Modulatory Effects of Myomodulin on the Excitability and Membrane Currents in Retzius Cells of the Leech

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Wang, Yong, Judith A. Strong, and Christie L. Sahley. Modulatory effects of myomodulin on the excitability and membrane currents in Retzius cells of the leech. *J. Neurophysiol.* 82: 216–225, 1999. Ion channel modulation by the peptide myomodulin (MM) has been demonstrated in a wide variety of organisms including *Aplysia*, *Lymnaea*, and *Pleurobranchaea*. This neural and muscular modulation has been shown to be important for shaping and modifying behavior. In this paper, we report that MM modulates several distinct ionic channels in another species, the medicinal leech *Hirudo medicinalis*. Experiments have focused on the Retzius cell (R) because the R cell is a multifunction neuron that has been implicated in a number of behaviors including feeding, swimming, secretion, thermal sensing, and the touch elicited shortening reflex and its plasticity. Previous work had identified a MM-like peptide in the leech and demonstrated that this peptide modulated the excitability of the R cell. Using combined current- and voltage-clamp techniques to examine the effects of MM on the R cell, we found that in response to a step pulse, MM increased the excitability of the R cell such that the cell fires more action potentials with a shorter latency to the first action potential. We found that this effect was mediated by the activation of a Na^+-mediated inward current near the cell resting membrane potential. Second, we found that MM differentially modulated the potassium currents I_A and I_K. No effect of MM was found on I_A, whereas MM significantly reduced both the peak and steady-state amplitudes of I_K by 49 ± 2.9% and 43 ± 7.2%, respectively (means ± SE). Finally we found that MM reduced the amplitude of the Ca^{2+} current by ~20%. The ionic currents modulated by MM are consistent with the overall effect of MM on the cellular activity of the R cell. An understanding of the cellular mechanisms by which MM modulates the activity of the R cell should help us to better understand the roles of both MM and the R cell in a variety of behaviors in the leech.

INTRODUCTION

The myomodulin (MM) family of peptides has been identified in a broad range of organisms including several molluscan species (Fujiwara-Sakata and Kobayashi 1992; Greenberg et al. 1997; Santama et al. 1994a,b), arthropods (Christie et al. 1994; Evans 1994; O'Brien and Taghert 1998), annelids (Keating and Sahley 1996; Takahashi et al. 1994), and one mammal (Vilim and Ziff 1994). Further, the MM peptides have been shown to play an important role in the modulation of the properties of both muscles (Brezina et al. 1994a,b) and neurons (Critz et al. 1991), including those associated with specific behaviors such as feeding (Cropper et al. 1987a,b), locomotion (Evans 1994), reproduction (van Golen et al. 1996), and molting (O'Brien and Taghert 1998). In *Aplysia*, where MM first was isolated and purified (Cropper et al. 1987b), multiple forms of MM were found to be localized in many neurons including feeding motoneurons (Brezina et al. 1995; Cropper et al. 1987b, 1991; Miller et al. 1993). There they serve as cotransmitters modulating neuromuscular transmission. The effects of MM have been seen as a potentiation (Brezina et al. 1995; Cropper et al. 1987b, 1991) or depression of neuromuscular transmission (Brezina et al. 1995; Cropper et al. 1988, 1990; Vilim et al. 1994). The specific net modulatory effect of the neuropeptides depends on the ratio of different MMs and other transmitters and modulators involved (Brezina et al. 1995, 1996; Cropper et al. 1987a,b). All nine MMs (A–I) have a potentiation effect on the contraction of the accessory radular closer muscle at low concentrations (10 nM), but seven of the nine MMs (MM_A and $MM_{D\text{-}I}$) produce depression at concentrations of 1–10 μM. Moreover,the potentiation and depression appear to have different time courses (Brezina et al. 1995).

Voltage-clamp analysis has revealed that potentiation of buccal muscle contraction in *Aplysia* is primarily due to the MM enhancement of an L-type Ca^{2+} current (Brezina et al. 1994a; Scott et al. 1997). Depression of the contraction is due to the activation of the modulator-induced K^+ current (Brezina et al. 1994a; Scott et al. 1997). Interestingly, MMs differ in their effectiveness in activating K^+ currents, whereas they are equally effective in activating the Ca^{2+} current (Brezina et al. 1994a,b, 1995; Scott et al. 1997). Different MMs activate the K^+ current with variable efficacy at their corresponding maximal concentrations, and additive effects can be seen with selective combinations of different MMs (Brezina et al. 1995). Therefore the effect of MMs on the buccal muscle contraction is a result of complex temporal interplay of the modulation of these various currents (Brezina et al. 1995; Scott et al. 1997).

In addition to its action on muscles, MM has been found to modulate neurons. MM modulation of neural properties also has been analyzed. In *Aplysia*, for example MM opens both the S-K current ($I_{K,S}$) and the voltage-gated K^+ current ($I_{K,V}$) of the tail sensory neurons (Critz et al. 1991), resulting in a decrease in the excitability of these neurons. In the leech, MM transiently depolarizes the resting membrane potential and increases the firing rate of the Retzius cell (Wang et al. 1998).

A set of putative MM-containing neurons in the CNS of the leech has been identified (Keating and Sahley 1996). MM immunoreactivity is distributed across cells within neural circuits mediating several distinct behaviors including cardiovascular function and the touch-elicited shortening reflex. Within the shortening reflex circuit, the anterior Pagodas (AP), Leydig cells, longitudinal motor neuons (L), S cells, and their coupling interneurons are all immunoreactive for MM (Keating and

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Sahley 1996). Thus MM could potentially be important in the expression and modulation of the touch-elicited shortening reflex. Moreover we recently observed a putative peptidergic synapse between the S and the Retzius (R) cell (Wang, unpublished observations). Given the MM immunoreactivity observed in the S cell, R cell may be a physiologically relevant target of MM modulation.

Several of the ionic currents in the R cell have been characterized extensively in intact leech ganglia as well as in culture (Johansen et al. 1987; Schirrmacher and Deitmer 1991; Stewart et al. 1989b; for review, see Kleinhaus and Angstadt 1995). These include the voltage-dependent Na^+ current (Johansen and Kleinhaus 1987; Kleinhaus and Prichard 1976; Nicholls and Baylor 1968), and two major components of the K^+ currents, I_A, a rapidly activating and inactivating transient current, and I_K, a delayed rectifier current (Johansen and Kleinhaus 1986). In addition, a single class of Ca^{2+} current has been revealed in R cells by using both single-channel and voltage-clamp recordings (Bookman and Liu 1987; Johansen et al. 1987; Schirrmacher and Deitmer 1991). In this report, we characterized the ionic basis of the MM-induced cellular property changes in the R cell. We report that MM increases the excitability of the R cell such that the cell fires more action potentials or shortens the latency to the first action potential in response to a step current pulse; MM induces a small Na^+-mediated inward current near the cell resting membrane potential, which can account for the small depolarization observed when cells are monitored in current-clamp mode; MM has little or no effect on the rapidly activating and inactivating I_A current but significantly reduces the delayed rectifier I_K current; and MM decreases the amplitude of the Ca current. Taken together, the modulatory effects of MM on the ionic currents are consistent with the excitatory effect of MM on R cells.

METHODS

Adult leeches, *Hirudo medicinalis* (3–5 g) were obtained from Leeches USA (Westbury, NY) and maintained in plastic containers [28 (L) × 20 (W) × 8 (H) cm] filled to the depth of ~4 cm with artificial leech pond water [0.5 g of *Hirudo* salt (Leeches USA) dissolved in 1 L of ddH_2O] at room temperature with 12/12 h light/dark cycle. Segmental ganglia, excluding the sex ganglia (ganglia 5 and 6), were dissected and pinned down in a custom-made Plexiglass (200 μl in volume) recording chamber containing silicone elastomer (Sylgard; Dow Corning, Midland, MI). The capsule covering each ganglion was removed carefully by fine scissors or forceps.

Cells were impaled with borosilicate microelectrodes (1 mm OD, 0.75 mm ID) (Sutter Instrument, Novato, CA) filled with 3 M potassium acetate with 100 mM KCl (10–20 MΩ). An Axoclamp 2A (Axon Instruments, Foster city, CA) was used for intracellular recording, current-clamp and single- and two-electrode voltage clamp (SEVC and TEVC). Standard intracellular recording in bridge mode was used to monitor the basic effect of MM on the R cells. For the excitability test, conventional discontinuous current clamp was used. The excitability was measured ~1.5–2 min after MM application, i.e., after the initial burst and depolarization were over. The membrane potential of the R cell was monitored and maintained between −55 and −65 mV by manually adjusting the constant DC output of the current-passing electrode during the excitability test. Bursts of action potentials were elicited by injecting a series of 90-ms depolarizing current pulses. The amplitudes of these depolarizing pulses range from 0.5 to 3 nA and were increased incrementally in steps of 0.5 nA. For all voltage-clamp experiments, the tips of the microelectrodes were coated with Sigmacote (Sigma, St Louis, MO). SEVC (some with a ramp protocol) was used to characterize the MM-induced current. Sample rates were set between 3 and 7 kHz, and the clamp gain was set at 25–50 nA/mV. For optimum capacitance neutralization, capacitance neutralization control was advanced until the 10 mV/mV MONITOR waveform on a second oscilloscope decayed most rapidly to a horizontal baseline without any overshoot or undershoot. All K^+ current studies were carried out using standard TEVC. The gain for TEVC was set between 800 and 2,500 vol/vol. The output bandwidth was set at 0.3 kHz. Both SEVC and TEVC were used in Ca^{2+}-current experiments. Data were digitized by a Digidata 1200 converter (Axon Instrument). All voltage steps were generated, stored, and analyzed by a Gateway P5–133 computer using pClamp 6.0 software (Axon Instruments). Further filtering was applied during data analysis and figure plotting. Data were accepted only if the step changes in voltage-clamp potential were fast and no voltage sag was detected (Critz et al. 1991), and the electrode drift at the end of the experiments was within ± 5 mV of the starting value (Nadim and Calabrese 1997). For subtraction, leakage current was obtained by using hyperpolarizing voltage steps. Leakage current from the hyperpolarizing pulse was multiplied to the correct magnitude on the assumption of a linear leakage and then used for subtraction.

All drugs and chemicals were purchased from Sigma unless noted otherwise. Normal leech saline containing (in mM) 115 NaCl, 4 KCl, 1.8 $CaCl_2$, and 10 HEPES with pH adjusted to 7.4 was used for ganglia preparation, excitability, and MM-induced current experiments. Other solutions were made by equimolar substitution (unless otherwise noted) of the above formula with *N*-methyl-D-glucamine replacing Na^+, and Co^{2+} (or Mg^{2+}) replacing Ca^{2+}, respectively. In some experiments, Ca^{2+} was replaced by Ba^{2+} as charge carrier. Stock solutions of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) were made in ddH_2O at 1 M each. TEA or/and 4-AP were added to the working solutions so that their final concentrations were 25 and 8 mM, respectively. We were not able to block voltage-gated Na^+ current in the R cell with either pharmacological methods (Kleinhaus and Prichard 1976) or by somata ligation (Acosta-Urquidi et al. 1989; Johansen and Kleinhaus 1986). Myomodulin A (PMSMLRL-NH_2) (*Aplysia*) peptide was purchased from Peninsula Laboratories (Belmont, CA). Leech MM peptide GMGALRL-NH_2 (Wang et al. 1998) was synthesized by Research Genetics (Huntsville, AL). The peptides were dissolved in ddH_2O to make 1 M stock and kept at −80 or −20°C in small-volume aliquots. Because the effects of *Aplysia* myomodulin A and leech MM were indistinguishable (Wang et al. 1998), the data for excitability and the MM-induced current were pooled from experiments using either *Aplysia* or leech MMs. Modulatory effects on I_A, I_K, and I_{Ca} were done using *Aplysia* myomoduolin A. We found that the effect of MM was saturated between 25–50 μM, which was similar to the range of concentrations used to study MM modulation of *Aplysia* neurons (Critz et al. 1991). Before use, the stock solutions were thawed, diluted to working concentration of 50 μM in appropriate recording solutions, and applied to the cells by bath superfusion.

Statistical comparisons were of the within-preparation differences produced by the treatment, using a paired Student's *t*-test. The criterion for statistical significance was $P < 0.05$. All averaged data were expressed as means ± SE.

RESULTS

MM effect on excitability

The response of the R cell to MM application is presented in Fig. 1*Aa*. As seen in the figure and as reported previously (Wang et al. 1998) MM modulates the activity of the R cell causing a small depolarization accompanied by a train of action potentials. This response of the R cell to MM application is subject to rapid desensitization. The responsiveness of R cell to repetitive applications of MM decreased dramatically as shown

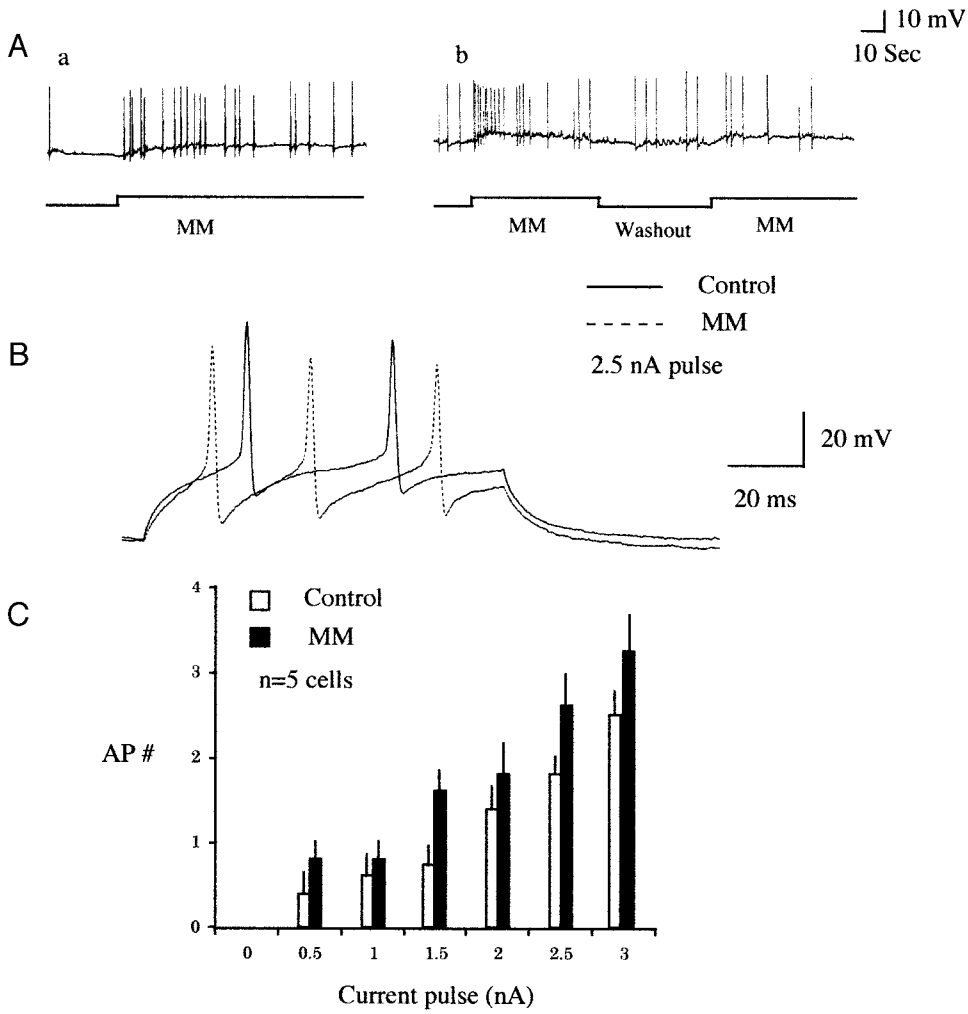

FIG. 1. Effects of myomodulin on Retzius cell (R) cell excitability. *Aa*: 50 μM of the synthetic leech myomodulin-like peptide was applied to the R cell by bath superfusion. Cell membrane potential was monitored by standard intracellular recording in BRIDGE mode. *Ab*: desensitization of the myomodulin effect on the R cell. After the 1st application of 50 μM, myomodulin (MM) was washed out and a 2nd dose of 50 μM MM was applied. Responsiveness of the R cell declined dramatically after the 1st application of myomodulin. *B*: excitability, measured as the number of action potentials produced by injecting depolarizing currents. This was done ~1.5–2.0 min after myomodulin A (*Aplysia*) application, i.e., after the initial burst and depolarization was over. For a given cell, at a given value of current injection, the number of action potentials tended to increase, and the time to the 1st action potential tended to decrease. *C*: excitability group data. Cells were injected with a series of depolarizing pulses in steps of 0.5 nA. A consistent increase in number of action potentials was observed after myomodulin A application.

in Fig. 1*Ab*. In addition, as presented in Fig. 1*B*, MM appears to increase the excitability of the R cell. That is, a given value of depolorizing current pulse elicited a consistent increase in number of action potentials, a decreased latency to fire, and a decreased interspike interval after MM application (Fig. 1*B*). For example, for a 2.5-nA current pulse, the average number of action potentials before and after MM application were 1.8 $\pm$ 0.2 and 2.7 $\pm$ 0.4, respectively ($t = 3.16$, $P < 0.05$, $n = 5$). In addition, there was a decrease of the latency time for the first spike from 25 $\pm$ 4.8 to 19 $\pm$ 4.8 ms, a reduction of 23 $\pm$ 5.5%. There was also a consistent reduction in interspike interval time between the first and the second spikes from 48 $\pm$ 6.0 to 28 $\pm$ 8.3 ms ($n = 3$). Excitability data from five cells are plotted in Fig. 1*C*. No apparent changes in action potential shape such as duration or afterhyperpolarization were observed, although sometimes a small reduction of the action potential amplitude was noticed.

MM induced a small Na current

Using voltage-clamp procedures, we analyzed the specific ion current modulation underlying the MM-induced changes in the R cell. Desheathed leech ganglia were bathed in normal leech saline. R cells were clamped at a holding potential of -60 mV, a potential slightly hyperpolarized from rest. As seen in Fig. 2*B*, superfusion of 50 μM of MM induced a small inward current (Fig. 2*B*). In the nine cells studied with this protocol, the average MM-induced current was -0.70 ± 0.08 nA. The induced inward current was not completely inactivated at 1 min after MM application. Because the currents near the resting potential were so small, it was difficult to determine whether the current was sustained or slowly decayed over longer time periods.

As shown in Fig. 2*C*, in a Na-free saline, no MM-induced inward current was observed ($n = 7$). An apparent, small transient outward current was seen occasionally during the application of MM. This small current was very similar to that sometimes observed during the application of control Ringer, thus it was considered to be a superfusion artifact. The MM-induced current persisted in bath solutions with lowered [Ca^{2+}] (Ca^{2+} = 0.9 mM) Ringer. The amplitude of the MM-induced current in 0.9 mM Ca^{2+} Ringer solution was relatively larger than the same current observed in normal Ringer, 1.11 $\pm$ 0.35 nA ($V_H = -60$ mV, $n = 5$), although that increase was not statistically significant ($t = -1.5$, $P > 0.05$, df = 12). However, the MM-induced current was blocked by extracellular Co^{2+} ions. As shown in Fig. 2*D*, when the R cell was bathed in normal Ringer with 1.8 mM Co^{2+} substituting for Ca^{2+}, the MM-induced current was reduced significantly to 0.23 $\pm$ 0.13 nA ($n = 7$; $t = 3.2$, $P < 0.01$, df = 14). MM-induced currents

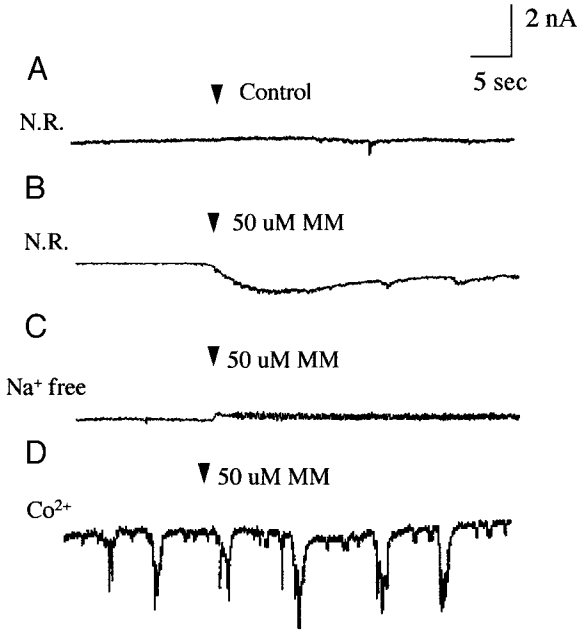

FIG. 2. MM rapidly activates an inward Na^+-mediated current near the resting membrane potential. Single-electrode voltage clamp (''switching clamp'') was used to investigate the ion current activated immediately after myomodulin application. R cell membrane was clamped near the resting potential −60 mV while MM was bath superfusion applied. *A*: superfusion of normal Ringer. *B*: superfusion of 50 μM of myomodulin. *C*: superfusion of 50 μM MM while cells were bathed in Na^+-free Ringer solution. *D*: superfusion of 50 μM MM while the cells were bathed in 1.8 mM Co^{2+} Ringer solution.

in four of seven cells showed complete blockade by extracellular Co^{2+}.

Results from the previous experiments revealed a lack of time dependence in the MM-induced current. Thus we were able to study the voltage dependence of the current using a ramp protocol. We applied a voltage ramp from −85 to −55 mV in normal leech Ringer followed by an identical ramp in MM containing Ringer solution. Figure 3*A* shows the total current-voltage (*I-V*) curves before and after application of MM. The MM-induced *I-V* relationship presented in Fig. 3*B* was acquired by subtracting the total current with MM from the total current without MM.

MM effect on K currents

TOTAL K. MM peptides have been demonstrated to differentially modulate several distinct K^+ currents in *Aplysia* (Brezina et al. 1994a,b, 1995; Critz et al. 1991; Scott et al. 1997). Because modulation of K^+ currents has a dramatic effect on neuronal excitability (Critz et al. 1991), we also examined the MM effect on the K^+ currents in R cells. Using two-electrode voltage clamp, we first examined the MM effect on the total K^+ current. Cells were bathed in both Na^+- and Ca^{2+}-free saline. The results from a series of step pulses from −70 to 40 mV in 10-mV increments applied from the holding potential of −60 mV are presented in Fig. 4. As seen in Fig. 4, *C* and *D*, a general decrease of total K^+ current was observed. At 20 mV, there was a 25 ± 3.5% ($t = 5.9$, $P < 0.005$, $n = 5$) reduction in the peak current and a 27 ± 2.2% ($t = 6.8$, $P < 0.005$, $n = 5$) reduction in the steady-state current. The difference current was obtained by subtracting the total current after MM application from the total current before MM and presented in Fig. 4*B*. The difference current appears to resemble the I_K current (see following text) in that it has a smaller peak current (compared with I_A) and a long-lasting steady-state current.

The voltage-sensitive K^+ currents in the R cell consist of at least two distinct components that can be distinguished readily by their voltage-sensitive inactivation kinetics and/or selective sensitivity to K^+ channel blockers, TEA and 4-aminopyridine (4-AP) (Acosta-Urquidi et al. 1989; Johansen and Kleinhaus 1986). By analogy with similar currents in other preparations, these two currents have been termed I_A and I_K, respectively (Johansen and Kleinhaus 1986).

I_A. I_A can be distinguished from I_K by its specific sensitivity to 4-AP but not TEA. To study the effect of MM on I_A current, 25 mM of TEA was added to the Na^+-, Ca^{2+}-free bath solution to block I_K. Results from a set of incremental (10 mV) 300-ms depolarizing steps, preceded by a 500-ms conditioning prepulse to −90 mV from a holding potential of −60 mV are presented in Fig. 5*B*. Command voltage steps to elicit I_A were kept at or below 0 mV to minimize the possible contribution of a small TEA-resistant fraction of I_K because there is potentially an overlap of these two currents at V_M values more positive than 0 mV (Acosta-Urquidi et al. 1989; Johansen and Kleinhaus 1986). As seen in Fig. 5*B*, MM had no effect on the peak amplitude of I_A ($t = 0.92$, $P > 0.4$, $n = 5$) within the voltage range examined. For example at 0 mV, the peak I_A before and after MM application was 25.2 ± 1.4 and 25.9 ± 1.4 ($t = 0.94$, $P > 0.3$, $n = 5$). Moreover, no effect on the inactivation time constant (τ_{off}) was observed. Values of τ_{off} pre- and post-MM application for currents elicited by voltage stepping to 0 mV from V_H of −60 mV were 42 ± 2.5 ms and 37 ± 4.8 ms ($t =$

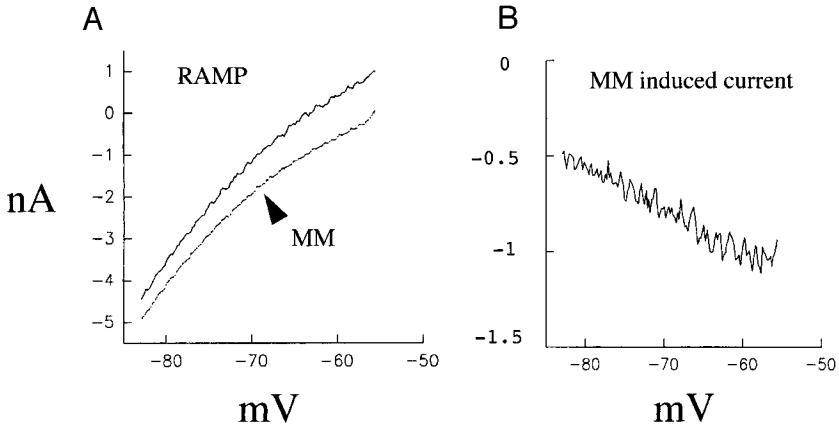

FIG. 3. Single-electrode voltage clamp (SEVC) with voltage-ramp protocol was used to determine the voltage dependence of the MM-induced current. Desheathed ganglia were bathed in normal leech saline. R cell membrane potential was ramped from the holding (−55 mV) to more negative values (−85 mV). *A*: MM activated an inward current throughout the negative potential range monitored, and the conductance increase was larger at the more positive potentials. *B*: *I-V* relation for the difference current was acquired by subtracting the total current with myomodulin from the total current without myomodulin. Voltage-ramp to more positive potentials could not be achieved because the Na^+ channels underlying the action potential in the leech R cell are insensitive to all known voltage-gated Na-channel blockers.

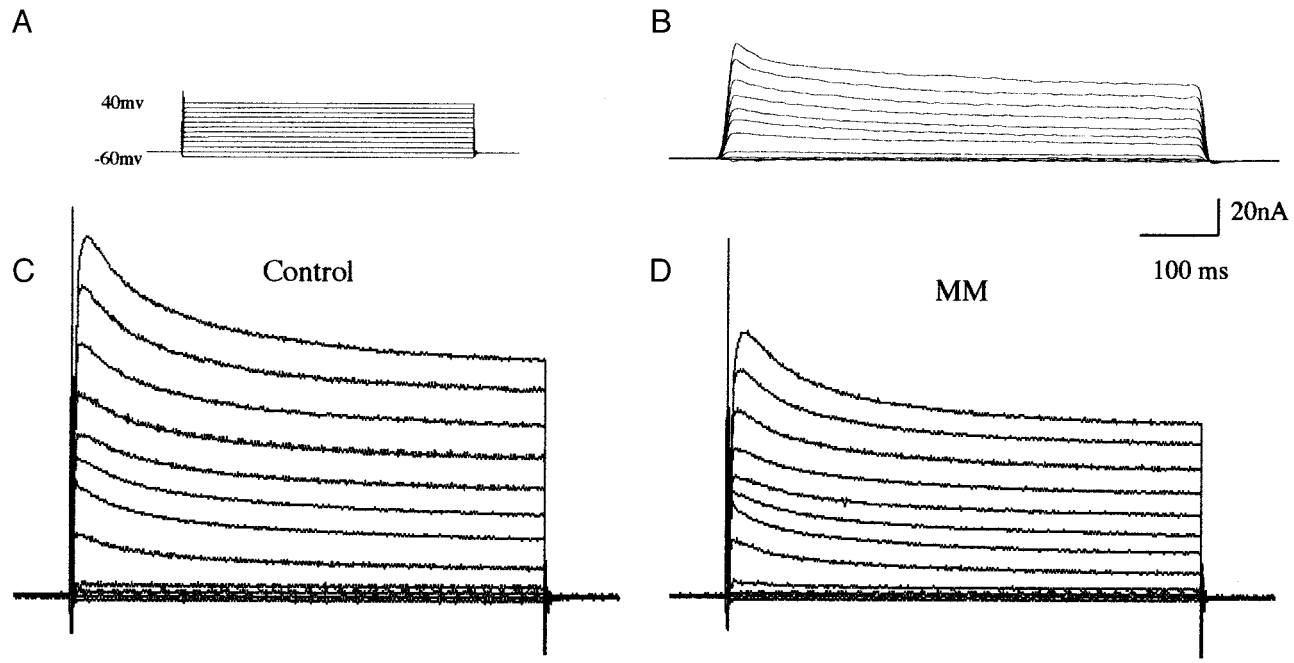

FIG. 4. MM reduces the total voltage-activated K^+ currents. Voltage-activated K^+ currents were examined by 2-electrode voltage clamp (TEVC) using Na^+-free (*N*-methyl-D-glucamine-substituted), Ca^{2+}-free (Co^{2+} substituted) bath solution. A series of step pulses from −70 to 40 mV in 10-mV increments were applied from the holding potential of −60 mV. *A*: cell membrane potentials following the voltage-clamp protocol. *B*: difference currents obtained by subtracting currents after MM from those before the application of MM at each potential. Modulated current has the property of I_K current. *C* and *D*: sample currents from a single cell before and after MM application.

1.8, $P > 0.1$, $n = 5$). Representative unsubtracted sample records before and after the application of 50 μM MM from a single cell were shown in Fig. 5, *C* and *D*. An *I-V* plot constructed from leak-subtracted currents in the presence (●) and absence (○) of MM is plotted in Fig. 5*B*.

I_K. I_K was separated from I_A by adding 8 mM of 4-AP to the Na^+-, Ca^{2+}-free saline. Current responses were evoked by command voltage steps in increments of 10 mV from a V_H of −50 mV. In contrast to the lack of effect on I_A, 50 μM MM consistently suppressed both the peak and steady-state amplitudes of I_K in the R cells (Fig. 6). Sample records from a single cell with and without MM are presented in Fig. 6, *A* and *B* (leak currents not subtracted). The *I-V* plot constructed from five cells for the peak current value for each voltage step is presented in Fig. 6*C*. These data indicated that there was a consistent decrease in peak current beginning at about −40 mV and reaching its maximum at 0 mV. The average reduction at 0 mV for peak I_K was 18.7 ± 4.3 nA (control, 37.7 ± 8.3 nA; MM, 18.9 ± 4.2 nA). This represents a mean decrease of 49 ± 2.9% ($t = 4.3$, $P < 0.01$, $n = 5$). Similarly, there was a consistent decrease of steady state I_K for all the voltage steps examined. Steady-state I_K at 0 mV was reduced by 7.1 ± 2.0 nA (control, 15.7 ± 3.1 nA; MM, 8.6 ± 1.4 nA). This represents a mean decrease of 43 ± 7.2% ($t = 3.5$, $P < 0.05$, $n = 5$; Fig. 6*D*).

MM effect on Ca current

Both patch-channel and voltage-clamp recordings have revealed only a single type of voltage-gated Ca^{2+} current in R cells of the adult leech (Bookman and Liu 1987; Johansen et al. 1987). We examined the effect of MM on this voltage-gated Ca^{2+} current. The effect of MM on the Ca^{2+} current is presented in Fig. 7, *C* and *D*, respectively. To isolate the Ca^{2+} current, we used Na^+-free Ringer with the addition of TEA (25 mM) and 4-AP (8 mM) to block the K^+ currents with Ca^{2+} or Ba^{2+} (at 2.8 mM) as the charge carrier. Current traces were leak-subtracted using conventional methods. Consistent with previous reports, we found that from a holding potential of −60 mV, the current first appeared with steps to about −20 mV, peaked between 5 and 10 mV, then decreased until it reversed ~40 mV (Johansen et al. 1987; Schirrmacher and Deitmer 1991). An *I-V* plot from a sample recording is presented in Fig. 7*B*. As seen in Fig. 7, *C* and *D*, MM induced a significant decrease in peak current at 0 mV ($V_H = -60$ mV). With calcium as the charge carrier, the current at 0 mV in the presence of MM was reduced to 78 ± 4% (control, 23.5 ± 1.3 nA; MM, 18.0 ± 0.9 nA) of the pre-MM amplitude ($t = 4.6$, $P < 0.002$, $n = 9$). With barium, the post-MM current at 0 mV was reduced to 83 ± 5% (control, 17.4 ± 1.6 nA; MM, 14.6 ± 1.7 nA) of the pre-MM value ($t = 3.5$, $P < 0.05$, $n = 5$; Fig. 8). No change in the shape of I_{Ca} and I_{Ba} before and after MM application was apparent, indicating that the kinetics of the current was not altered.

DISCUSSION

The results presented above extend the analysis of the mechanisms of the neuropeptide MM as a neuromodulator to an additional invertebrate species, the medicinal leech *Hirudo medicinalis*. Due to the peptide's localization to specific neurons within well-characterized and behaviorally relevant neural circuits in the leech, the role of MM in the expression of several specific behaviors can be analyzed. Understanding the

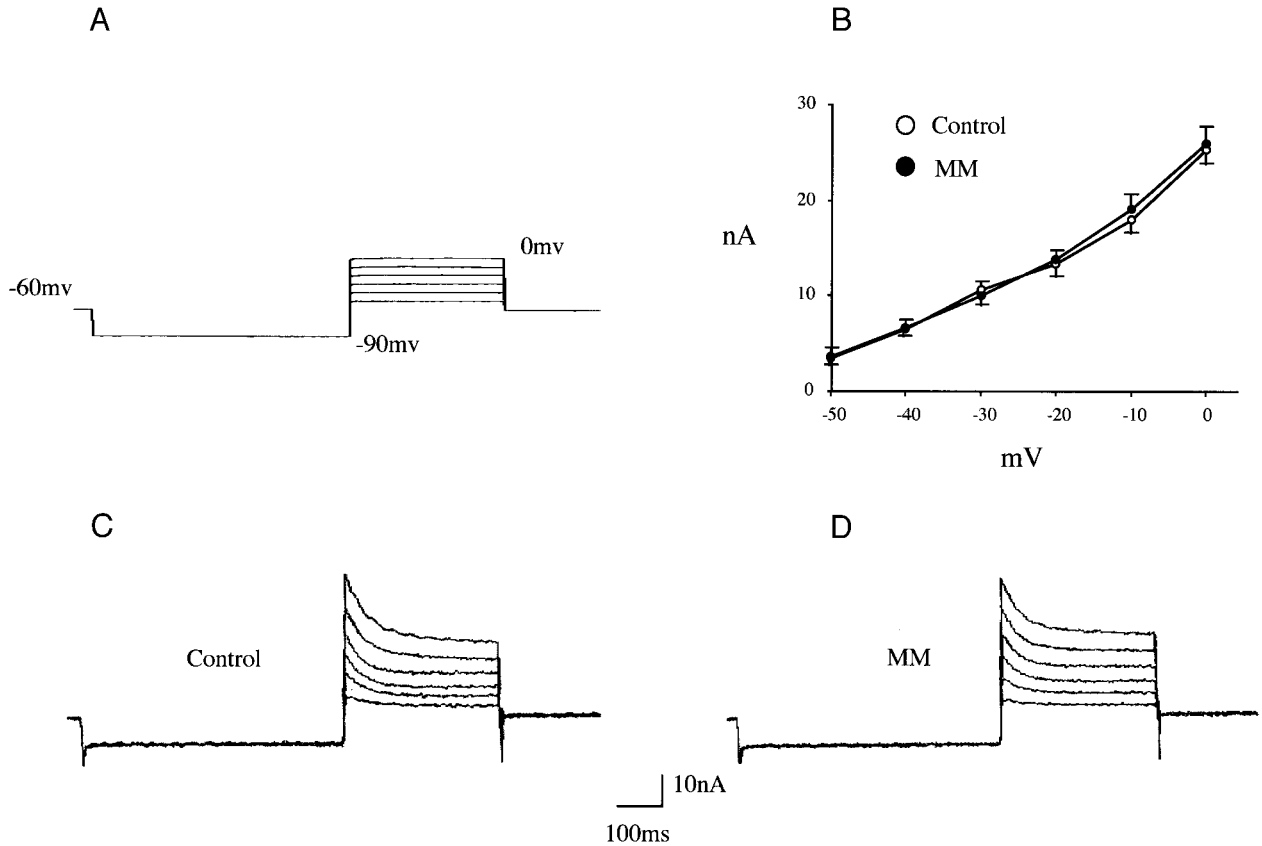

FIG. 5. MM has no effect on I_A in R cell. Currents were evoked by 300-ms depolarizing steps in 10-mV increments, following a 500-ms conditioning prepulse to −90 mV from a holding potential of −60 mV. Bath saline was Na^+- and Ca^{2+}-free with 25 mM TEA added to remove I_K contamination. *A*: cell membrane potentials following command voltage protocol. *B*: *I-V* plot of the peak amplitudes of the I_A currents (leakage corrected, see METHODS) before (○) and after (●) MM application. No change of peak I_A currents were observed throughout the voltage range examined. *C* and *D*: exemplary traces recorded from a single cell before (*C*) and after (*D*) application of 50 μM myomodulin. Leakage current was not subtracted from the records.

actions of MM will facilitate our understanding as to how neuropeptides modulate behaviors.

Although we previously had purified a MM-like peptide from the leech (Wang et al. 1998) and demonstrated that bath application of MM produced an excitatory response in the R cell, the ionic basis of this effect was not known. Here we present evidence that the MM effect on the R cell consists of the activation of a Na^+-mediated current near rest as well as the modulation of I_A and $I_{Ca^{2+}}$.

The characteristics of the small, inward current induced by MM near rest are well suited to account for the initial, rapid depolarization seen when MM is applied in current-clamp conditions. Because the current disappeared in Na^+-free Ringer and persisted in low Ca^{2+} Ringer, it appears that the current was carried predominantly by sodium, although we cannot rule out the possibility that calcium and/or potassium are also permeable.

Voltage steps around the resting potential did not reveal any new time-dependent components induced by MM, hence we were able to use voltage ramps study the current-voltage relation of this current. Voltage ramps revealed an unusual current-voltage relationship for the current. Over the narrow range of potentials examined, the current grew larger at more positive potentials even though the driving force should be smaller at these potentials. We were unable to examine this phenomenon at potentials more positive than −50 mV because of our inability to specifically block the voltage-gated, TTX-resistant sodium current that underlies the action potential in the R cell.

A very similar current induced by the neuropeptide FMRFamide near the cell resting potential was reported in the heart interneurons of the leech (Schmidt et al. 1995). There are several striking similarities between the MM-induced current in the R cell and the FMRFamide-induced current in the heart interneuron. Both currents have similar *I-V* characteristics at the corresponding voltage range tested and both are blocked by extracellular Co^{2+}. Moreover effects of the peptides on the corresponding cells desensitize rapidly. Finally, neuromodulator currents with similar characteristics that are carried mainly by Na^+ have been reported in a variety of other systems including proctolin in the lateral pyloric neurons of the crab (Golowasch and Marder 1992) and FMRFamide in the R14 neuron of *Aplysia* (Ichinose and McAdoo 1988). Interestingly, in these other systems, inward currents mainly mediated by Na^+ were blocked when extracellular Ca^{2+} concentration was elevated (Gillette and Green 1987; Golowasch and Marder 1992; Ichinose and McAdoo 1988). The effects of divalent cations on those currents suggests that there may be an instantaneous block of the currents by extracellular calcium. Although the MM-induced current in the R cell appears to be larger in low Ca^{2+} saline, the difference is not significant. However, the MM-induced current was blocked efficiently by extracellular Co^{2+} ions. This effect is similar to the FMRF-

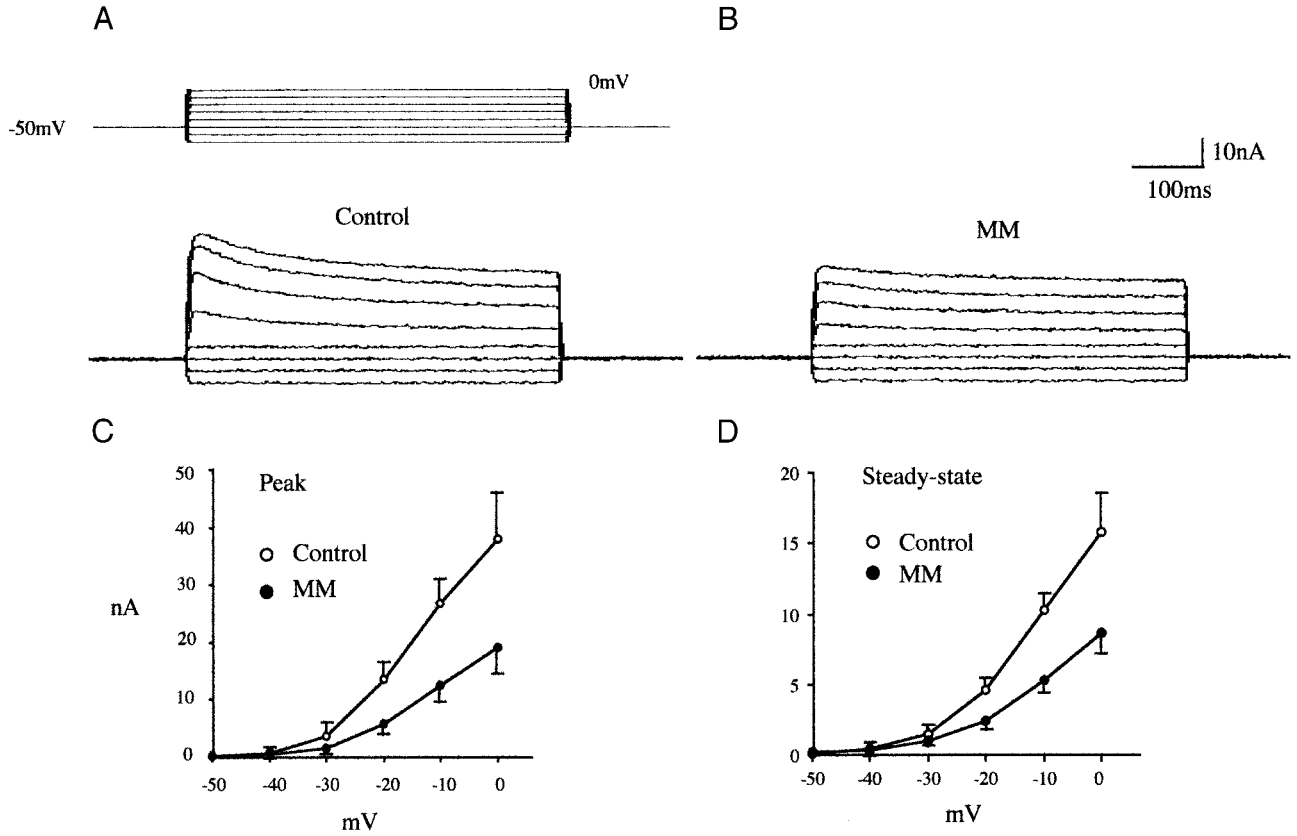

FIG. 6. MM suppresses I_K in R cell. Current responses were evoked by command voltage steps in increments of 10 mV from a V_H of −50 mV. Bath saline was Na^+- and Ca^{2+}-free with 8 mM 5-aminopyridine (4-AP) added to remove I_A. *A* and *B*: sample traces (leak unsubtracted) recorded from a single cell before (*A*) and after (*B*) application of 50 μM of myomodulin. *Inset*: cell membrane potentials following the command voltage protocol. I_K responses appear to have reached steady state at the end of the command pulse (550-ms duration). *C* and *D*: *I-V* plots of peak (*C*) and steady-state (*D*) I_K amplitudes (leak subtracted, see METHODS) before and after myomodulin application. There was a consistent decrease in both peak and steady-state I_K currents within the voltage range examined. Average decrease of peak I_K at 0 mV was 49 ± 2.9% ($t = 4.3$, $P < 0.01$, $n = 5$). Average decrease of steady-state I_K at 0 mV was 43 ± 7.2% ($t = 3.5$, $P < 0.05$, $n = 5$)

amide-induced current in leech heart interneuron (Schmidt et al. 1995), the proctolin-induced current in crab neurons (Golowasch and Marder 1992), and the serotonin-induced current in snail neurons (Gillette and Green 1987; Sudlow and Gillette 1995). The *I-V* characteristics of the MM-induced current seem to resemble the persistent Na^+ current, I_p, in the leech heart interneuron (Opdyke and Calabrese 1994). If the MM-induced current and I_p were the same, then the effects of Co^{2+} and MM on the current would be mutually exclusive.

The reduction of I_K also could contribute to the MM-induced increase in excitability. MM reduces I_K to about half its normal value. It is likely that the modulation of I_K contributes to the increase in excitability because I_K begins to activate around −35 mV (Figs. 4*B* and 6, *C* and *D*), a value that is within the normal range of the R cell resting membrane potential (−30 to −60 mV) (Hagiwara and Morita 1962; Leake 1986). Although other investigators further divide peak and steady-state I_K into I_{K1} and I_{K2} (Hodgkin and Huxley 1952; Simon et al. 1992; Stewart et al. 1989b), we did not attempt this distinction because we found that MM reduced both peak and steady-state I_K equally effectively. No MM-induced kinetic alteration of I_K was observed.

MM had no effect on IA both in terms of current amplitude and inactivation time constant. Interestingly, the I_A τ_{off} in the *Hirudo* R cells measured in these experiments is smaller than what was reported in the R cell in *Macrobdella* (Acosta-Urquidi et al. 1989; Johansen and Kleinhaus 1986). This, perhaps, reflects the differences in the voltage-gated I_A between the two species. In addition we found that the total K currents measured in these experiments in *Hirudo* were larger than those in *Macrobdella* (100 vs. 40 nA at 0 mV) (Johansen and Kleinhaus 1986).

As a multifunction neuron, activity in the R cell varies depending on the particular behavior the animal is expressing. These behaviors range from feeding (Lent and Dickinson 1984), to swimming (Kristan and Nusbaum 1983; Willard 1981), to mucus secretion (Lent 1973). It appears that changes in the R cell may be the result of its modulation by a number of neurotransmitters and neuropeptides including serotonin (5-HT) (Acosta-Urquidi et al. 1989), FMRFamide (Sahley et al. 1993; Strong et al. 1996), and the small cardiac peptide B (SCPB) (Kleinhaus and Sahley 1989). Analysis of the modulatory effects of these various neurotransmitters indicates that several distinct and sometimes overlapping ion channels are modified. One dramatic example of the relationship between R cell activity and behavior is the onset of patterned bursting of the R cell that accompanies swimming (Friesen 1989). This R cell bursting can be mimicked by the application of FMRFamide (Sahley et al. 1993).

In contrast to MM and FMRFamide, serotonin has an im-

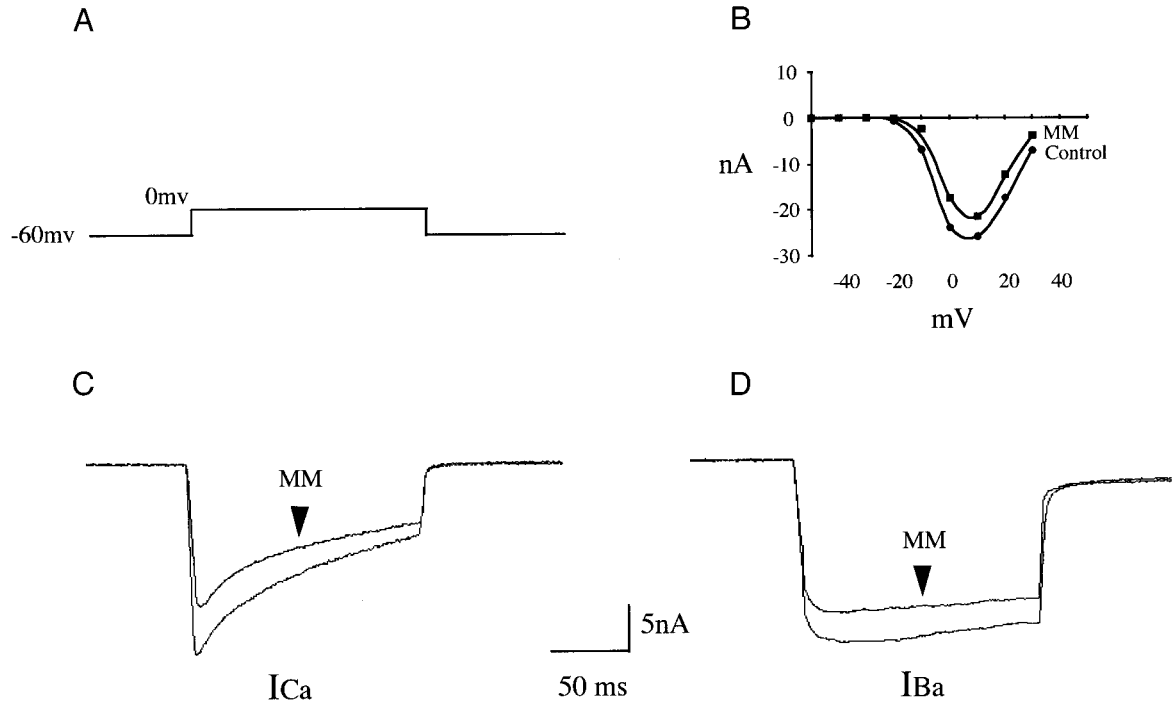

FIG. 7. MM reduces the voltage-activated Ca^{2+} current. Voltage-activated Ca^{2+} current was examined by both SEVC and TEVC. Bath saline was Na^+-free Ringer with either Ca^{2+} or Ba^{2+} as the charge carrier, and TEA and 4-AP were added to block K^+ currents. Membrane potential was stepped from −60 to 0 mV for 250 ms. *A*: command voltage protocol. *B*: *I-V* relation for peak I_{Ca} with and without myomodulin. *C* and *D*: sample records of I_{Ca} and I_{Ba} at 0 mV before and after myomodulin application. These current traces have been leak-subtracted by using leakage current resulting from hyperpolarizing pulse multiplied to the correct magnitude assuming a linear relation for the leakage current.

mediate transient inhibitory effect on Retzius cell because of the enhancement of a chloride conductance (Walker and Smith 1973). In addition, a more long-lasting effect of 5-HT has been characterized by Acosta-Urquidi et al. (1989) in which 5-HT enhances I_A while suppressing I_K. There is evidence that the effect of 5-HT on leech neurons is mediated at least partially by elevated cAMP level and accompanied by protein kinase A (PKA) activity (Biondi et al. 1982; Garcia-Gil et al. 1993). Although a systematic characterization of the second-messenger pathways underlying 5-HT and MM modulation is still lacking, the fact that MM has different effect on the I_A and I_K compared with 5-HT indicates that a different second-messenger mechanism may be involved.

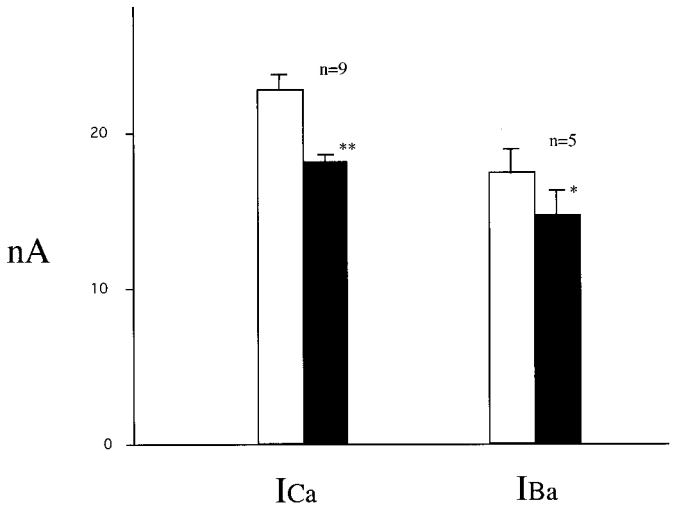

FIG. 8. Group data plot of the *ICa* and I_{Ba} reductions. Mean reduction for I_{Ca} at 0 mV was 22 ± 4% (** $t = 4.6$, $P < 0.002$, $n = 9$; 5 cells SEVC and 4 cells TEVC). Mean reduction for I_{Ba} at 0 mV was 17 ± 5% (* $t = 3.5$, $P < 0.05$, $n = 5$; SEVC).

Although cells express different types of voltage-gated Ca^{2+} channels (Hille 1992), single-channel and voltage-clamp recordings have revealed only one class of Ca^{2+} channel in leech R cells (Bookman and Liu 1987; Johansen et al. 1987; Schirrmacher and Deitmer 1991). The R cell Ca^{2+} channel permeability is $Sr^{2+}>Ba^{2+}>Ca^{2+}$, and the Ca^{2+} current shows characteristic Ca^{2+} dependent inactivation (Bookman and Liu 1987; Schirrmacher and Deitmer 1991; Stewart et al. 1989b). Voltage-gated Ca^{2+} (Ba^{2+}) currents, measured in Na^+-free and K^+-channel-blocked solution, showed characteristics of Ca^{2+} currents previously measured in adult leech ganglion cells (Johansen et al. 1987; Stewart et al. 1989b). Activation occurs at potentials more positive than −20 mV and peaks between 5 and 10 mV. Our data showed a moderate but significant reduction of the Ca^{2+} current after MM application. Peak I_{Ca} reduction is ~10–20% of the total I_{Ca}, with little alteration of the activation and inactivation kinetics of the Ca^{2+} current. The functional consequence of this modulation on the excitability of the R cell is not known. The effect of MM on I_{Ca} is expected to have minimum effect on the cell excitability because R cell action potential is exclusively Na^+ dependent in the absence of artificial perturbation of the endogenous Na^+ and K^+ conductances (Kleinhaus and Prichard 1975, 1976). In addition, like others (Johansen and Kleinhaus 1986) we did not see evidence for a Ca^{2+}-activated $I_{K(Ca)}$ component (data not shown) even after prolonged step depolarization (>400 ms)

although $I_{K(Ca)}$ has been detected in cultured R cells (Stewart et al. 1989b) as well as in R cells from specialized sex ganglia (Merz 1995). However, it is likely that the effect of MM on I_{Ca} could have an impact on the Ca^{2+}-mediated signal transduction and transmitter releases (Stewart et al. 1989a).

In the *Aplysia* buccal musculature, MM enhances the L-type Ca^{2+} current by an average of 50–70% via a cAMP-mediated second-messenger pathway (Brezina et al. 1994b; Hooper et al. 1994; Scott et al. 1997). FMRFamide, which does not activate cAMP in the buccal muscle, causes a 10–20% reduction of the Ca current (Cropper et al. 1994; Scott et al. 1997). We do not know if the reduction of I_{Ca} in the R cell by MM is mechanistically comparable with that in *Aplysia* by FMRFamide.

Taken together, our results on the MM modulation of the various ionic currents are consistent with the overall excitable effect of MM on the R cell. What could be the potential role of MM modulation on leech behaviors? One possibility is that it may participate in modulating the touch-elicited shortening reflex circuit in the leech. Recently we have obtained evidence that indicates a peptidergic synapse exists between the S and R cells (Wang, unpublished data). Previous work has indicated that both the S cell (Modney et al. 1997; Sahley et al. 1994) and the R cell (Ehrlich et al. 1992; Sahley 1994) are important for the expression of learning dependent changes in the behavior. Thus the S to R synapse could be an important site of modulation. Studies are underway to explore the role of modulation by MM in learning.

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