Multiple Presynaptic and Postsynaptic Sites of Inhibitory Modulation by Myomodulin at ARC Neuromuscular Junctions of Aplysia

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Multiple presynaptic and postsynaptic sites of inhibitory modulation by myomodulin at ARC neuromuscular junctions of Aplysia. J Neurophysiol 89: 1488–1502, 2003; 10.1152/jn.00140.2002. The functional activity of even simple cellular ensembles is often controlled by surprisingly complex networks of neurotransmitters and neuromodulators. One such network has been extensively studied in the accessory radula closer (ARC) neuromuscular system of Aplysia. The ARC muscle is innervated by two motor neurons, B15 and B16, which release modulatory peptide cotransmitters to shape ACh-mediated contractions of the muscle. Previous analysis has shown that key to the combinatorial ability of B15 and B16 to control multiple parameters of the contraction is an asymmetry in their peptide modulatory actions. B16, but not B15, releases myomodulin, which, among other actions, inhibits the contraction. Work in single ARC muscle fibers has identified a distinctive myomodulin-activated K current as a candidate postsynaptic mechanism of the inhibition. However, definitive evidence for this mechanism has been lacking. Here, working with the single fibers and then motor neuron-elicited excitatory junction potentials (EJPs) and contractions of the intact ARC muscle, we have confirmed two central predictions of the K-current hypothesis: the myomodulin inhibition of contraction is associated with a correspondingly large inhibition of the underlying depolarization, and the inhibition of both contraction and depolarization is blocked by 4-aminopyridine (4-AP), a potent and selective blocker of the myomodulin-activated K current as a candidate postsynaptic mechanism of the inhibition. However, definitive evidence for this mechanism has been lacking. Here, working with the single fibers and then motor neuron-elicited excitatory junction potentials (EJPs) and contractions of the intact ARC muscle, we have confirmed two central predictions of the K-current hypothesis: the myomodulin inhibition of contraction is associated with a correspondingly large inhibition of the underlying depolarization, and the inhibition of both contraction and depolarization is blocked by 4-aminopyridine (4-AP), a potent and selective blocker of the myomodulin-activated K current. However, in the intact muscle, the experiments revealed a second, 4-AP-resistant component of myomodulin inhibition of both B15- and B16-elicited EJPs. This component resembles, and mutually occludes with, inhibition of the EJPs by another peptide modulator released from both B15 and B16, buccalin, which acts by a presynaptic mechanism, inhibition of ACh release from the motor neuron terminals. Direct measurements of peptide release showed that myomodulin also inhibits buccalin release from both B15 and B16 terminals. At the level of contractions, nevertheless, the postsynaptic K-current mechanism is responsible for much of the myomodulin inhibition of peak contraction amplitude. The presynaptic mechanism, which is most evident during the initial build-up of the EJP waveform, underlies instead an increase of contraction latency.

INTRODUCTION

The functional activity of cellular ensembles is integrated by neurotransmitters and neuromodulators. However, the integration is often, at first sight, surprisingly complex. Even very simple ensembles—two neurons, or a neuron and a muscle cell—often incorporate complex networks of many transmitters and modulators with multiple effects (Brezina and Weiss 1997a). This complexity appears to be related to the complex, multidimensional behavior that even such simple ensembles can produce. Multiple dimensions of the integrative network are needed to control multiple dimensions of the functional output (Brezina and Weiss 1997a,b; Brezina et al. 1996). In a neuromuscular system, for example, multiple modulatory actions are needed to independently control multiple parameters of the shape of the contraction waveform (Brezina and Weiss 2000; Brezina et al. 2000b,c).

We have been studying these issues in the model ARC neuromuscular system of Aplysia (for reviews, see Hooper et al. 1999; Kupfermann et al. 1997; Weiss et al. 1992, 1993). The accessory radula closer (ARC) muscle (Cohen et al. 1978), one of the muscles of the animal’s buccal mass, participates in consummatory feeding behaviors such as biting, swallowing, and rejection of unsuitable food. The muscle is innervated by two motor neurons, B15 and B16, which release their classical transmitter, ACh, to depolarize and contract the muscle (Cohen et al. 1978; Kozak et al. 1996). In addition, however, the motor neurons release modulatory peptide cotransmitters of several different families that shape the ACh-induced contractions. Motor neuron B15 releases the small cardioactive peptides (SCPs) and the buccalins; B16 releases the myomodulins and the buccalins (Brezina et al. 1995; Cropper et al. 1987a,b, 1988, 1990a, 1991; Lloyd et al. 1984; Vilim et al. 1994, 1996b, 2000). Each of these peptides exerts a distinct yet overlapping spectrum of modulatory actions at the B15- and B16-ARC neuromuscular junctions and on the muscle itself. The buccalins inhibit contractions of the muscle, the available evidence suggests, purely presynaptically by inhibiting the release of ACh from the B15 and B16 terminals (Cropper et al. 1988, 1990a; Vilim et al. 1994). The SCPs and the myomodulins, on the other hand, have been thought to act primarily postsynaptically, on the ARC muscle itself, in three main ways. They potentiate contractions by enhancing the muscle’s L-type Ca current that supplies Ca$^{2+}$ essential for contraction (Brezina et al. 1994d, 1995; Cropper et al. 1987a,b, 1991; Lloyd et al. 1984; Whim and Lloyd 1990); they inhibit contractions by...
activating a distinctive, modulator-specific K current in the muscle (Cropper et al. 1987b, 1991; Brezina et al. 1994a, 1995); and they accelerate the relaxation rate of the muscle probably by modulating its contractile machinery (Cropper et al. 1990b; Whim and Lloyd 1990; Brezina et al. 1995; Probst et al. 1994). The combinatorial interaction of these effects, activated by different combinations of the peptides released by different patterns of firing of the motor neurons B15 and B16, is thought to optimize multiple parameters of the shape of the ARC muscle contractions for efficient performance in each feeding behavior (Brezina et al. 1996, 2000c).

In previous work (Brezina et al. 1996), we analyzed the postsynaptic interaction of the SCPs and the myomodulins. Key to their combinatorial control was the fact that the spectrum of their actions, while in most ways similar, differs in one major respect. The myomodulin spectrum includes a much stronger component of inhibition of ARC muscle contractions than does the SCP spectrum. It appeared very likely that this was because the myomodulins activate to a much greater degree the modulator-specific K current in the muscle.

The idea that the myomodulins inhibit contractions by activating the K current is very plausible, but the evidence for it has been largely correlational (Brezina et al. 1994a, 1995; see also Cropper et al. 1994). Furthermore, most of the evidence was obtained in dissociated muscle fibers, which, quite apart from the possibility of dissociation artifacts, lack the physiological structure, indeed the entire presynaptic element, of the intact system. The motivation for the present study was therefore to test, on the single fibers but more importantly in the intact ARC neuromuscular system, two key predictions of the K-current hypothesis. First, if the K current is involved, it presumably acts by opposing the ACh-induced depolarization and so activation of the L-type Ca current and Ca$^{2+}$ influx. If so, the myomodulin inhibition of contractions should be obligatorily accompanied by an inhibition of the underlying depolarization of the single fibers and of the motor neuron-elicited excitatory junctional potentials (EJPs) in the intact system. Second, 4-aminopyridine (4-AP), a potent and selective blocker of the K current (Brezina et al. 1994e) should block the myomodulin inhibition of EJPs and contractions.

We test these predictions here. We find that, indeed, activation of the K current is responsible for a large component of the myomodulin inhibition. Unexpectedly, however, the experiments reveal several additional novel effects of myomodulin, for a still more complex, but more complete, picture of the network of modulation in the ARC neuromuscular system.

**METHODS**

Methods were as in previous work in the ARC neuromuscular system. A brief summary of the methods used follows.

**Membrane voltage and contractions of single ARC muscle fibers**

Single fibers were dissociated from the ARC muscle by collagenase treatment. Fibers were isolated at one end by a suction pipette or, more usually, impaled close to the end with an intracellular microelectrode for voltage recording and current injection and stretched straight in a fast-flowing stream of solution. ACh was puffed onto the fiber from an upstream puff pipette; MM$_A$ and 4-AP were added to the main solution stream. The length of the fiber was continuously measured with an on-line video/computer system. All experiments were done in normal artificial sea water (ASW) at room temperature. See Brezina (1994) and Brezina et al. (1994a,d) for further details.

**Motor neuron-elicited EJPs and contractions in the intact ARC muscle**

Details of the standard preparation and methods used in these experiments have been given, e.g., by Cohen et al. (1978), Weiss et al. (1978), Cropper et al. (1987a), Vilim et al. (1994), and Brezina et al. (1995). Briefly, the preparation consisted of the buccal ganglia, an ARC muscle, and the connecting nerve 3 through which the buccal motor neurons B15 and B16 innervate the muscle. The muscle was placed in a separate subchamber to which the peptides and 4-AP were applied. Experiments were done at room temperature.

Most experiments were done with motor neuron B15. The motor neuron was impaled with a double-barreled microelectrode and stimulated with brief current injections to fire spikes in the desired pattern. Membrane voltage in the ARC muscle was recorded intracellularly with a single-barreled microelectrode. To study EJPs, B15 was fired at relatively low frequency (4–14 Hz) in short (1 s) bursts every 20 s to elicit robust EJP waveforms yet only minimal contractions. Alternatively, contractions were monitored with an isotonic transducer. To study contractions, B15 was fired at higher frequency (>10 Hz) for 1.5 s every 30 s to elicit contractions of medium amplitude. In sets of experiments where the effect of a peptide was to be compared under different conditions (e.g., Figs. 5, 6C, 7C, and 11, B and C), the frequency of B15 firing was adjusted at the beginning of each experiment so that the effect of the peptide was always measured at a similar level of EJP depolarization or contraction. All these patterns of B15 firing release ACh but not the neuron’s endogenous peptide cotransmitters (Cropper et al. 1990b; Vilim et al. 1996a) and so provided stable EJP waveforms and contractions for testing of the exogenously applied peptides. Similar, although less comprehensive, experiments were done with motor neuron B16.

**Buccal release from motor neuron B15**

A variant of the ARC-buccal ganglion preparation described in the preceding text was used (for details see Vilim et al. 1996a; Karhunen et al. 2001). The ARC muscle was perfused through its artery with ASW at 20 µl/min. Every 2.5 min, a drop of the outflowing perfusate was allowed to fall into a test tube in which the buccalin content was subsequently determined by radioimmunoassay (RIA) (Vilim et al. 1996b). Motor neuron B15 was fired at 12.5 Hz for 3.5 s every 7 s, in 10-min blocks separated by 15-min rest periods. MM$_A$ was added to the muscle perfusate. The preparation was maintained at 15 ± 0.5°C.

**RESULTS**

**Myomodulin inhibits depolarization and contraction of single ARC muscle fibers by activating the modulator-specific, 4-AP-sensitive K current**

We monitored the membrane voltage and length of single ARC muscle fibers and recorded the depolarization and contraction elicited by brief puffs of ACh or, in some experiments, by equivalent brief injections of depolarizing current (cf. Brezina and Weiss 1993), before and after application of MM$_A$, which is likely to be the functionally most significant myomodulin (Brezina et al. 1995). We compared the effects of MM$_A$ under control conditions and in the presence of 100 µM 4-AP, which at this concentration completely blocks the modulator-specific K current (Brezina et al. 1994e) but has little effect on any other K current in the ARC muscle fibers.
A representative experiment is shown in Fig. 1, group data in Fig. 2.

As reported previously (Brezina et al. 1994a; Kozak et al. 1996), the puffs of ACh (and the equivalent current injections) depolarized fibers from their usual resting potential between −60 and −70 mV typically to between −30 and −20 mV. These depolarizations were accompanied by sizable contractions (control in Figs. 1 and 2, A and B).

Application of 10 μM MM_A immediately depressed the peak depolarization by >20 mV, to around −50 mV (“MM_A” in Figs. 1 and 2, A and C, left). In some fibers, there was also a hyperpolarization, of up to several millivolts, of the resting potential. At the same time, MM_A greatly depressed, indeed often completely abolished, the corresponding contraction (MM_A in Figs. 1 and 2, B and C, right).

All of these effects of MM_A were blocked by 100 μM 4-AP. When applied after MM_A, 4-AP immediately restored the MM_A-depressed depolarization to nearly its control level. At the same time, it restored the MM_A-depressed contraction, not merely to the control level, but significantly above it (MM_A + 4-AP in Figs. 1 and 2, A–C).

Presumably, block of the depressive effect unmasked the underlying MM_A-induced potentiation of contractions that has been well documented in the ARC muscle fibers and in the intact ARC muscle (Cropper et al. 1987b, 1991; Brezina et al. 1994d,e, 1995). When the order of application of MM_A and 4-AP was reversed, 4-AP alone had no effect on contractions, and, in its presence, MM_A did not depress contractions but rather potentiated them (Fig. 2B, right).

**FIG. 1.** Myomodulin inhibits depolarization and contraction of single accessory radula closer (ARC) muscle fibers by activating the modulator-specific, 4-AP-sensitive K current. Representative experiment. The fiber’s membrane voltage (bottom) and length (top) were monitored while brief puffs of 10 μM ACh were applied in control solution (left), after addition of 10 μM MM_A (middle), and after further addition of 100 μM 4-AP (right).

**FIG. 2.** Myomodulin inhibition of depolarization and contraction in single ARC muscle fibers: group data. Fibers were depolarized with brief puffs of ACh as in Fig. 1 (measurements A in A and B) or with equivalent brief injections of depolarizing current (measurements C in A and B). A: the peak depolarization (the most positive absolute voltage; symbolized V_peak below) attained in 3 fibers (lines connect measurements from the same fiber) in control solution, after addition of 10 μM MM_A and after further addition of 100 μM 4-AP. B, left: the corresponding contraction of the fiber (decrease in length expressed as a percentage of the resting length; symbolized Cn below) in the same 3 conditions as in A. Right: reversing the order of addition of the MM_A and 4-AP. C: summary of the MM_A effect. Left: depression of peak depolarization by MM_A in the absence and presence of 4-AP. Plotted are means ± SE (n indicated) of the differences V_peak,control − V_peak,MM_A and V_peak,control − V_peak,MM_A + 4-AP computed for each of the fibers in A. Right: the corresponding depression of contraction. Means ± SE (n indicated) of the results of the computations 100 × (Cn,control − Cn/MM_A)/Cn,control and 100 × (Cn,control − Cn/MM_A + 4-AP)/Cn,control for the fibers in B, left and right.
When added to other available information (see DISCUSSION), these results argued strongly that all of the inhibitory effects of myomodulin that are seen in the single ARC muscle fibers are due to activation of the modulator-specific, 4-AP-sensitive K current.

Myomodulin inhibits motor neuron B15-elicited EJPs in the intact ARC muscle

We now sought to extend the single-fiber results to the intact ARC muscle. We began by recording intracellularly from fibers in situ in the intact muscle and examining the ACh-mediated depolarization produced by firing of motor neuron B15. We stimulated B15 to fire in bursts; each burst elicited a complex facilitating and summating EJP waveform in the muscle. Figure 3, 1-control, shows a typical example. We used B15 for most experiments because, at physiological firing frequencies, the EJPs within the waveform summate more slowly and so are easier to resolve than those of B16 (Cohen et al. 1978) (see below).

Application of 1 μM MMA to the muscle hyperpolarized the resting potential by ±10 mV and, at the same time, strongly depressed the EJP waveform (Fig. 3, “2-MM A”). In six muscles, the peak depolarization attained during the EJP waveform, EJP peak, was depressed by MMA from -43.3 ± 2.9 to -55.0 ± 2.9 mV, by 11.7 ± 0.4 mV (means ± SE given throughout).

Myomodulin inhibition of EJPs is partly, but not completely, blocked by 4-AP

We repeated the experiments just described in the presence of 100 μM 4-AP. When applied in the presence of 4-AP, MMA did not hyperpolarize the resting potential of the muscle. Furthermore, it depressed the B15-elicited EJP waveform much less (Fig. 4A). In eight muscles, 1 μM MMA depressed EJP peak by only 5.7 ± 0.9 mV as compared to the 11.7 ± 0.4 mV in the absence of 4-AP. This difference was statistically significant (see Fig. 5). Thus part of the MMA depression was blocked by 4-AP. But, unlike in the single muscle fibers, significant depression still occurred in the presence of 4-AP.

4-AP-resistant component of myomodulin inhibition resembles inhibition by buccalin

Examining the EJP waveforms closely, we noticed that often MMA did not simply scale down the whole waveform but also changed its shape. MMA depressed disproportionately strongly the first few individual EJPs in the waveform so that the build-up of the waveform was slowed and delayed.

The depression of the build-up persisted in the presence of 4-AP and indeed became especially obvious when 4-AP largely blocked the depression of the peak of the waveform (Fig. 4A). Thus the depression of the build-up appeared to represent a distinct, 4-AP-resistant component of myomodulin depression. This component apparently was not present in the experiments on the single muscle fibers. Because the single fibers represent just the postsynaptic element of the B15-ARC neuromuscular junction, this could be explained if the second component were due to a presynaptic action of myomodulin, on the terminals of motor neuron B15 to inhibit release of ACh onto the muscle.

Cropper et al. (1988, 1990a) and Vilim et al. (1994) have
provided evidence that the buccalins, modulatory peptide cotransmitters released by both motor neurons B15 and B16, act in precisely this way at the B15-ARC neuromuscular junction (see Discussion). We therefore carried out another series of experiments with a representative buccalin, BUC\textsubscript{A}. The BUC\textsubscript{A} depression of the B15-elicited EJP waveform (Fig. 4\textit{B}) indeed strikingly resembled just the second, 4-AP-resistant component of MM\textsubscript{A} depression. In seven muscles, 1 \mu M BUC\textsubscript{A} depressed EJP\textsubscript{peak} by 6.2 \pm 1.2 mV, a value similar to that found for the 4-AP-resistant MM\textsubscript{A} depression. Furthermore BUC\textsubscript{A} strongly depressed the build-up of the EJP waveform (Fig. 4\textit{B}). Finally, the BUC\textsubscript{A} depression was completely resistant to 4-AP (Fig. 4\textit{C}). In six muscles in the presence of 100 \mu M 4-AP, 1 \mu M BUC\textsubscript{A} depressed EJP\textsubscript{peak} by 5.5 \pm 1.2 mV, a value not significantly different from that in the absence of 4-AP (see Fig. 5).

For a more rigorous analysis, we needed to quantify the depression of the build-up of the EJP waveform. The depression of the build-up was consistently robust around the point where the EJP waveform rose to its half-maximal amplitude (see Fig. 4), whereas the precise voltage, the number of individual EJPs, and the time it took to reach that point all varied from muscle to muscle. Analogously to EJP\textsubscript{peak}, we therefore defined EJP\textsubscript{1/2}, the depolarization attained by the individual EJP—usually the third, fourth, or fifth in the waveform—that came closest to the voltage level half-way between the resting potential and EJP\textsubscript{peak}. We then measured the depression of EJP\textsubscript{1/2} as the depression of that particular EJP in the waveform.

Figure 5 summarizes our measurements of the depression of EJP\textsubscript{peak}, together with corresponding measurements of the depression of EJP\textsubscript{1/2} from the same experiments, for an overall comparison of the inhibitory effects of myomodulin and buccalin on the motor neuron B15-elicited EJPs. The two asterisked differences are statistically significant, but none of the other differences that would be meaningful reach significance. In particular, the depressive effects of myomodulin and buccalin on EJP\textsubscript{1/2} are not significantly different and are not significantly blocked by 4-AP.

The simplest interpretation of Fig. 5 is that there are, indeed, two components of depression. The first, seen at depolarized voltages near the peak of the EJP waveform, is exerted only by myomodulin. It is blocked by 4-AP and is due to postsynaptic activation of the modulator-specific, 4-AP-sensitive K current. The second, 4-AP-resistant component of depression is exerted equally by myomodulin and buccalin. This component also contributes to the depression of the peak of the EJP waveform, but it is solely responsible for the depression of the build-up of the waveform at more negative voltages. Our hypothesis is that this second component, for myomodulin as for buccalin, reflects presynaptic inhibition of ACh release from B15 terminals onto the ARC muscle.

**Mutual occlusion of the myomodulin and buccalin inhibition of EJPs**

If myomodulin and buccalin indeed inhibit EJPs, in part, by a common mechanism, their effects might occlude each other. To examine this, we performed two kinds of occlusion experiments. In one series of experiments (Figs. 6, 7, A and B), we applied the two peptides cumulatively. The first peptide was applied at 10 \mu M, a high concentration likely to saturate its effect; the second peptide was then applied at the usual concentration of 1 \mu M. In the second series of experiments (Figs. 6\textit{C} and 7\textit{C}), we simply measured the effect of the test peptide in the background presence of the other peptide, having adjusted the initial size of the EJP waveform (see Methods) so that the measurements were made in the same voltage range and could directly be compared to those already made for each peptide alone.

We first examined whether myomodulin occluded the inhibition of EJPs by buccalin (Fig. 6). Indeed, following MM\textsubscript{A}, BUC\textsubscript{A} had very little additional effect of any kind. This was true in both series of occlusion experiments. In the cumulative experiments (Fig. 6, A and B), once MM\textsubscript{A} had depressed the EJP waveform, BUC\textsubscript{A} did not depress it significantly further, even though, as the EJP\textsubscript{1/2} plot in Fig. 6\textit{B} shows, in the absence of MM\textsubscript{A}, it was capable of depressing EJPs at considerably more negative voltages. The occlusion was equally pronounced in the absence and in the presence of 100 \mu M 4-AP (Fig. 6\textit{B}). In the second series of occlusion comparisons (Fig. 6\textit{C}), too, MM\textsubscript{A} occluded equally well all BUC\textsubscript{A} depression, of EJP\textsubscript{peak} as well as of EJP\textsubscript{1/2}, in the absence as well as in the presence of 4-AP. All this argues that the component of EJP inhibition that is exerted by buccalin is also exerted by myomodulin, presumably because the two peptides act convergently, perhaps through the very same mechanism. Furthermore, because the occlusion persists in 4-AP, this is presumably the second, 4-AP-resistant mechanism, the presynaptic inhibition of ACh release.

We then examined, conversely, whether buccalin occluded the

**FIG. 5.** Summary of the inhibitory effects of myomodulin and buccalin on motor neuron B15-elicited EJPs. EJP\textsubscript{peak}; summary of data given in text. Means \pm SE (n indicated) of the difference EJP\textsubscript{peak,control} − EJP\textsubscript{peak,MM\textsubscript{A}}, and EJP\textsubscript{peak,control} − EJP\textsubscript{peak,BUC\textsubscript{A}} (with 1 \mu M MM\textsubscript{A}, BUC\textsubscript{A}) in the absence and presence of 100 \mu M 4-AP. EJP\textsubscript{1/2} depression of EJP\textsubscript{1/2} (for definition, see 4-AP-resistant component of myomodulin inhibition resembles inhibition by buccalin) from the same experiments. Statistical significance was assessed with Student’s t-test; **P < 0.01, *P < 0.05, throughout.
inhibition of EJPs by myomodulin (Fig. 7). Again, we found significant occlusion, although, as expected, the picture was more complex. Especially in the case of the large MM_A depression of EJP peak in the absence of 4-AP, although a significant part of the depression was occluded by BUC_A, a large part remained unoccluded (Fig. 7C). This presumably reflects the fact that MM_A, but not BUC_A, depresses EJP peak also by the first mechanism, the postsynaptic 4-AP-sensitive K current. The smaller depressive effects of MM_A on EJP peak in the presence of 100 μM 4-AP and on EJP_1/2 in the absence and presence of 4-AP were all significantly occluded by BUC_A (Fig. 7, A–C). Even in these cases, however, a larger part of the MM_A effect remained unoccluded by BUC_A than conversely in Fig. 6C. It may be that, even with respect to the second, presynaptic mechanism, MM_A is a stronger depressor than BUC_A.

Another member of the myomodulin family does not inhibit EJPs

In addition to MM_A, we tested another member of the myomodulin family, MM_B, which functionally is probably relatively insignificant in the ARC system but which is a useful tool in that it activates much less than MM_A, the modulator-specific K current in the ARC muscle (Brezina et al. 1994e, 1995, 1996). It should therefore inhibit EJPs much less. Indeed, 1 μM MM_B had little or no effect on the B15-elicited EJP waveform and did not occlude its subsequent inhibition by MMA (Fig. 8). Interestingly, MM_B had no effect either on EJP peak or EJP_1/2, suggesting that it shares neither the postsynaptic nor the second, presynaptic action of MM_A. MM_B thus apparently lacks the inhibitory components of MM_A action while fully retaining its potentiation of contraction amplitude and relaxation rate (Brezina et al. 1994d, 1995, 1996; Cropper et al. 1991).

Myomodulin inhibits motor neuron B16-elicited EJPs

The ARC muscle’s other motor neuron, B16—which releases, as cotransmitters, the endogenous myomodulins in the system—also elicits ACh-mediated EJPs in the muscle. We examined whether these EJPs, too, are inhibited by myomodulin. Indeed, in eight muscles, 1–10 μM MM_A significantly
depressed the B16-elicited EJP waveform (Fig. 9). As was sometimes the case also with B15, the relaxation of the EJP waveform back to the resting potential after the end of the B16 burst was accelerated by MM_A (Fig. 9, 2, scaled). MM_B had no obvious effect on the EJPs (4 muscles).

When motor neuron B16 is fired at physiological frequencies, which are higher than those for B15, the B16-elicited EJPs facilitate and summate to peak much more rapidly than those of B15 (Cohen et al. 1978). This made it difficult to dissect the myomodulin inhibition as we did for B15. Nevertheless, myomodulin strongly depressed the build-up of the EJP waveform as well as its peak (Fig. 9), suggesting that, in addition to activating the postsynaptic K current that would be expected to provide inhibition common to both B15 and B16, myomodulin may well presynaptically inhibit ACh release from B16 as from B15. In the case of B16, too, there is a similar presynaptic inhibition by buccalin (Vilim et al. 1994).

Myomodulin inhibits peptide release from motor neuron B15

The terminals of motor neuron B15 release not only ACh but also the peptide cotransmitters, the buccalins and the SCPs. Therefore if myomodulin presynaptically inhibits the release of ACh, it might also inhibit the release of the peptide cotransmitters. Indeed, this has already been shown for the other presynaptic inhibitor, buccalin (Vilim et al. 1996a).

Because all of the peptide cotransmitters of B15 are contained in the same vesicles and obligatorily coreleased (Vilim et al. 1996b), it is sufficient to measure the release of one of them. We measured the release of buccalin, as in previous work (Karhunen et al. 2001; Vilim et al. 1996a,b) by direct radioimmunoassay of ARC muscle perfusate while stimulating B15 to fire in a quasi-physiological pattern. As Fig. 10 shows, application of 1 μM MM_A significantly inhibited the release of buccalin, by ~40%.
Myomodulin inhibits motor neuron B15-elicited ARC muscle contraction

The ARC is a nonspiking muscle in which the EJP depolarization directly translates into contraction (Cohen et al. 1978). However, the translation is highly nonlinear and, especially in its dynamics, not well understood. It was thus important to determine to what extent the myomodulin inhibition of EJPs, and each of the two components of the inhibition, actually translates to an inhibition of contraction.

Each burst of motor neuron B15 firing of sufficient frequency elicited, as well as the EJP waveform, a corresponding contraction. As reported previously (Brezina et al. 2000c; Cropper et al. 1991), myomodulin, at higher concentrations, inhibited these contractions. At 1 μM, MM_A profoundly depressed the contractions, indeed in some experiments completely abolished them (Fig. 11, A, top, and B). Often, MM_A also induced a baseline relaxation of the muscle (see, e.g., Fig. 12, A, top left) (Brezina et al. 1995). There were similar effects on motor neuron B16-elicited contractions (see Brezina et al. 1995; Cropper et al. 1987b, 1991).

To determine the relative contributions of the two components of myomodulin inhibition, we used the same strat-
egy as with the EJPs. We examined the degree to which the depression of contractions was blocked by the postsynaptic blocker 4-AP. We further examined the degree of occlusion between the depressive effects on contractions exerted by myomodulin and buccalin. Buccalin inhibits motor neuron B15-elicited contractions of the ARC muscle (Brezina et al. 2000c; Cropper et al. 1988, 1990a; Vilim et al. 1994) presumably through its presynaptic effect, the inhibition of ACh release.

In these experiments, there was evidence that the second, presynaptic component of myomodulin inhibition made a contribution to the inhibition of contractions. A substantial part of the MM A depression of contractions remained unblocked by 100 μM 4-AP (Fig. 11B). Also, MM A significantly occluded the depression of contractions by 1 μM BUC A (Fig. 11C). Occlusion at the level of EJPs (Fig. 6C) thus translated to occlusion at the level of contractions.

On the other hand, there was significant block of the MM A depression by 4-AP (Fig. 11B). Furthermore, in the converse occlusion experiment, BUC A failed to significantly occlude the depression of contractions by 1 μM MM A (Fig. 11C). This suggested that the myomodulin effect not shared by buccalin, the activation of the postsynaptic K current, had a dominant influence. It alone could account for most of the total myomodulin depression, whereas the presynaptic effect could not. Accordingly, the occlusion was stronger (although still not quite significant) when 4-AP had blocked the K current (Fig. 11C).

Altogether, then, it appeared that both components of the myomodulin inhibition of EJPs make a contribution to the inhibition of contractions but that the postsynaptic mechanism, the activation of the modulator-specific, 4-AP-sensitive K current, is more important in this respect.

Underlying these inhibitory effects, and complicating any very precise interpretation of them, is the potentiation of contractions by myomodulin. As in the single muscle fibers, we sometimes saw a net potentiation, especially with lower concentrations of MM A, in the intact muscle when we had applied 4-AP, or 4-AP and BUC A, to block a substantial part of the inhibition (e.g., Fig. 11A, bottom).

FIG. 11. Myomodulin inhibits motor neuron B15-elicited ARC muscle contraction. A: representative experiment. Top: depression of contractions by 100 nM and 1 μM MM A. Bottom: in the presence of 1 μM BUC A and 100 μM 4-AP, the MM A depression is blocked and even turned into potentiation. Two ARC muscles from the same animal. B: group data summarizing the depression of contractions by 1 μM MM A and the block of this effect by 100 μM 4-AP. Means ± SE (n indicated). C: group data summarizing mutual occlusion of the depressive effects of myomodulin and buccalin on contractions. Same approach as in the second series of EJP occlusion experiments in Figs. 6C and 7C. The depressive effect of 1 μM BUC A was measured alone and in the background presence of 10 μM MM A; the effect of 1 μM MM A was measured alone and in the presence of 10 μM BUC A, in the absence and presence of 100 μM 4-AP. (The “MM A” and “MM A, in 4-AP” data repeat, for convenience, the data in B.) Means ± SE (n indicated).
Myomodulin increases contraction latency

It seemed from the results just described that the presynaptic component of the myomodulin inhibition of EJPs had only a minor impact on contraction. However, the peak contraction amplitude is by no means the only functionally significant parameter of contraction. The slowing and delay of the build-up of the EJP waveform by myomodulin, as well as buccalin, suggested that these peptides might also significantly slow and delay the development of the contraction.

Indeed, myomodulin, as well as buccalin, had a pronounced effect of this kind on the motor neuron B15-elicited contractions. Figure 12A, top right, shows, superimposed, a control contraction (1 - control), a MM$_A$-depressed contraction (2 - MM$_A$), and the latter scaled up to the same peak amplitude as the former (2, scaled). MM$_A$, clearly increased, in this case by 34%, the latency from the beginning of the B15 burst (bar B15 below) to the rising phase of the contraction. For convenience, as indicated in the figure, we chose to measure the latency to the time at which the contraction reached its half-maximal amplitude. At the very foot of the contraction, the increase in latency was even larger.

Figure 12A shows two representative experiments, and Fig. 12, B and C, summarizes measurements of contraction latency from the same experiments as in Fig. 11, B and C. For contraction latency, the results were very different than for peak contraction amplitude. The increase in latency by MM$_A$ was not at all blocked by 4-AP (Fig. 12, A, bottom, and B). The effect of MM$_A$ occluded that of BUC$_A$, but also, conversely, the effect of BUC$_A$ occluded the effect of MM$_A$, whether 4-AP was present or not (Fig. 12C). Although, as expected, there was a general trend for larger increases in latency to be associated with larger depression of contraction amplitude, it was possible to find comparable muscles where the two effects could be somewhat dissociated. In the pair of muscles in Fig. 12A, for example, while 4-AP partially blocked the MM$_A$ depression of contraction amplitude, the increase in latency was not blocked and may even have been enhanced.

Thus while the postsynaptic component of myomodulin...
inhibition may be important for depressing peak contraction amplitude, the presynaptic mechanism, the presumed inhibition of ACh release, appears to be largely responsible for the increase in contraction latency.

**Discussion**

**Postsynaptic mechanism of inhibition: activation of the modulator-specific K current**

The ARC is a nonspiking muscle in which the EJP waveform directly translates into contraction, although in a highly nonlinear manner. Contraction typically begins only when the ACh-induced depolarization exceeds the threshold, around −40 mV, for activation of the muscle’s voltage-dependent L-type Ca current (Brezina et al. 1994c) that supplies Ca\(^{2+}\) essential for contraction (Brezina and Weiss 1993, 1995a). A significant amount of additional Ca\(^{2+}\) can enter through the ACh-receptor channels themselves (Kozak et al. 1996; Vilim et al. 1994). All this Ca\(^{2+}\) probably functions largely to trigger the release of further Ca\(^{2+}\) from intracellular stores (Brezina and Weiss 1995a,b). The likely complexity of integration of these various fluxes of Ca\(^{2+}\) as the EJP waveform develops makes it difficult, as yet, to predict quantitatively the size and shape of the contraction that will correspond to any particular EJP waveform. Qualitatively it is clear, however, that just above its threshold, the Ca current increases very steeply with depolarization, making the Ca current, Ca\(^{2+}\) influx, and contraction highly sensitive to changes in the level of depolarization.

The modulator-specific K current is a powerful depressor of the level of depolarization and hence of contraction. It too is voltage dependent, small around the muscle’s resting potential but increasing steeply at more depolarized voltages in opposition to the Ca current (Brezina et al. 1994c). This voltage profile explains why the K current depresses the EJP depolarization, and the contraction, most powerfully at its peak. The size of the current is such that even modest activation of it can depress the depolarization significantly, and its fully activated size is sufficient to explain the large depressions, of tens of millivolts in some cases, that we have observed here. The observed depression of depolarization is in turn sufficient to explain the corresponding depression of contraction (Brezina and Weiss 1993, 1995a). Thus, unambiguously in the single fibers where this mechanism operates alone but apparently even in the intact muscle (Fig. 11C, MM\(_A\) in BUC\(_A\)), the fully activated current can completely abolish contraction because it depresses the depolarization to below the threshold of the Ca current. The depression of depolarization is not only sufficient, but necessary; preliminary experiments in single fibers show that when the depression of depolarization is prevented under voltage clamp, so is the depression of contraction (Brezina et al. 1993).

There is good correlation, within the myomodulin family—the extremes being MM\(_A\) and MM\(_B\)—but also for other postsynaptic ARC-muscle modulators including the SCPs, 5-HT, and the FRF peptides, between activation of the K current and the depression of contraction. Modulators that activate large K currents depress contractions strongly; those that activate small K currents depress only weakly (Brezina et al. 1994e, 1995; Cropper et al. 1994). Finally, as we have shown here, when the K current is blocked by 4-AP, the postsynaptic component of the depression of depolarization and of contraction, in the single fibers as well as in the intact muscle, is blocked. Taken together, all this constitutes very strong evidence that the postsynaptic modulators of the ARC muscle, and the myomodulins in particular, inhibit its contractions by activating the modulator-specific K current.

**Presynaptic mechanism: inhibition of ACh release**

In the intact muscle, we have discovered a second component of myomodulin inhibition of the EJP waveform and of contraction. This component has a very different profile and properties: in particular, it is not blocked by 4-AP. It closely resembles the inhibitory effect of buccalin, and indeed the myomodulin and buccalin effects, on the EJP waveform as well as on contraction, mutually occlude, suggesting a closely converging, if not the same, mechanism of action.

Buccalin is thought to act by presynaptic inhibition of ACh release from the motor neuron terminals. What is the evidence that buccalin, and so too myomodulin, acts presynaptically? Buccalin, while depressing motor neuron-elicited contractions, fails to depress contractions elicited by direct application of ACh to the muscle (Cropper et al. 1988; Vilim et al. 1994). Along the same lines, we have here failed to observe the second component of myomodulin depression with ACh-elicited contractions of the single fibers. Buccalin has been reported to inhibit the release of radioabeled ACh from motor neuron B15 (Phares and Lloyd 1992). Buccalin acts presynaptically, probably at least in part by depressing voltage-dependent Ca currents, to inhibit ACh release at central synapses of Aplysia (Baux et al. 1993). Perhaps the strongest evidence for the presynaptic action of buccalin, however, is the fact that it also inhibits the directly measured release of the peptide co-transmitters from motor neuron B15 (Vilim et al. 1996a). We have shown the same here for myomodulin. The dual inhibition of ACh and peptide release is consistent with possible action on presynaptic Ca currents, presynaptic Ca\(^{2+}\) dynamics, or one of the other aspects of the release process that classical transmitters and peptide release share (e.g., Mansvelder and Kits 2000; Morgan and Burgoyne 1997; Verhage et al. 1994; Zucker 1996). At the very least, however, the inhibition of peptide release clearly demonstrates the presence of presynaptic buccalin and myomodulin receptors.

The presynaptic component of myomodulin inhibition, like the buccalin inhibition, has essentially the opposite voltage profile to the postsynaptic component. Rather than at the peak of the EJP waveform, the largest depression occurs during its initial build-up, which is slowed and delayed. In large part this voltage profile probably reflects simply the driving force of the ACh-activated current that generates the EJP waveform. This current is cationic but with an admixture of Cl current (Kozak et al. 1996); as a result, it decreases very rapidly with depolarization until its reversal, already around −30 or −20 mV, fixes the peak of the EJP waveform and renders it relatively insensitive to how much, or how little, ACh is being released (Vilim et al. 1994; Kozak et al. 1996).

At the level of contractions, the presynaptic action of myomodulin, and of buccalin, underlies a corresponding slowing and delaying of the contraction waveform, or an increase in contraction latency. For this, to a first approximation, the
altered shape of the EJP waveform appears a sufficient explanation. However, when the contributions of the various fluxes of Ca\(^{2+}\) are quantitatively understood, it may be found that a more subtle factor also plays a role: if some of the Ca\(^{2+}\) for contraction enters through the cationic ACh-receptor channels themselves, driving-force considerations suggest that this should make the greatest contribution, and a reduction in it may be most significant, at negative voltages during the initial build-up of the EJP waveform and of the contraction (cf. Brezina and Weiss 1995c).

**Integration of myomodulin modulation**

In addition to the two kinds of inhibition, myomodulin also potentiates contraction of the ARC muscle, postsynaptically, by enhancing the L-type Ca current in the muscle (Brezina et al. 1994d, 1995, 1996; Cropper et al. 1987b, 1991). This does not require, and is not associated with, any significant change in the depolarization of the single fibers or, presumably, in the EJP waveform in the intact muscle.

Whether there is net potentiation or inhibition of contraction is then determined by the balance of the various competing mechanisms. The observed outcome is net potentiation at low concentrations of myomodulin, from \(-1\) nM to between 100 nM and 1 \(\mu\)M, and net inhibition at higher concentrations. Nevertheless the postsynaptic inhibition, at least, makes a contribution at even the lowest concentrations, where it makes the net potentiation smaller than it otherwise would have been (Brezina et al. 1995, 1996).

At the same time as it exerts these effects on contraction amplitude and on contraction latency, myomodulin accelerates the relaxation rate of the contractions (Brezina et al. 1995, 1996; Cropper et al. 1991). Altogether, myomodulin has the most complex spectrum of effects of all the modulators that have been studied so far in the ARC or indeed other Aplysia buccal muscles. In addition to B16, myomodulin is expressed by a number of other motor neurons (Church and Lloyd 1991) that innervate other buccal muscles, some of which—notably, the I2 (Hurwitz et al. 2000), I7–I10 (Evans et al. 1999; Scott et al. 1997), and I3a (Church et al. 1993) muscles—are modulated by myomodulin with a very similar spectrum of effects as in the ARC. At the same time, the individual effects of myomodulin are shared, in various combinations, by the other modulators of the ARC (as we have demonstrated again here) and other buccal muscles (Church et al. 1993; Evans et al. 1999; Fox and Lloyd 1997, 1998, 2000; Hurwitz et al. 2000; Keating and Lloyd 1999; Lotshaw and Lloyd 1990; Scott et al. 1997). The spectrum of myomodulin effects in the ARC system thus provides a good test case to consider the broader functional significance of the complexity of such modulation.

*Functional logic of inhibitory modulation at ARC neuromuscular junctions*

Figure 13 summarizes our current knowledge, from this and previous work, of the intrinsic modulation—by modulators released from the motor neurons themselves—at the B15- and B16-ARC neuromuscular junctions. The SCPs from B15 and the myomodulins from B16 potentiate contractions postsynaptically. However, the rest of the modulation is inhibitory and largely presynaptic. The buccalins from both B15 and B16 inhibit ACh release from B15 and B16 and well as release of the peptide cotransmitters—presumably of all of the coreleased peptides, of the SCPs as well as the buccalins—from B15. We have now found that the myomodulins, in addition to inhibiting contractions postsynaptically, share all of these presynaptic inhibitory actions of the buccalins. It is not yet known whether the buccalins and myomodulins inhibit release of the peptide cotransmitters also from B16. The SCPs, in contrast, do not appear to act presynaptically (Vilim et al. 1992).

Figure 13 shows a complex network of diverging and converging effects. In our previous analysis of the postsynaptic part of the network, we discussed the likely general functions—integration and, conversely, uncoupling of effects—of such divergence and convergence and emphasized how their superimposition allows multiple input parameters to control, through such a network, multiple output parameters (Brezina and Weiss 1997a,b, 2000; Brezina et al. 1996, 2000b,c). These principles remain applicable when the network is enlarged to include the presynaptic effects. It is striking, for instance, how the pre- and postsynaptic effects of myomodulin are complementary in their location, their opposite voltage dependence, and their control of distinct contraction parameters.

The quantitative details of the presynaptic myomodulin effects, and of their interaction with the rest of the network, are not yet known. Furthermore, because of the mechanical complexity of the buccal mass and its movements in behavior (Drushel et al. 1997, 1998; Orekhova et al. 2001), it is not yet clear exactly what significance should be attributed to changes in different contraction parameters of any one muscle, such as the ARC, embedded within it (see Brezina and Weiss 2000). For these reasons, it would be premature to speculate about ultimate behavioral significance. However, several themes already emerge in Fig. 13 concerning, notably, the duality of pre- and postsynaptic control, and homo- and heterosynaptic modulation. These themes have parallels of considerable interest at central synapses (see, e.g., Bailey et al. 2000; Hawkins et al. 1993; Malenka and Nicoll 1999; Turrigiano 1999).

What is the significance of the dual pre- and postsynaptic modulation by myomodulin? Dual pre- and postsynaptic control is ubiquitous in synaptic physiology. In the ARC muscle itself, it has been suggested also for the extrinsic modulators, FMRFamide and the FRF peptides (Cropper et al. 1994). There are strong indications of it in other buccal-mass muscles of
Aplysia (Church et al. 1993; Fox and Lloyd 1997, 1998; Keating and Lloyd 1999; Lotshaw and Lloyd 1990). It occurs at many other invertebrate neuromuscular junctions (see reviews by, e.g., Calabrese 1989; Evans and Myers 1986; Harris-Warrick et al. 1989, 1998; Watson and Groome 1989; Worden 1998). Centrally, in both vertebrates and invertebrates, almost every synapse appears to be modulated presynaptically as well as postsynaptically (Nicoll et al. 1990; Clarac and Cattaert 1996; Nusbaum et al. 1997; Wu and Saggau 1997).

Suggestions as to the function of the duality are generally of the following kind: presynaptic modulation acts locally to gate and balance the individual inputs to the system, whereas postsynaptic modulation acts globally to regulate the overall output of the system.

Indeed, the postsynaptic modulation by the myomodulins from motor neuron B16, and by the SCPs from B15, appears to be global in character. The peptides’ delocalized sites and slow dynamics of release (Brezina et al. 2000a; Karhunen et al. 2001; Vilim et al. 1996b, 2000) suggest that the peptides accumulate and act rather diffusely within the muscle, which, to a first approximation, appears to be uniformly responsive. Thus in contrast to ACh, the peptides act by volume, as opposed to wiring, transmission (Zoli and Agnati 1996). More directly, Whim and Lloyd (1990) have shown that firing of motor neuron B15 can postsynaptically modulate contractions elicited by B16—even though the terminals of B15 and B16 within the muscle may be tens of micrometers apart (Vilim et al. 1996b)—and vice versa. In these experiments, Whim and Lloyd examined net potentiation and increased relaxation rate of the contractions, but it is likely that also the third postsynaptic effect of myomodulin, the activation of the modulator-specific K current, similarly provides widespread inhibition.

If the K current is indeed activated even between the more localized sites of ACh-elicited depolarization, it will sharpen and restrict the extent of the depolarization both temporally (we saw some evidence for this in Fig. 9) and spatially, within the three-dimensional electrically coupled syncytium of the muscle (see, e.g., Holman et al. 1990; Jack et al. 1983; Lotshaw and Lloyd 1990; Tomita 1970). This will make the inhibition even more powerful than would appear simply from the depression of the peak depolarization.

In contrast, the presynaptic inhibition, by the myomodulins as well as the buccalins from both B15 and B16, will presumably be most powerful homosynaptically because the peptides will be most concentrated near the terminals that released them. The presynaptic inhibition thus appears well suited to provide, individually for each motor neuron, homosynaptic feedback control of release of the kind that has been suggested at many synapses (Starke et al. 1989; Kalsner and Westfall 1990), and perhaps, through the mutual inhibition of myomodulin, buccalin, and ACh release, a dynamic control of the balance between the cholinergic and peptidergic “channels” of neuromuscular transmission (Vilim 1993; Vilim et al. 1996a). A presynaptic locus of action, interacting with intrinsic components of plasticity such as facilitation, also provides opportunities for temporal filtering of the input to the muscle (see, e.g., Fortune and Rose 2001; O’Donovan and Rinzel 1997), signs of which we have found here.

However, there is also evidence of presynaptically mediated heterosynaptic interaction between the motor neurons B15 and B16. We have shown here that myomodulin, released from B16, can act presynaptically also on B15. Furthermore, Vilim (1993) has reported that, in a presynaptic analogue of the experiments of Whim and Lloyd (1990) mentioned in the preceding text, firing of B16—through release of the buccalins or, it now appears, myomodulins—inhibited release of the peptide cotransmitters from B15. Thus the presynaptic inhibitory actions may also regulate the overall level of input to the muscle and distribute it dynamically between B15 and B16. Such coordination of inputs might, indeed, be required for optimal control of the muscle (Brezina et al. 2000b,c).

Our previous postsynaptic analysis showed that it is the asymmetry between B15 and B16, due to the fact that the myomodulins, but not the SCPs, activate the K current in the muscle, that allows the two motor neurons to combinatorially control multiple parameters of muscle contraction (Brezina et al. 1996). The presynaptic asymmetry, due to the fact that the myomodulins, but apparently not the SCPs, are presynaptic inhibitors, is likely to have a similar significance.

Thus the motor neurons B15 and B16 shape the contractions of the ARC muscle in very different ways by means of their different peptide modulators. Here we have substantially extended our knowledge of the actions of a key modulator, myomodulin. This will permit, in future work, a more complete functional analysis of the surprisingly complex modulatory network controlling this model neuromuscular system.

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