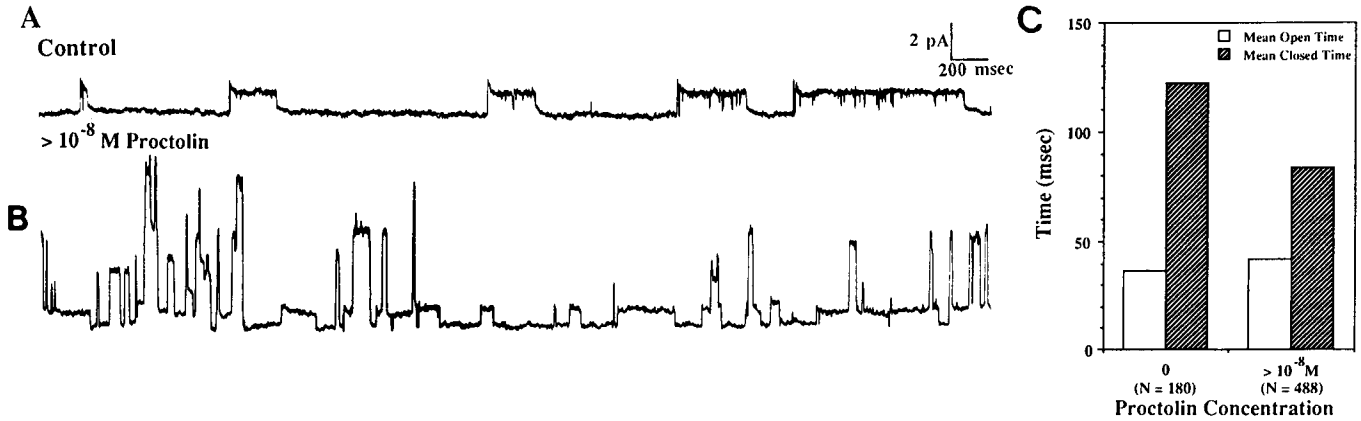

Figure 5. Proctolin also increased the open probability of 14 pS Ca^{2+} channel. *A,* Current record from the *most active* inside-out patch in the control bath. Clamp potential in this and the following record was 50 mV. *B,* Current record from an inside-out patch in $>10^{-8}$ M proctolin, showing the channel to be more active than the most active example in the control bath. *C,* The increased activity of the smaller channel was also due largely to a decrease in the mean closed time.

was reflected in a very long mean closed time and short mean open time and a very small single-channel open probability, P_o, of 0.006. When 5×10^{-9} M proctolin was present in the bath, there was generally an increase in P_o and an increase in the number of active channels in a single patch. Figure 4*B* represents a *typical* channel record seen in 22 patches in 5×10^{-9} M proctolin. In this patch, there were at least 2 active channels present; P_o increased to 0.12, which is 20 times greater than control. The increase in P_o was mainly due to a 20-fold decrease in mean closed time (Fig. 4*D*). In the 17 patches with active channels, P_os in 5×10^{-9} M proctolin (measured at clamp potentials between 25 and 50 mV) ranged from 0.005 to 0.30, with most having values greater than 0.10. A conservative interpretation of these results shows a 20-fold increase ($p < 0.01$; t test) in mean P_o in 5×10^{-9} M proctolin (mean P_o, 0.10 ± 0.01 SEM) compared to control (mean P_o, 0.005 ± 0.002 SEM). Interestingly, when even higher levels of proctolin ($\geq 10^{-8}$ M) were present in the bath, the mean closed time was not as short as in 5×10^{-9} M proctolin (Fig. 4*D*). This usually resulted in a lower P_o and probably indicates desensitization at these higher concentrations. Desensitization is also seen in tension records (see below). Figure 4*C* represents the *most active* record of 5 patches in 10^{-8} M proctolin.

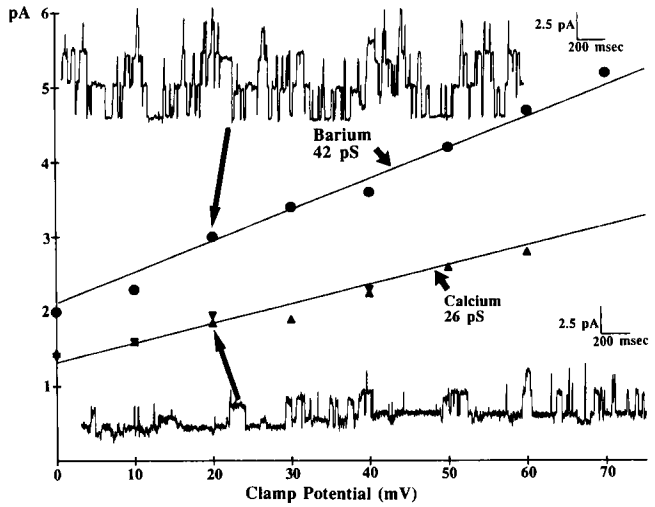

Figure 6. Large Ca^{2+} channel conducts Ba^{2+} about 40% better than Ca^{2+}: comparison of *I–V* curves for large Ca^{2+} channel in 137 mM Ba^{2+} and 137 mM Ca^{2+}. Channel conductance, as indicated by the slopes of the fitted lines, was 42 pS in Ba^{2+} and 26 pS in Ca^{2+}. *Circles* (Ba^{2+}) and *triangles* (Ca^{2+}) show currents obtained from a single patch that was transferred from a Ba^{2+}-containing bath to a Ca^{2+}-containing bath. *Top* (Ba^{2+}) and *bottom* (Ca^{2+}) records show channel activity at a clamp potential of 20 mV for this patch. *Inverted triangles* are from a second patch experiment and demonstrate the consistency of the data. Each *data point* was determined from the Gaussian fit of an amplitude histogram generated from at least 8, and usually more than 50, events. Points have been fitted with lines with an intercept of −50.5 mV, to correspond to the intercept measured in Figure 2*B*.

Proctolin increased the activity of the small Ca^{2+} channel within a patch. The activity of the small conductance Ca^{2+} channel has not been as thoroughly analyzed as that of the large channel. This is, in part, due to its small size, which allows the channel to be easily obscured by noise and the activity of the large channels. However, a comparison of the small Ca^{2+} channel's activity in patches taken in either control or proctolin-containing baths was attempted using higher concentrations ($>10^{-8}$ M) of proctolin, where, because of desensitization, the large Ca^{2+} channel was not as active as in lower concentrations of the peptide. Figure 5 compares a recording of the small Ca^{2+} channel in the control patch where it was *most active* (*A*) and a recording at a high proctolin concentration (*B*). Channel activity for the small channel in the control bath was considerably higher (P_o = 0.23) than for the large Ca^{2+} channel. Channel activity for the small channel did, however, still increase in the presence of proctolin (P_o increased about 40% to 0.33). As with the large channel, increased P_o in the presence of proctolin was due mainly to a decrease in closed time (Fig. 5*C*).

Figure 5 also demonstrates that the small Ca^{2+} channel is probably an independent channel and not a subconductance state of the larger channel. This is indicated in the appearance of one channel and not the other in some patches (e.g., Fig. 5*A*), in the additive amplitudes of the 2 channels when they occur in the same patch (Fig. 5*B*), and in the distinctly different kinetics of the channels (Fig. 5*B*).

Proctolin did not increase Cl^- channel activity. A comprehensive analysis of Cl^- channel activity has yet to be undertaken. Nonetheless, a visual survey of the records yielded no case of a dramatic enhancement in Cl^- channel activity in the presence

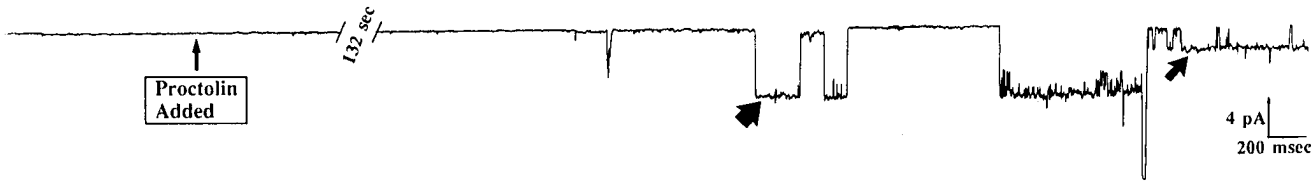

Figure 7. Evidence that proctolin activates Ca^{2+} channel activity via intracellular messenger. Current record from a cell-attached patch held at −50 mV hyperpolarized. The pipet solution was 137 mM $BaCl_2$. No channel activity was detected in the absence of proctolin, even after several depolarizing steps of up to 100 mV. About 2 min after proctolin (5×10^{-9} M) was perfused onto the muscle, both large (*large arrow*) and small (*small arrow*) channels were detected. Because the channels were not directly exposed to the peptide (they were isolated from the proctolin-containing bath by the patch pipet), we inferred they were activated by an intracellular messenger. The time course for channel activation was consistent with the time it took to initiate tension amplification after proctolin application (Bishop et al., 1987).

of proctolin, such as seen for the large Ca^{2+} channel. Indeed, a decrease in channel activity was seen in some experiments.

Thus, the above results together demonstrate that proctolin selectively enhances Ca^{2+} channel activity. In the next section, we describe some additional properties of these proctolin-modulated channels.

Properties of the proctolin-modulated Ca^{2+} channels in excised patches

A remarkable property of these proctolin-modulated Ca^{2+} channels is that high levels of activity were often seen in excised patches, with no obvious diminution, for as long as the patches were held (up to 1 hr). We were able to take advantage of this phenomenon and compare the amplitude of the large Ca^{2+} channel in barium and in calcium using a single patch. The patch was transferred from a bath containing 137 mM Ba^{2+} to a bath containing 137 mM Ca^{2+}, as described in Materials and Methods. Proctolin (5×10^{-9} M) was added to the Ba^{2+} bath prior to seal formation to enhance the activity of the channel. *I–V* curves were constructed first in the Ba^{2+} bath and then in the Ca^{2+} bath from amplitude histograms generated at various clamp potentials (Fig. 6). Use of the same patch in each bath allowed unambiguous identification of the channel in each condition. In 137 mM bath Ca^{2+}, conductance of the channel was 26 pS; it increased to 42 pS in 137 mM Ba^{2+}.

Reversal of the single-channel Ca^{2+} currents was never detected in our experiments when negative clamp potentials were applied (Fig. 2). Because the patch pipet contained almost exclusively Na^+, this suggests that the proctolin-modulated Ca^{2+} channels do not conduct Na^+ under these conditions.

Few pharmacological manipulations have yet been undertaken. However, we did not see either the large or the small Ca^{2+} channel in patches in the presence of proctolin when 1–6.75 mM $CoCl_2$ was included in the pipet solution ($n = 3$; $p < 0.01$ for difference between Co^{2+} plus proctolin versus proctolin alone). These results support the conclusion that the 2 proctolin-modulated channels are indeed selective Ca^{2+} channels.

Proctolin appears to regulate Ca^{2+} channels via an intracellular messenger

Under the experimental regimens we have tried thus far, on-cell recordings were not easily obtained. Usually, gigaseals could be formed but not maintained in these experiments. We presume that this was due to muscle contraction occurring during the course of experiments. Occasionally, we were able to maintain on-cell patches. (In these experiments, the 137 mM $BaCl_2$-containing solution described in Materials and Methods was placed in the patch pipet.) In 2 of these experiments, proctolin was introduced to the bath only after seal formation (see Materials and Methods). Results from one of these experiments are shown in Figure 7. In this recording, the patch was held hyperpolarized at −50 mV; no channel activity was seen even after depolarizing steps up to 100 mV were applied. After a 10-min wait, 5×10^{-9} M proctolin was perfused onto the muscle; large and small barium currents appeared about 2 min after peptide application without further depolarization. The time course for the initiation of channel activity was consistent with the time course for tension enhancement after proctolin exposure (Bishop et al., 1987). A similar result was obtained in the second on-cell patch experiment from a second animal, except that channel became active after proctolin application only after the patch was depolarized by 20 mV. These experiments make 3 points: First, they indicate that proctolin can act through an intracellular messenger to activate the Ca^{2+} channels, because direct contact between the peptide and the channels was prevented by the gigaseal of the pipet. Second, they suggest that the depolarization requirement may be much lower for the on-cell patch than for the excised patch. Finally, they show that the proctolin-modulated channels have no appreciable K^+ conductance, because no outward K^+ current was seen with depolarization.

Four additional on-cell experiments (using muscles from 4 different animals) suggest that the peptide acts exclusively via an intracellular messenger and not directly on the channels. In these experiments, the patch pipets also contained 5×10^{-10} M proctolin (just above threshold). This level of proctolin was chosen because some leakage of the pipet solution onto the muscle is inevitable since positive pressure is applied continuously as the pipet tip is lowered onto the muscle surface. We assume that dilution would bring the concentration of proctolin below threshold everywhere except inside the pipet tip. In the 4 cell-attached patches under these conditions, no Ca^{2+} channel activity was detected even after depolarizations of 100 mV.

Also, in experiments ($n = 3$) where proctolin (5×10^{-9} M) was added to the bath after excision, no channel activity was ever seen, even after 100 mV depolarization. These results, together, indicate that proctolin probably acts via an intracellular messenger and not directly on the calcium channels from either the extracellular or cytosolic side.

Attempts to identify intracellular messenger or messengers mediating Ca^{2+} channel regulation led us to compare Ca^{2+} channel activity in control patches excised from muscles in our standard bath solution with patches excised from muscles that had been bathed in 50 μM of the membrane-permeable cAMP analog 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (CPT-cAMP; Evans, 1984). The large Ca^{2+} channel was detected in 58% (7/12) of depolarized patches after exposure to CPT-

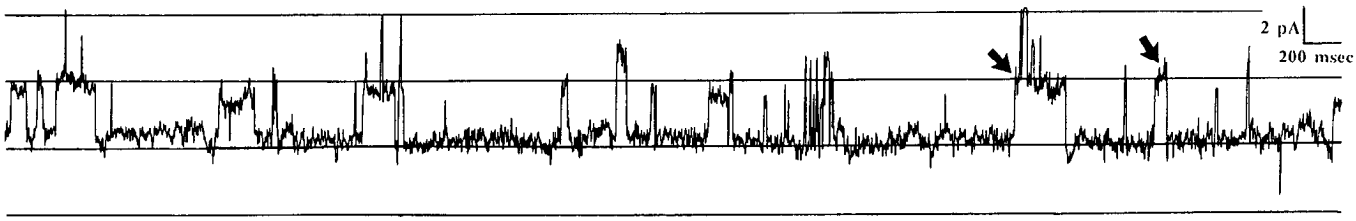

Figure 8. Treatment with CPT-cAMP resulted in significant increase in proportion of patches having active, 38 pS Ca^{2+} channels. A current record from an inside-out patch at a clamp potential of 50 mV shows activity in 2, possibly 3, channels. This patch was from a muscle exposed to 50 μM CPT-cAMP prior to seal formation and shows the most active patch from 7 CPT-cAMP-treated patches that contained the 38 pS channel (*arrows*). Other Ba^{2+} conductances were seen in this patch that were not apparent in our other experiments.

cAMP (Fig. 8), compared to 17% (5/29) in control ($p < 0.01$). This result suggests that cAMP may be at least partially involved in proctolin's channel activity potentiation.

Ca^{2+} channel activation in excised patches has a similar dose-dependent response to proctolin as tension enhancement in muscle

The above results indicate that 2 plasma membrane calcium channels are activated by the peptide cotransmitter in this system. The depolarization requirement of these channels is consistent with the peptide's effect on tension. Also consistent was the dose dependence to the peptide. Figure 9 compares Ca^{2+} channel activity (expressed as the percentage of patches containing active channels) with the effect of peptide dose on tension. Both tension and channel activity increased in 5×10^{-10} M proctolin. (The increase for the small channel, however, was not significant.) All responses reached a maximum at 5×10^{-9} M proctolin, and all responses fell off in higher desensitizing proctolin concentrations. Thus, the proctolin dose–response of the channels closely parallels that for tension.

Discussion

We have shown that proctolin increases the activity (both the percentage of patches with active channels and the single-channel open probability) of 2 channels (38 and 14 pS in 137 mM Ba^{2+}) in the plasma membrane of the crayfish tonic flexor muscle. These channels do not appear to conduct Na^+ (inside-out experiments) or K^+ (cell-attached experiments) and are therefore thought to be Ca^{2+} selective. In addition, both channels are blocked by Co^{2+}. Also, channel conductance for the larger channel is higher in Ba^{2+} than in Ca^{2+}, a characteristic that has been described for other Ca^{2+}-selective channels (Hille, 1984). (Channel conductance comparisons in Ba^{2+} and Ca^{2+} have yet to be performed for the smaller channel.) In our experiments, enhancement of channel activity by proctolin was selective for these 2 Ca^{2+} channels and did not occur for the Cl^- channels that were also present in our patches. An inhibitory effect of proctolin on the open probability of the anion channels cannot yet be ruled out. (Although no extensive ion selectivity studies of these anion channels have yet been undertaken, we have called them Cl^- channels because Cl^- is the most likely ion they would conduct in the living animal.)

The conductances of the proctolin-modulated Ca^{2+} channels fall within the middle to large end of the range of conductances described for various Ca^{2+}-selective channels to date (Lux, 1983; Reuter, 1983; Velasco et al., 1988). They are unusual in their maintained activity in excised patches.

We have found that proctolin channel modulation in the tonic

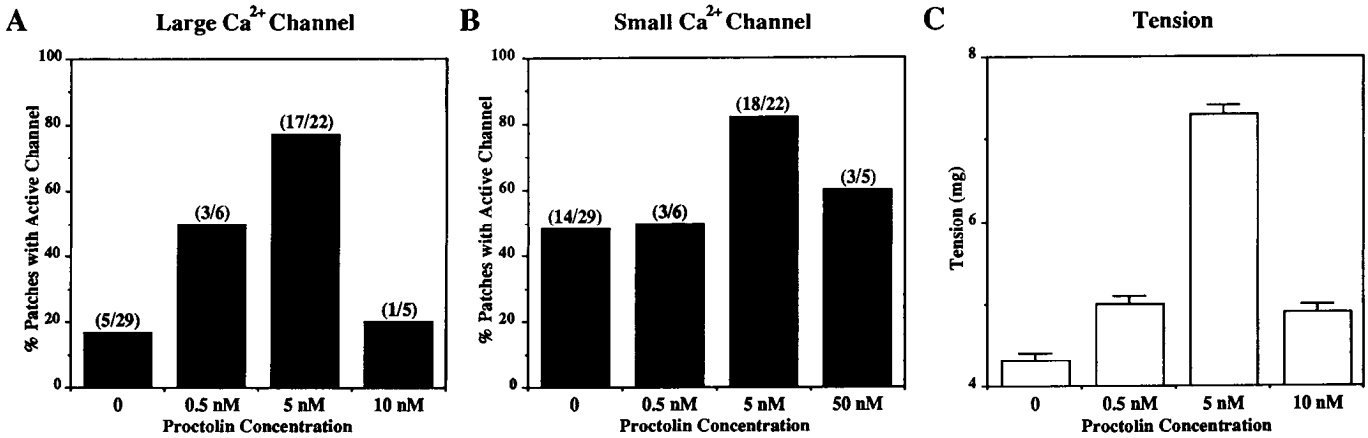

Figure 9. Parallel pattern of proctolin dose–responses for Ca^{2+} channel activation and for tension enhancement in muscle. Percent depolarized patches with active channels [either large Ca^{2+} (*A*) or small Ca^{2+} (*B*); the fraction of total sample is given in *parentheses* above each bar] at varying concentrations of proctolin are compared to a typical dose–tension experiment (*C*). Tension was generated by current injected directly into the muscle fiber. In this experiment, 575-msec current pulses, producing 25-mV peak depolarizations, were delivered every 4 sec ($n = 3$ for each peptide concentration). Occurrence of the large Ca^{2+} channel and tension both increased significantly with the addition of 5×10^{-10} M proctolin. Little increase occurred with the small Ca^{2+} channel at this concentration. However, occurrence of both channels, as well as tension, was enhanced by the addition of 5×10^{-9} M proctolin. This peptide concentration produced the highest channel occurrence, as well as the most tension. Channel occurrence and tension declined at higher proctolin concentrations (10^{-8} M).

flexor muscle is depolarization dependent and also dose dependent in a manner consistent with the peptide's enhancement of tension in the muscle. This dose-dependent response includes a decline at higher ($\geq 10^{-8}$ M) proctolin concentrations. These correlations support a connection between proctolin enhancement of Ca^{2+} channel activity and muscle tension. Thus, proctolin-induced tension potentiation in the tonic flexor muscle can be modeled as an enhanced influx of extracellular Ca^{2+} resulting in increased cytosolic free Ca^{2+} (Fig. 10). However, enhanced Ca^{2+} influx is probably not the only mechanism by which proctolin amplifies tension in this muscle. We have shown previously that proctolin can still enhance tonic flexor muscle tension in a Ca^{2+}-free, Co^{2+}-substituted bath after treatment with the Ca^{2+} ionophore A23187 (tension amplitudes were reduced, however; Bishop and Wine, 1986); because we have presented evidence that Co^{2+} blocks the Ca^{2+} channels reported here, these results indicate that proctolin may also be able to generate some degree of tension in the absence of functional plasma membrane Ca^{2+} channels.

The involvement of Ca^{2+} influx in muscular proctolin responses has been suggested in a number of different preparations. In barnacle muscle fibers, for example, a proctolin response (in this case, a proctolin-stimulated, ouabain-insensitive Na^{2+} efflux) was found to be eliminated with Cd^{2+} and Co^{2+} and correlated with increased cytosolic free Ca^{2+} (which was measured with aequorin; Bittar and Nwoga, 1989). Similar Ca^{2+} channel blockers were found to block proctolin-induced contractures in a number of other muscle preparations, including *Limulus* heart muscle (Watson and Hoshi, 1985), lobster opener muscle (Schwartz et al., 1980), and cockroach hyperneural muscle (Hertel and Penzlin, 1986). In other preparations, for example, the insect oviduct (Holman and Cook, 1985), cockroach hindgut (Cook and Holman, 1985), and locust extensor tibia muscle (O'Shea et al., 1985), the proctolin response is relatively unaffected by removal of extracellular Ca^{2+} or addition of Ca^{2+} channel blockers. Thus, as our results also suggest, plasma membrane Ca^{2+} channels may not be the sole means by which proctolin can influence muscle tension.

Our results suggest that proctolin potentiates Ca^{2+} channel activity in the tonic flexor muscle via an intracellular messenger, and cAMP may be involved. Thus, the peptide appears to be binding to a specific membrane receptor and may phosphorylate either the channel itself or an associated regulatory molecule. We hypothesize that this, in turn, increases the channel's sensitivity to depolarization.

It is interesting to note that in neither excised nor on-cell experiments was there ever substantial large Ca^{2+} channel activity without proctolin, even when large levels of depolarization were applied. This channel appears to require both proctolin and depolarization to become active. The smaller channel, on the other hand, seems to be reasonably active in excised patches without proctolin. The 2 channels may therefore serve different roles in the regulation of Ca^{2+} entry into the muscle fiber.

A number of questions remain to be answered concerning these Ca^{2+} channels. One such question is whether junctional and extrajunctional proctolin-sensitive channels differ. Our patches were chosen at random over the dorsal surface of the muscle, and though multiple proctolinergic terminals are located along the extent of these muscle fibers (Bishop et al., 1984), most of our patches were probably extrajunctional. With regard to kinetics, high levels of depolarization were required to activate these Ca^{2+} channels in excised patches. This may be due to distorted channel kinetics resulting from exposing the cytoplasmic face of our patches to millimolar concentrations of Ca^{2+}. An interesting alternative is that excision may be removing some cellular component influencing the depolarization threshold. That this high level of depolarization is associated with excision is supported by the cell-attached experiments, where lower levels of depolarization appeared able to activate the channels. We have not yet determined the voltage dependence of open and closed states beyond showing that depolarization induces activity. When we compared P_os at different voltages in 3 patches with active channels, we saw a decrease in P_o with depolarization that could be due to voltage-dependent inactivation, but the protocol that we had established to assess proctolin sensitivity (step-wise voltage changes; see Materials and Methods) did not allow us to prove this point. Finally, it remains to be determined how these results relate to whole-cell currents as measured by voltage clamp.

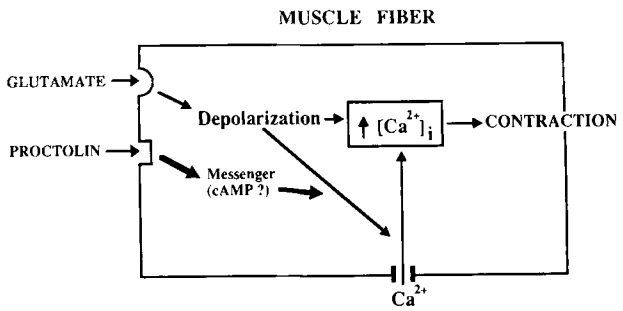

Figure 10. Model for peptide amplification of depolarization–contraction coupling. Motoneuron terminals corelease L-glutamate and proctolin, which bind to their respective receptors on the muscle membrane. L-Glutamate increases the conductance of cation channels, depolarizes the muscle fiber, increases cytosolic Ca^{2+} levels, and produces tension. Proctolin leads to the generation of an intracellular messenger, possibly cAMP, which acts in an as yet unknown way to amplify depolarization activation of 2 plasma membrane Ca^{2+} channels, resulting in enhanced influx of Ca^{2+} and, hence, an increased amount of tension for a given level of depolarization.

It will be important to pursue these questions, because regulation of cytosolic free Ca^{2+} via modulation of plasma membrane Ca^{2+} channels is a powerful means by which neuromodulators may control cellular functions. A commonly cited example is the control of cytosolic free Ca^{2+} and tension in the vertebrate heart by noradrenaline (Reuter, 1983, 1984). The evidence presented here indicates that this regulation is probably important for the proper function of invertebrate skeletal muscles, as well. Other neuromodulators have been shown to potentiate tension in arthropod muscle (e.g., Kravitz, 1988), and it should be possible to determine how their mechanism of action compares to that of proctolin. The control of the large Ca^{2+} channel by proctolin in the tonic flexor muscle is clearly multifaceted. We recently found that proctolin's modulation of the large Ca^{2+} channel can be absent during winter months (Bishop et al., in press), but modulation by proctolin can be restored when octopamine is also present, even though octopamine alone does not affect channel activity (Bishop et al., 1990). These points, combined with the large single-channel conductance, the long channel lifetime in excised patches, the high density of channels, and the simplicity of the preparation, strongly recommend this system for further study.

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