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Cholinergic Modulation of the Locomotor Network in the Lamprey Spinal Cord

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Abstract

Acetylcholine (ACh) was found here to be a strong modulator of swimming activity in the isolated spinal cord preparation of the adult lamprey (*Ichthyomyzon unicuspis*). During fictive swimming induced with either d-glutamate or *N*-methyl-d-aspartate, addition of ACh (200 μM) significantly reduced the cycle period of ventral root bursts to 54%, intersegmental phase lag to 32%, and ventral root burst proportion to 80% of control levels. Effects of ACh were apparent at concentrations as low as 1 μM. Both nicotinic and muscarinic receptors are involved, in that application of either nicotinic or muscarinic agonists alone significantly reduced cycle period. There is sufficient endogenous ACh in the spinal cord to modulate ongoing fictive swimming, as shown by application of the cholinesterase inhibitor eserine (physostigmine). Eserine (20 µM) significantly reduced the cycle period to 78% and phase lag to 58% of control levels, and these effects were reversed with the addition of cholinergic blockers. Addition of only a nicotinic or muscarinic antagonist, mecamylamine (10 µM) or scopolamine (20 µM), respectively, to the spinal cord during fictive swimming produced significant increases in cycle period and phase lag, suggesting that both types of cholinergic receptors participate in endogenous cholinergic modulation. It is concluded that ACh is an endogenous modulator of the locomotor network in the lamprey spinal cord and that ACh may take part in the regulation of cycle period, intersegmental coupling, and ventral root burst duration.

INTRODUCTION

Neuronal networks are under the influence of both extrinsic and intrinsic neuromodulators that provide a mechanism for tuning and adapting networks to changing conditions (Marder and Thirumalai 2002). Although invertebrate nervous systems have provided the best preparations for understanding neuromodulation at the cellular, synaptic, and network levels, the nervous system of the lamprey, a lower vertebrate fish, is an advantageous model for vertebrates. The isolated lamprey spinal cord expresses the neuronal correlate of swimming (fictive swimming), and many of the cellular, synaptic, and network mechanisms of the locomotor network have been described (Buchanan 2001; Grillner 2003). A variety of endogenous neuromodulators of locomotion have been investigated in the lamprey spinal cord including serotonin (Harris-Warrick and Cohen 1985; Wallén et al. 1989), dopamine (McPherson and Kemnitz 1994; Svensson et al. 2003), substance P (Parker and Grillner 1998; Parker et al. 1998), GABA (Tegner et al. 1993), and neurotensin (Barthe and Grillner 1995). We report here the effects of acetylcholine on fictive swimming.

Acetylcholine (ACh) has been implicated in locomotor network function in many vertebrate preparations. In the embryonic *Xenopus* spinal cord, ACh can change the frequency of occurrence of swim episodes by muscarinic receptors (Panchin et al. 1991). In addition, collateral motoneuron axons from *Xenopus* motoneurons provide nicotinic excitatory postsynaptic potentials (EPSPs) on other motoneurons and locomotor interneurons in a positive feedback mechanism that may help sustain the swim episode. However, this feedback is not necessary for rhythm generation because the rhythm continues in the presence of a nicotinic antagonist (Perrins and Roberts 1994, 1995a–c; Zhao and Roberts 1998). In the isolated spinal cord of the mudpuppy, application of ACh, acting by muscarinic receptors, disrupts locomotor activity (Fok and Stein 2002). In turtles (Guertin and Hounsgaard 1999) and in the neonatal rat spinal cord (Cowley and Schmidt 1994; Kiehn et al. 1996), ACh can induce or facilitate the induction of rhythmic activity in motoneurons. In the decerebrate cat, cholinergic commissural spinal neurons located in the intermediate gray of the lumbar spinal cord are rhythmically active during fictive locomotion (Huang et al. 2000).

In the lamprey, cholinergic neurons have been mapped in the brain using choline acetyltransferase (ChAT) immunohistochemistry (LeRay et al. 2003; Pombal et al. 2001). In addition to somatic motoneurons, ChAT immunoreactivity reveals numerous groups of interneurons. One such group is located near the mesencephalic locomotor region (MLR), and it has been proposed that these cholinergic neurons provide a major output of the MLR to the reticular formation (LeRay et al. 2003). In support of this proposal, stimulation of the MLR produces excitatory nicotinic synaptic potentials on reticulospinal neurons, and activation of reticulospinal neurons with ACh can induce locomotor activity (LeRay et al. 2003). Less is known about cholinergic actions in the lamprey spinal cord. Spinal motoneurons stain for acetylcholinesterase (Wachtler 1974) and ChAT immunohistochemistry (Pombal et al. 2001), and there are some spinal neurons that stained positive for ChAT yet have a morphology and localization that does not match that of the motoneurons (Pombal et al. 2001; Wallén et al. 1985). As in *Xenopus*, locomotor activity in the isolated lamprey spinal cord is not blocked by application of cholinergic antagonists (Grillner et al. 1981).

In the present study we have examined the effects of ACh on locomotor activity induced in the isolated lamprey spinal cordby d-glutamate or *N*-methyl-d-aspartate (NMDA). We have found powerful modulatory effects of ACh on cycle period and intersegmental phase lag acting by both nicotinic and muscarinic receptors. In addition, there is sufficient endogenous ACh within the isolated spinal cord to provide an ongoing modulation of the lamprey locomotor network.

METHODS

Seventy-eight adult silver lampreys (*Ichthyomyzon unicuspis*) between 18 and 38 cm in length were used for these experiments. Before use, the animals were kept in aerated and filtered fresh water aquaria at 5°C. The experiments were conducted in conformity to the American Physiological Society's *Guiding Principles in the Care and Use of Animals* and were approved by the Marquette University Institutional Animal Care and Use Committee. The animals were anesthetized by immersion in a solution of tricaine methane sulfonate (~250 mg/l) until responses to tail pinch were lost. The spinal cord/notochord preparation was dissected as previously described (Rovainen 1974) using the midbody region from the caudal gills to the beginning of the first dorsal fin. Care was taken to remove muscle fibers from the notochord by initially stripping or cutting the bulk of the muscle from the notochord and then by scraping the surface of the notochord with the tips of microscissors. Two types of preparations were used for the experiments (Fig. 1): *1*) short spinal cord preparations (7–20 segments) with fictive locomotion induced by NMDA and *2*) long spinal cord preparations (30–40 segments) with fictive locomotion induced by d-glutamate. Ringers solution contained (in mM): 91 NaCl, 2.6 $CaCl_2$, 2.1 KCl, 1.8 $MgCl_2$, 4.0 glucose, 20 $NaHCO_3$, 8 HEPES (free acid), 2 HEPES (sodium salt) (pH = 7.4). Ringers solution in the experimental chamber was kept cooled (7–10°C). When not in use, the tissue was stored in Ringers at 4°C and was used within 3 days of dissection. All chemicals were obtained from Sigma-Aldrich.

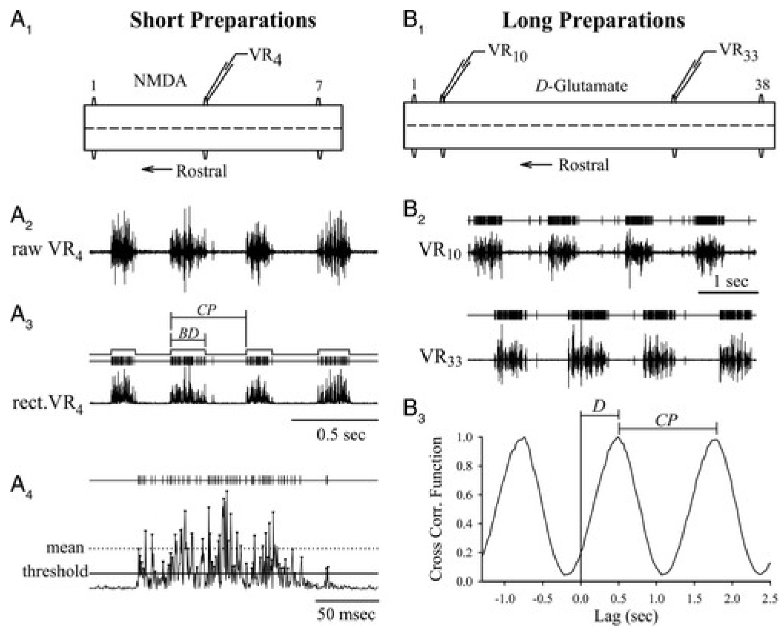

FIG. 1. Experimental setup and data analysis. *A*$_1$: short spinal cord preparations consisted of 7–20 segment lengths of isolated spinal cord perfused with 0.15–0.2 mM *N*-methyl-d-aspartate (NMDA) in Ringers. *A*$_2$: sample of rhythmic bursting recorded with a glass suction electrode on a ventral root. *A*$_3$: ventral root activity was digitally rectified and spike event times detected by voltage increasing through a threshold level. Time between onsets of consecutive bursts was the cycle period (CP) and the time from burst onset to offset was the burst duration (BD). *A*$_4$: burst intensity was defined as the product of the mean instantaneous spike frequency within the burst and the mean amplitude of the detected peaks. Peaks were detected as the highest point between the time of voltage increasing through a threshold and then decreasing below the threshold level. *B*$_1$: long spinal cord preparations consisted of 30–40 segment lengths of isolated spinal cord perfused with 0.5 mM d-glutamate. *B*$_2$: bursting activity recorded in 2 ipsilateral ventral roots separated by 23 segments. *B*$_3$: cross-correlograms were made between the event times of the 2 ventral root recordings in 10- to 20-s windows. Time between adjacent correlogram peaks is the cycle period (CP) and the delay (D) of the main peak from zero lag was used to determine the phase lag as described in methods.

Short spinal cord preparations

In the short spinal cord/notochord preparations (7–20 segments), the notochord was cut down its ventral midline to optimize visibility, spread laterally, and pinned to the Sylgard-lined (Dow Corning) floor of the experimental chamber. One or 2 ventral root recordings were made from the middle region of the spinal cord piece using glass suction electrodes (Fig. 1*A*$_1$). To induce fictive swimming, the bath was perfused with Ringers solution containing NMDA (0.15–0.2 mM). After the swim rhythm had stabilized (∼1 h after start of perfusion), the perfusion of the bath was stopped for 5 to 10 min, and the experiments were then performed using the nonperfused, static bath. After obtaining control recordings, ACh was applied to the bath by adding a small volume (0.001 × the bath volume) of Ringers containing ACh at a concentration 1,000 × the final desired concentration (1–1,000 µM). The bath was then rapidly stirred by drawing the fluid of the experimental chamber into a syringe and then expelling the volume back into the chamber. This stirring was accomplished in 5–10 s, thus providing a rapid concentration step compared with the time required for adding ACh through the perfusion system. The slower perfusion application produced less robust and consistent effects because of desensitization. Stirring the bath without adding ACh produced no significant change in the swim cycle period (Fig. 2).

In these preparations, nicotine was applied in the same manner as ACh because it showed desensitization similar to that of ACh. Mecamylamine, dihydro-β-erythroidine hydrobromide, scopolamine, and eserine (physostigmine) were applied by bath perfusion (1–4 ml/min). Oxotremorine was applied either to the static bath or by perfusion.

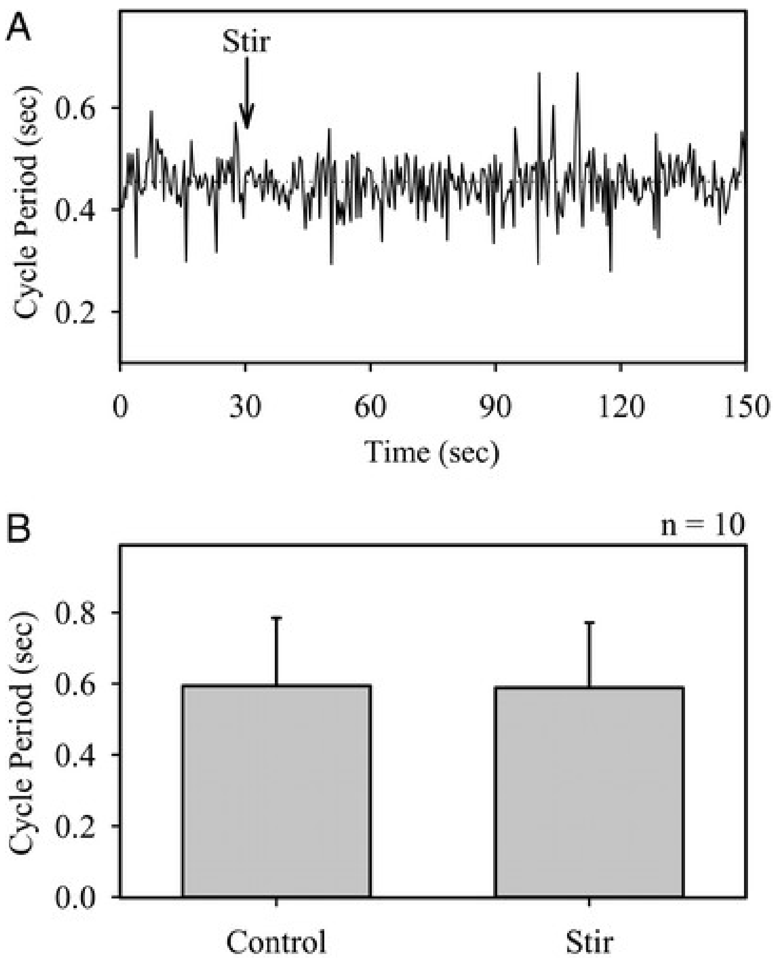

FIG. 2. In NMDA-induced fictive swimming, stirring the static bath does not significantly alter the cycle period. *A*: cycle-by-cycle plot of cycle period with the time of stirring indicated by the arrow. Dotted line indicates prestir mean of cycle period. *B*: summary of 10 preparations showing mean and SD of cycle period before and after stirring. There was no significant change in the cycle period ($P = 0.56$).

Long spinal cord preparations

Long spinal cord preparations (30–40 segments) were used to make accurate measurements of phase lag and to assess endogenous ACh modulation of the swim rhythm. In these preparations, continuous perfusion (2–3 ml/min) of d-glutamate (0.5 mM) was used to induce fictive locomotion because d-glutamate gave more regular rhythms in long spinal cord preparations than NMDA. The long spinal cord/notochord preparation was pinned to the Sylgard chamber floor. The notochord was not split in these preparations as a matter of convenience because of the long lengths used. Ventral root recordings (2–4 roots) were made from ventral roots on one side of the spinal cord (Fig. 1B_1). As described above for the short preparations, a small volume of ACh at a high concentration was added to the bath followed by rapid stirring to achieve the final desired concentration. In the long preparations, however, bath perfusion was continuous and on addition of ACh the perfusion was switched immediately to d-glutamate Ringers containing the same final ACh concentration. In contrast to the NMDA experiments, stirring of the bath in the absence of ACh when d-glutamate was used as the excitatory amino acid agonist did produce a significant decrease in swim cycle period but no effect on phase lag (Fig. 9, *C* and *D*). However, this decrease was smaller and of shorter duration than that produced by the addition of ACh. Other cholinergic drugs in the long preparation were applied by perfusion (eserine, mecamylamine, scopolamine, and atropine).

Recording techniques

Ventral root recordings were made with glass suction electrodes filled with Ringers solution. The tip of the electrode was placed on a ventral root near its exit point from the spinal cord. The signals were amplified (10,000×) and filtered (10 Hz high-pass; 1 kHz low-pass) with an AC differential amplifier (A-M Systems). The signals were digitized (2 kHz) using a micro1401 computer interface and Spike2 data acquisition software (Cambridge Electronic Design, CED) and stored on disk for later analysis.

Data analysis

Data analysis was done using Spike2 software (CED). Two types of data analysis were used: *1*) cycle-by-cycle analysis for the short spinal cord preparations (Fig. 1A_3) and *2*) cross-correlational analysis for the long spinal cord preparations (Fig. 1B_3). For cycle-by-cycle analysis, the ventral root bursts were digitally rectified, and ventral root spikes detected by thresholding (Fig. 1, A_3 and A_4). Thresholds were set above the noise level of the ventral root recording, and were kept constant throughout each experiment. Event times of the spikes were then used to mark the onsets and offsets of ventral root bursts using a custom script in Spike2 software. The marked onsets and offsets of each burst were then checked manually for accuracy. The cycle period was measured as the time interval from the onset of one burst to the onset of the subsequent burst (Fig. 1A_3). Burst duration was the time from burst onset to offset and was normalized to its cycle period to obtain burst proportion. Burst intensity was defined as the product of mean instantaneous spike frequency within the burst and the mean amplitude of the peaks of the rectified spikes (Fig. 1A_4). The peaks were detected as the highest data point between the time when voltage ascended through a threshold level until it descended back through the threshold level. Beginning at the maximal effect, ≥30 successive cycles of swimming were used to provide means and SDs of cycle period, burst proportion, and burst intensity under each condition.

The long spinal cord preparations were used to measure the effect of ACh on cycle period and phase lag. For these measurements, Spike2 (CED) software was used to create cross-correlograms of spike event times between 2 ventral roots separated by 15 to 25 segments (Fig. 1, B_2 and B_3). Windows of data 10 to 20 s in duration were analyzed to produce each cross-correlogram. The cross-correlograms provided data on both cycle period and the rostral–caudal delay between bursts in 2 ipsilateral ventral roots (Fig. 1B_3). For phase lag, the delay between bursts was normalized to the cycle period and to one segment (by dividing delay by the number of segments between the ventral root recordings) and the result expressed as a percentage.

Statistical analysis

SigmaStat software (SPSS) was used for statistical analysis. Mean values of cycle period before, during, and after ACh application were analyzed for significance using a Student's paired *t*-test. *P* values of 0.05 and under were considered significant, and data were checked for normality ($P > 0.01$) and equal variance. To analyze the effects of increasing ACh concentrations on cycle period (Fig. 3*D*), a one-way ANOVA using Tukey's procedure was used with a *P* value of 0.05 to determine significance. Mean percentage change in the cycle period and phase lag calculated in Fig. 11, B_3 and B_4 and 11A_3 also used ANOVA with Tukey's procedure. Because the data of percentage change in phase lag for Fig. 11A_4 did not pass the normality test for Tukey's procedure, a Student–Newman–Keuls method ANOVA was used to determine significance.

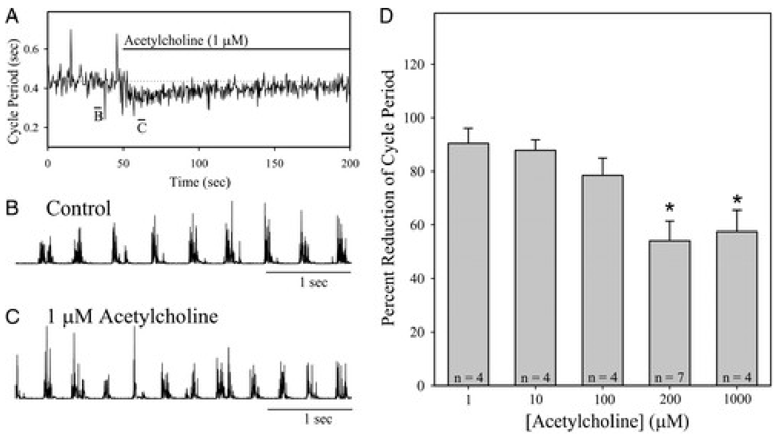

FIG. 3. Acetylcholine (ACh) produces a dose-dependent decrease in fictive swimming cycle period. *A*: when stirred into the bath during fictive swimming, 1 µM ACh decreased cycle period as shown in the cycle-by-cycle plot of cycle period. Dotted line represents the mean of the control cycle period. *B*: sample of rectified ventral root bursting before ACh was added in region indicated in *A*. *C*: sample of bursting during ACh application in region indicated in *A* showing shortened cycle periods. *D*: summary of results of addition of various concentrations of ACh in different preparations. Using one-way ANOVA, concentrations of 200 and 1,000 µM ACh were found to elicit significant decreases in the cycle period ($P < 0.001$).

RESULTS

Short spinal cord preparations

Our initial experiments applied ACh by bath perfusion (data not shown). Although the results of these first experiments were consistent with those described here, the effects were weaker and more variable because of strong desensitization. Therefore a more rapid application technique for ACh was used as described in methods. Control experiments of stirring the bath during NMDA-induced fictive swimming in the absence of added ACh were done in short spinal cord preparations. An example experiment (Fig. 2*A*) showed little or no change in mean cycle period after the stir. Combined results from 10 preparations (Fig. 2*B*) showed no significant change in mean cycle period ($P = 0.56$).

When the bath was stirred after the addition of ACh, there was a significant reduction in the cycle period. An example experiment is shown in Fig. 3, *A*–*C*. As can be seen in the cycle-by-cycle plot of the cycle period in Fig. 3*A*, addition of 1 µM ACh to the bath induced a small but clear reduction in the cycle period (to about 85% of control). The peak reduction in cycle period occurred about 20 s after the stir, and there was a slow return toward control levels over several minutes, which may have been a result of the ACh breakdown by cholinesterases as well as receptor desensitization. Desensitization could also be demonstrated by a diminished response to a second application of ACh within the next 2 h, although the swim parameters returned to normal within the next hour. Therefore to determine the dose responsiveness of the effects of ACh on cycle period, each ACh concentration was applied to a previously unexposed spinal cord preparation in each experiment. As shown in Fig. 3*D*, maximal effects on cycle period occurred with 200 µM (cycle period reduced to 54% of control).

To examine the effects of ACh on other swim parameters, 200 µM ACh was applied, and an example experiment is shown in Fig. 4. The cycle-by-cycle plots of swim parameters are shown in Fig. 4*A*, and samples of rectified ventral root recordings are shown in Fig. 4, *B*–*D*. With the addition of 200 µM ACh, the cycle period decreased to half of its control level, from 0.6 to 0.3 s. In the continued presence of ACh, the cycle period returned toward the control level, during which ACh's effect diminished to half-maximal in about 60 s. The swim rhythm also began to undergo a slow modulation with a period of about 10 to 20 s, marked by asterisks in Fig. 4*A*. A sample of raw data undergoing slow modulation is shown in Fig. 4*D*. Induction of slow modulation of the rhythm by ACh was observed in about half of the preparations. With the addition of ACh, burst proportion and burst intensity showed increased variation (Fig. 4*A*), and the mean burst proportion decreased slightly, whereas the mean burst intensity showed little or no change. A summary of 7 experiments in which 200 µM ACh was applied is shown in Fig. 5. Acetylcholine significantly reduced the cycle period to an average of 54% of control ($P < 0.001$; Fig. 5A_1) and burst proportion to an average of 80% of control ($P = 0.044$; Fig. 5B_1), whereas burst intensity did not show a significant change (Fig. 5C_1). The effects of ACh on cycle period and burst proportion generally returned to control levels after washing for 1 h. To quantify the increased variability of the swim parameters, the coefficient of variation was measured for 1 min after the peak ACh effect, and the results are summarized in Fig. 5, A_2, B_2, and C_2. There were significant increases in the coefficient of variation for all 3 parameters.

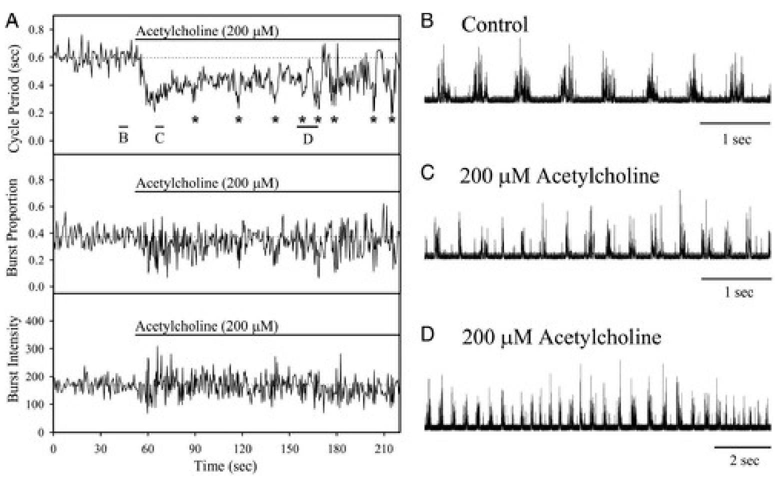

FIG. 4. Example of the effects of acetylcholine on fictive swim parameters. *A*: cycle-by-cycle plot of cycle period (*top*), burst proportion (*middle*), and burst intensity (*bottom*) with the addition of 200 µM ACh to the bath during fictive swimming. Dotted lines indicate the pre-ACh means. Asterisks mark the troughs of a slow modulation of the cycle period. *B*: sample of ventral root activity before ACh in region indicated in *A*. *C*: sample of ventral root activity at the peak of the ACh effect in region indicated in *A*. *D*: sample of slow modulation of ventral root bursting that appeared after addition of ACh.

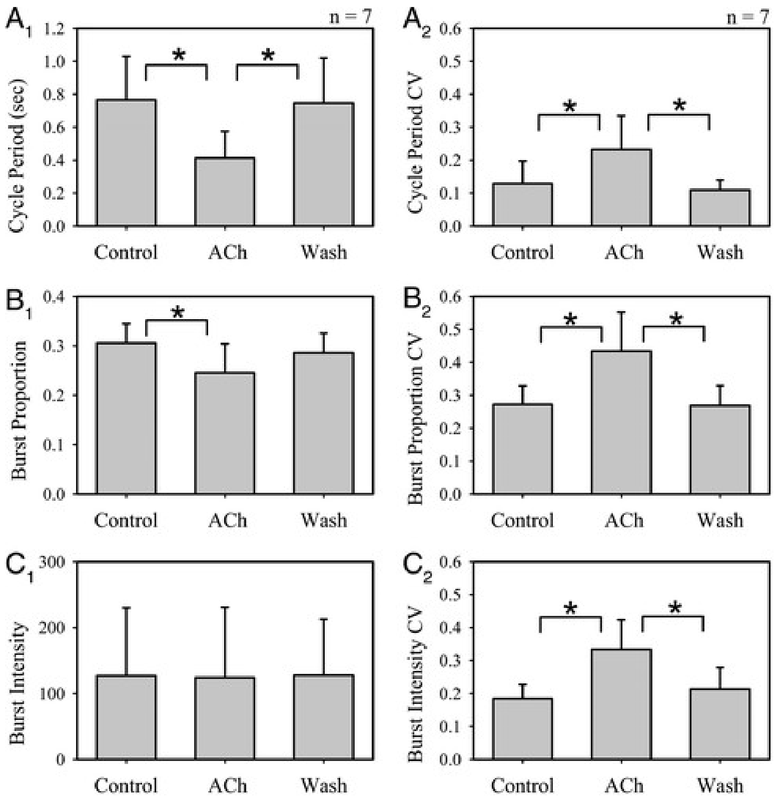

FIG. 5. Summary of changes in fictive swim parameters produced by 200 µM ACh in 7 preparations. A_1: cycle period decreased significantly to 54% of control ($P < 0.001$). A_2: variability of cycle period also increased significantly ($P = 0.004$), as indicated by the mean of the coefficient of variation (CV). B_1: burst proportion decreased significantly to 80% of control ($P = 0.044$). B_2: burst proportion variability increased significantly ($P = 0.014$). C_1: burst intensity did not change significantly with ACh ($P = 0.46$). C_2: variability of burst intensity increased significantly ($P = 0.001$). Significance was determined by paired *t*-test where $\alpha < 0.05$.

To examine the cholinergic pharmacology of the ACh effects (Figs. 6 and 7), we restricted our analysis to the cycle period because this parameter was most strongly altered by ACh. The experiments indicate that both nicotinic and muscarinic cholinergic receptors contribute to the ACh effects on the cycle period. Application of nicotine (200 µM) to the static bath, followed by stirring, produced a significant ($P = 0.03$) decrease in cycle period to 72% of control (Fig. 6*A*). This effect showed a decay similar to that of ACh, returning toward control levels soon after it was stirred into the bath. With prior application of a nicotinic antagonist, either 20 µM mecamylamine ($n = 4$) or 20 µM dihydro-β-erythroidine hydrobromide ($n = 3$), this effect was attenuated (cycle period reduced to only 92% rather than to 72% of control with nicotine addition) but the effect was still statistically significant compared with control ($P = 0.015$; Fig. 6*B*). Application of these nicotinic antagonists before 200 µM ACh application also attenuated, but did not eliminate, reduction of the cycle period produced by ACh (cycle period reduced to only 79% rather than to 54% of control with ACh application) (Fig. 6*C*), suggesting that muscarinic receptors are also involved.

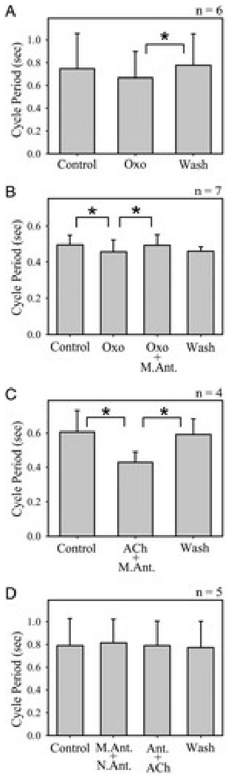

FIG. 7. Muscarinic contribution to changes in cycle period. *A*: addition of 20 µM oxotremorine, a muscarinic agonist, reduced the cycle period to 89% of control when stirred into a static bath, although the effect was not statistically significant (P = 0.099). *B*: bath perfusion of oxotremorine (20 µM) produced a significant reduction of cycle period to 92% of control (P = 0.017). This decrease was reversed with the additional perfusion of a muscarinic antagonist, scopolamine (10 µM) or atropine (2 µM) (P = 0.016). *C*: when scopolamine or atropine was bath perfused first, the addition of ACh decreased cycle period to 71% (P = 0.012) with the addition of ACh compared with a reduction to 54% of control with ACh without blocker (see Fig. 5A_1). *D*: bath perfusion of both a nicotinic and a muscarinic antagonist together prevented ACh from inducing any significant changes in cycle period (P = 0.86).

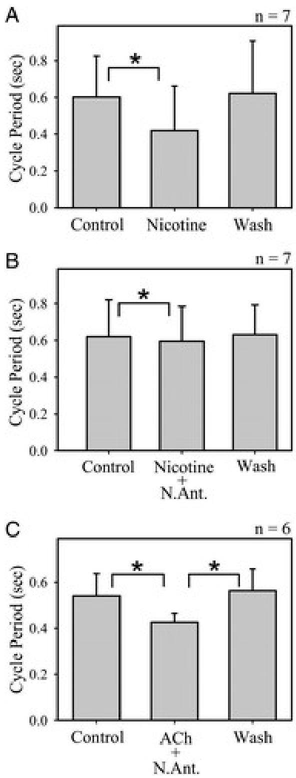

FIG. 6. Nicotinic contribution to changes in cycle period. *A*: addition of 200 μM nicotine significantly decreased cycle period to 72% of control ($P = 0.03$). *B*: prior 30-min perfusion of a nicotinic antagonist, mecamylamine (20 μM) or dihydro-β-erythroidine hydrobromide (20 μM), resulted in less nicotine-induced decrease of cycle period (to 92% of control vs. 72%), although the decrease was significant ($P = 0.015$). *C*: prior 30-min perfusion of a nicotinic antagonist resulted in an ACh-induced decrease of cycle period to 79% of control ($P = 0.019$), compared with a reduction to 54% of control with ACh alone (see Fig. 5A_1).

To test the muscarinic receptor contribution to the effects of ACh, a muscarinic agonist, oxotremorine (20 μM), was added to the static bath followed by stirring. This resulted in a small (to 89% of control) but nonsignificant reduction in the cycle period (Fig. 7*A*). Because effects induced by activation of muscarinic receptors might have longer latency than nicotinic actions and drugs were applied in the static bath for only 5 to 10 min, bath perfusion of oxotremorine was performed over a longer time period. With this method of application, oxotremorine again produced a small (to 92% of control) but, in this case, statistically significant reduction in cycle period ($P = 0.017$) after 20 min of perfusion (Fig. 7*B*). This reduction was reversed by addition of a muscarinic antagonist (10 μM scopolamine or 2 μM atropine) (Fig. 7*B*). Reduction of the cycle period by ACh was also attenuated by prior treatment with a muscarinic antagonist (cycle period reduced to 71% of control in the presence of the antagonist compared with 54% of control with the addition of just ACh) (Fig. 7*C*). Although neither nicotinic nor muscarinic antagonists alone could completely block the effect of ACh (Figs. 6*C* and 7*C*), when added together they eliminated the cholinergic reduction of cycle period (Fig. 7*D*). To look for evidence of endogenous ACh, the cholinesterase inhibitor eserine (20–100 μM) was also stirred into the static bath of short preparations, and a slight (97% of control) but nonsignificant ($P = 0.14$) decrease in the cycle period was observed ($n = 9$, data not shown). As with oxotremorine, the duration of the drug application in the static bath was likely insufficient to produce the significant changes that were seen in longer preparations with perfusion of the drugs over a longer period.

Long spinal cord preparations

To accurately determine the effect of ACh on intersegmental phase lag, longer spinal cord preparations were used (30 to 40 segments). Because NMDA did not always produce regular bursting in these preparations, d-glutamate (0.5 mM) was used to induce fictive swimming. As in the short preparations using NMDA to induce fictive swimming, rapid application of ACh in the longer d-glutamate preparations significantly decreased the cycle period. In addition, ACh produced a significant decrease in the intersegmental phase lag. These effects can be seen in an individual experiment in Fig. 8. Figure 8, *A* and *B* show the decrease in cycle period and phase lag, respectively, produced by the addition of 200 μM ACh to the bath. These decreases can also be seen in the sample ventral root records of Fig. 8, *C* and *D*. At the extreme, the phase lag in the experiment shown here became negative, indicating a reversal of the normal rostral-to-caudal propagation of the bursts. Because glutamate-induced fictive swimming was more sensitive to pauses in bath perfusion than NMDA-induced fictive swimming, perfusion of ACh–Ringers was initiated immediately after stirring the bath in the long preparations. Little desensitization, if any, is seen in the time course of the experiment shown in Fig. 8. However, in other experiments using the long preparation a decay in the response was observed, so that after 5 min of ACh exposure only half of the maximal effect remained. The longer time course of desensitization seen in long preparations could be attributable to the continued perfusion of ACh during the experiments, in contrast to the static bath experiments in which the applied ACh could be broken down locally by esterases without being replenished. Figure 9, *A* and *B* summarize the results of 12 experiments with the application of ACh to longer spinal cord preparations. Addition of ACh (200 μM) reduced the average cycle period to 45% of control ($P = 0.002$) and reduced the average phase lag to 32% of control ($P = 0.002$). Stirring the bath without the addition of ACh also significantly decreased the cycle period of fictive swimming induced with d-glutamate ($P < 0.001$; $n = 14$) but to a significantly lesser degree than with addition of ACh, to 88% of control without ACh versus to 45% of control with ACh ($P < 0.001$; Fig. 9*C*). Phase lag, however, was not significantly changed from control by stirring without addition of ACh ($P = 0.46$; Fig. 9*D*).

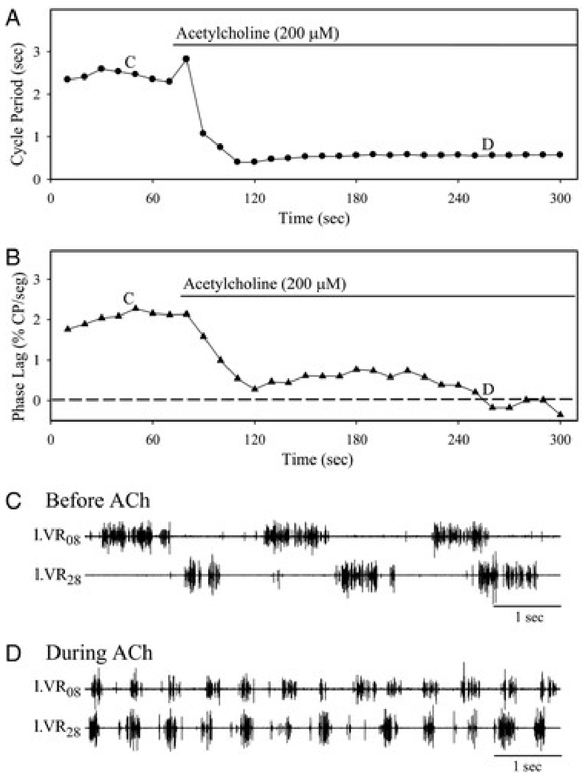

FIG. 8. Example of ACh effects on cycle period and phase lag in a long spinal cord preparation. *A*: rapid addition of ACh (200 μM) to the bath during d-glutamate–induced fictive swimming reduced cycle period from 2.4 to 0.4 s. *B*: phase lag also decreased dramatically with ACh, from 2.0 to 0.4% per segment. *C*: sample of ventral root bursting before addition of ACh. *D*: sample of ventral root bursting after addition of ACh showing that the bursting in the widely separated ventral roots became nearly synchronous.

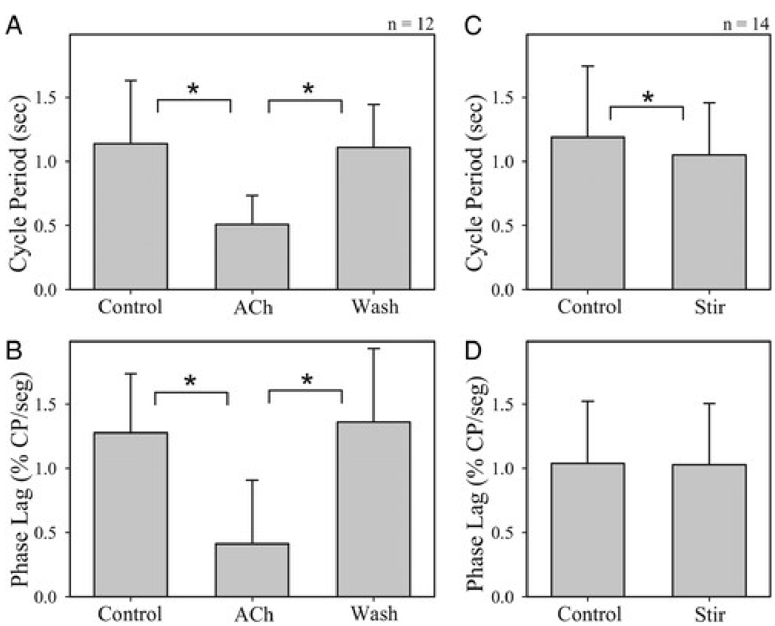

FIG. 9. Summary of effects of ACh (200 μM) in d-glutamate–induced fictive swimming in long spinal cord preparations. *A*: cycle period was reduced to 45% of control in 12 preparations ($P = 0.002$). *B*: phase lag was reduced to 32% of control ($P = 0.002$). *C*: stirring the bath of d-glutamate–induced fictive swimming resulted in a significant decrease of cycle period to 88% of control ($P < 0.001$). This is in contrast to the lack of stirring effect in NMDA-induced fictive swimming (see Fig. 2). *D*: phase lag was not changed significantly by stirring ($P = 0.46$).

Endogenous ACh modulation

Ventral root bursting during d-glutamate fictive swimming in the longer spinal cord preparations could be quite regular and thus offered the possibility to reliably measure small changes in the cycle period and phase lag. Therefore this preparation was used to determine whether there is a sufficient level of ACh within the spinal cord to produce ongoing modulation of the locomotor rhythm. If ACh is being released endogenously, then application of an acetylcholinesterase inhibitor would be expected to have similar effects as the addition of exogenous ACh. In an example experiment, when eserine (20 μM) was added to the bath perfusion, the cycle period (Fig. 10A_1) and the phase lag (Fig. 10A_2) were both clearly decreased. For all experiments (n = 7), eserine significantly decreased the cycle period to 73% of control (P = 0.007; Fig. 10A_3) and decreased the phase lag to 58% of control (P = 0.009; Fig. 10A_4). While eserine was still present, the nicotinic and muscarinic antagonists, mecamylamine (10 μM) and scopolamine (20 μM), were added together to the bath perfusion. The antagonists generally not only reversed the effects of eserine but increased both the cycle period and the phase lag beyond the control levels before adding eserine (Fig. 10, A_1–A_4). These observations suggest that in the control condition, the swim rhythm is under the influence of endogenous ACh. To test this possibility, the combined antagonists were bath perfused without the addition of eserine. As shown in an individual experiment, the combined blockers increased both the cycle period (Fig. 10B_1) and the phase lag (Fig. 10B_2). In 5 experiments, perfusion of the combined blockers significantly increased the cycle period to 119% of control (P = 0.034) and the phase lag to 143% of control (P < 0.001; Fig. 10, B_3 and B_4).

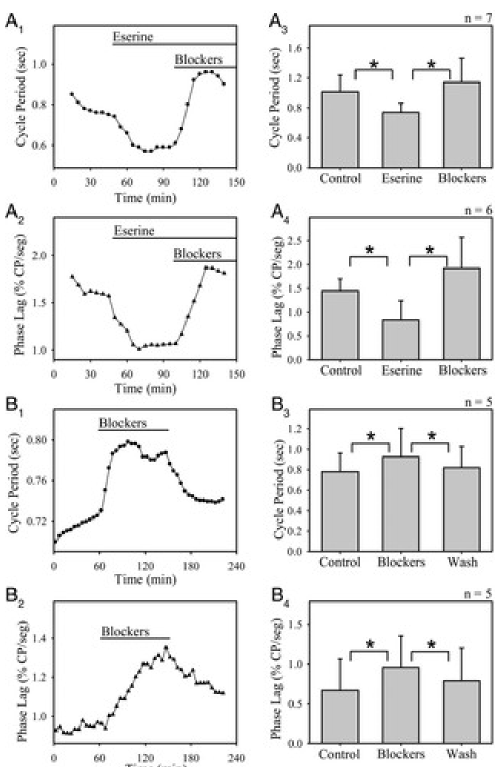

FIG. 10. Acetylcholinesterase inhibition and ACh receptor antagonism indicate endogenous release of ACh and ongoing modulation of fictive swimming. A_1: in an example experiment, the ACh esterase inhibitor eserine (20 μM) was added to the d-glutamate perfusion producing a small but clear reduction in cycle period. Addition of both nicotinic and muscarinic antagonists (10 μM mecamylamine and 20 μM scopolamine) to the perfusion increased the cycle period beyond the pre-eserine levels. A_2: same experiment shows similar effects of eserine and the blockers on phase lag. A_3: summary of the effects of eserine and combined blockers on cycle period in 7

preparations. Cycle period decreased to 73% of control with eserine (P = 0.007) and then increased to 154% of eserine levels with the addition of the blockers. A_4: summary of the effects of eserine and combined blockers on phase lag in 6 preparations. Phase lag decreased to 58% of control with eserine (P = 0.009) and then increased to 230% of the eserine level with the addition of the blockers. B_1: sample experiment showing that the addition of combined mecamylamine and scopolamine to the d-glutamate perfusion produced a small but clear increase in cycle period. B_2: same experiment shows a similar effect on phase lag. B_3: summary of the effects of combined blockers on cycle period in 5 preparations showing a significant increase to 119% of control (P = 0.034). B_4: summary of the effects of combined blockers on phase lag, showing a significant increase to 143% of control (P < 0.001).

To determine the relative contribution of nicotinic versus muscarinic receptors to the endogenous ACh effects, the cholinergic antagonists were added separately. In these experiments, eserine (20 µM) was applied first followed by increasing concentrations of one of the antagonists. An example experiment is shown for mecamylamine in Fig. 11, A_1 and A_2, in which step changes in cycle period and phase lag are discernible with the step increases in mecamylamine concentration. The summary of 6 experiments (Fig. 11, A_3 and A_4) shows that 10 µM mecamylamine significantly increased both the cycle period and the phase lag to 170 and 130% of the eserine levels, respectively. In an example experiment for scopolamine, the effects of step increases in scopolamine concentration can be seen in the cycle period and phase lag (Fig. 11, B_1 and B_2). The summary of 6 experiments (Fig. 11, B_3 and B_4) shows that at 25 µM scopolamine, both the cycle period and the phase lag increased to 144% of the eserine levels. Atropine (2 and 10 µM) produced effects similar to those of scopolamine (n = 2) (data not shown).

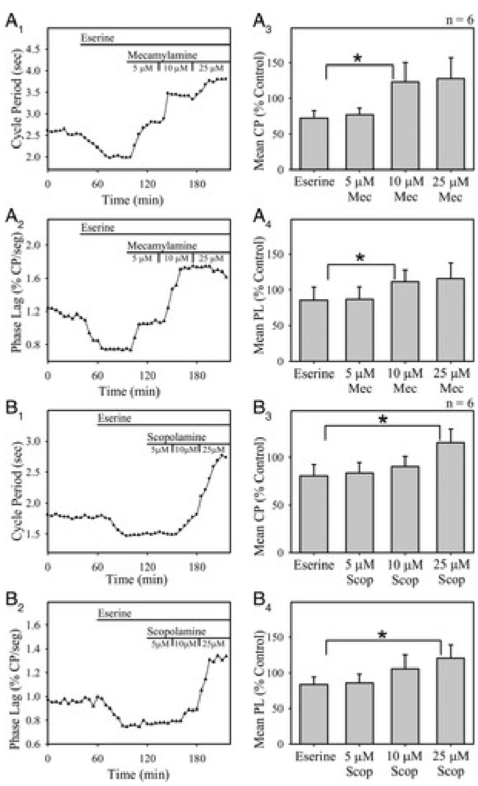

FIG. 11. Nicotinic and muscarinic contributions to endogenous ACh modulation. A_1: example experiment of adding increasing concentrations of mecamylamine after pretreatment with eserine. Each increase in mecamylamine concentration produced a clear increase in cycle period. A_2: same experiment showing the effect of mecamylamine on phase lag. A_3: summary of 6 preparations showing that 10 µM mecamylamine produced a significant increase of cycle period to 170% of eserine levels (P = 0.003). A_4: summary of 6 preparations showing

that 10 μM mecamylamine produced a significant increase of phase lag to 130% of eserine levels (P = 0.043). B_1: example experiment of adding increasing concentrations of scopolamine on cycle period after pretreatment with eserine. B_2: same experiment showing effect on phase lag. B_3: summary of 6 preparations showing that 25 μM scopolamine significantly increased cycle period to 144% of eserine levels (P < 0.001). B_4: summary of 6 preparations showing that 25 μM scopolamine significantly increased phase lag to 144% of eserine levels (P < 0.001). Significance was determined by α <0.05 using one-way ANOVA with Tukey's procedure (A_3, B_3, B_4) and SNK method (A_4).

DISCUSSION

This study has shown for the first time that ACh is a powerful modulator of the locomotor rhythm in the isolated lamprey spinal cord. Bath application of ACh produced significant reductions of cycle period, burst proportion, and phase lag. These effects were similar in short spinal cord preparations (7–20 segments) versus long spinal cord preparations (30–40 segments) and whether using NMDA or d-glutamate as the excitatory agonist to elicit fictive locomotion. The effects were mediated by both nicotinic and muscarinic receptors, although nicotinic receptors appear to be more effective. Application of cholinergic blockers without the addition of acetylcholine also produced significant changes in the cycle period and phase lag, suggesting that there is sufficient ACh within the spinal cord to provide an ongoing modulation of the locomotor network.

Possible sources of endogenous ACh

The endogenous acetylcholine producing the effects observed here could potentially originate from either motoneurons or from cholinergic interneurons. Acetylcholinesterase histochemistry (Wachtler 1974) and ChAT immunohistochemistry (Pombal et al. 2001) revealed brain and spinal neurons with morphology consistent with somatic motoneurons. Furthermore, muscle activity is blocked by the nicotinic antagonist curare (Teräväinen 1971), confirming that lamprey motoneurons are cholinergic. If endogenous ACh originates from motoneurons, 2 possible sites of origin would be the neuromuscular junction in the periphery or synaptic outputs of motoneuron axons within the spinal cord. The release of ACh from neuromuscular junctions seems unlikely as the source of endogenous ACh because muscle fibers were carefully removed from the notochord in the spinal cord/notochord preparation (see methods), and the preparation was continuously perfused with Ringers solution in the cholinergic blocker experiments (2–3 ml/min). As for output synapses of lamprey motoneuron axons within the spinal cord, collateral axon branches have been observed in lamprey (Wallén et al. 1985), and antidromic stimulation of ventral roots produces synaptic potentials within spinal neurons that are blocked by cholinergic antagonists (Buchanan 1999). Although antidromic stimulation of 1–3 ventral roots did not change the swimming rhythm (Wallén and Lansner 1984), perhaps firing of the motoneurons from only 3 ventral roots was not of sufficient strength to modify the rhythm of the entire spinal cord preparation (11–29 segments) during fictive swimming. A second possible source of endogenous ACh is cholinergic interneurons. Labeling of spinal neurons with ChAT immunohistochemistry revealed cells that by their size and location do not appear to be motoneurons (Pombal et al. 2001), although further investigation into the possible existence of nonmotoneuron cholinergic cells needs to be done. It is also possible that axons from cholinergic brain neurons could contribute to spinal ACh, given that descending cholinergic fibers have been followed into the rostral spinal cord (Pombal et al. 2001). These populations of cholinergic neurons, alone or in combination, could be the source of endogenous ACh.

Possible mechanisms of cholinergic modulation of fictive locomotion

Acetylcholine is known to have a variety of cellular and synaptic effects within the CNS. In lamprey, it is known that ACh has an excitatory effect on neurons by nicotinic receptors (LeRay et al. 2003), and may also have presynaptic inhibitory actions on EPSPs by muscarinic receptors (Quinlan and Buchanan 2003). In other preparations, both presynaptic facilitation of glutamatergic synapses by presynaptic nicotinic receptors (Girod et al. 2000) and presynaptic inhibition by muscarinic receptors (Fernandez de Sevilla and Buno 2003) have been shown. In addition, various cholinergic effects on firing properties have been described. In the turtle spinal cord, for example, ACh can regulate firing frequency by the M-current (Alaburda et al. 2002) and can up-regulate L-type calcium currents to promote plateau potentials (Perrier et al. 2000). Thus ACh could be altering locomotor networks by several possible cellular and synaptic mechanisms.

In lamprey, the locomotor network has been proposed to consist of a pair of half centers on either side of the spinal cord coupled by reciprocal inhibition (Buchanan 2001; Cangiano and Grillner 2003). An increase of the speed of the swim rhythm could be accomplished by any mechanism that reduces the duration of the activity of the half-center generators on each side. In the proposed model of the lamprey locomotor network (Buchanan and Grillner 1987), rhythmicity is generated in large part by reciprocal inhibition of commissural interneurons (CC INs) so that mechanisms that decrease the excitability of CC INs or weaken their synaptic outputs would lead to a speeding of the network (Buchanan 1992). An additional feature of the proposed model is an off-switch mechanism by which the duration of CC IN activity on one side is limited by inhibition from a class of ipsilaterally projecting inhibitory interneurons, the lateral interneurons. Intracellular recordings of CC INs during fictive swimming demonstrated that they have an earlier peak depolarization compared with that of nearby motoneurons (Buchanan and Cohen 1982) and an earlier beginning of their inhibitory phase (Kahn 1982). These 2 features are consistent with inhibition of CC INs from lateral interneurons. In addition to lateral interneurons there are small glycinergic interneurons that have local inhibitory input to motoneurons (Buchanan and Grillner 1988). These local inhibitory interneurons could be functioning as an off-switch to the half center on one side of the spinal cord. If cholinergic interneurons or cholinergic feedback from motoneurons provided part of the excitation of such off-switch cells, this could account for the shortening of cycle period and for the reduced burst proportion that is observed with the addition of ACh.

Less is known about the cellular mechanisms of intersegmental coupling underlying the delay of ventral root bursting in more caudal segments during forward swimming. Like burst duration, this delay is normally a constant fraction of the cycle period over a wide range of cycle periods during forward swimming (Wallén and Williams 1984) and is presumably regulated to maintain efficient swimming form. Acetylcholine has been shown here to modulate phase lag, and increasing the presence of ACh, either through addition of exogenous ACh or by blocking its normal breakdown with eserine, causes intersegmental phase lag to decrease from its normal value. Alteration of phase lag by ACh could be accomplished by changes in the excitability of the locomotor neurons or the coupling neurons or by changes in the synaptic strengths of the coupling signals among the rhythm generators.

The powerful modulatory effect of ACh on phase lag and the ongoing modulation of phase lag by endogenously released ACh suggest that ACh may be part of the mechanism responsible for controlling phase lag, maintaining it within certain limits, and adjusting it to varying swimming conditions found in a lamprey's natural environment, such as swimming upstream against a current versus swimming downstream with a current, as well as changing behavioral needs such as forward swimming versus backward swimming.

Previous studies of ACh actions in lamprey

As confirmed in the present study, activation of cholinergic receptors is not necessary for the expression of fictive swimming. In the isolated spinal cord preparation, swimming activity continues in the presence of combined nicotinic and muscarinic blockers (Grillner et al. 1981) and in many past experiments on lamprey the nicotinic antagonist curare was added to Ringers to block muscle contractions during in vitro experiments (Teräväinen 1971). Based on the results of the present study, one would expect that addition of curare to block muscle contractions would increase the cycle period and phase lag. In addition, ACh application alone is not capable of inducing locomotor activity (unpublished observations). At the brain stem level, however, ACh may be playing an important role in the initiation of locomotor activity. Using ChAT immunohistochemistry, cholinergic neurons have been found in the vicinity of the mesencephalic locomotor region (MLR) of lamprey (LeRay et al. 2003). In the presence of nicotinic antagonist, locomotor activity induced by MLR stimulation is greatly depressed as are the monosynaptic EPSPs produced in reticulospinal neurons by this stimulation. Local application of ACh to reticulospinal cells can induce swimming in a semi-intact preparation or fictive swimming in the isolated brain/spinal cord preparation, and it can speed ongoing swimming (LeRay et al. 2003).

Fictive swimming has been elicited in lamprey with a variety of glutamatergic agonists and in spinal cord preparations that range widely in length. In this study NMDA and d-glutamate were used in short and long preparations, respectively, because of previous observations that these combinations of agonists and spinal cord length produced the most regular fictive swimming activity (T. L. Williams, personal communication). The basic finding of a decreased cycle period of fictive swimming in both preparations and agonists indicates that the results are not dependent on these experimental differences. There were, however, some differences between the 2 preparations. With fictive swimming induced by NMDA, the swimming rhythm was not sensitive to stirring the bath, whereas with d-glutamate–induced fictive swimming, there was some sensitivity to stirring (Fig. 2*B* vs. Fig. 9*C*). The mechanism of this difference is not known but may reflect differences in agonist uptake and resulting changes in local agonist concentration. Another difference in the 2 preparations was the slower decay of the ACh effect in the long preparations. This may in part reflect the action of endogenous acetylcholine esterase. The continuous perfusion of ACh in the long preparation may serve to replenish ACh but the static bath short preparation may experience local ACh concentration decreases.

Locomotor network actions of ACh in other vertebrates

Acetylcholine is involved in locomotion in other vertebrates, although no consistent pattern of involvement has emerged. In the embryonic *Xenopus* spinal cord, ACh can change the frequency of occurrence of swim episodes by a muscarinic receptor (Panchin et al. 1991). In addition, *Xenopus* motoneuron axons provide nicotinic EPSPs on other motoneurons and locomotor interneurons in a positive feedback mechanism that may help sustain the duration of an episode of swimming. However, this feedback is not necessary for rhythm generation because the rhythm continues to be expressed in the presence of a nicotinic antagonist (Perrins and Roberts 1994, 1995a–c; Zhao and Roberts 1998). In the isolated spinal cord of the mudpuppy, walking activity induced with NMDA is disrupted by application of ACh while not changing the overall level of activity in flexor and extensor motoneurons. This action of ACh was blocked by atropine, suggesting that muscarinic receptors mediate the disruption, whereas atropine alone had no effect (Fok and Stein 2002). In turtles, rhythmic activity in motoneurons can be induced by muscarine, and this action appears to be dependent on enhancement of L-type calcium currents (Guertin and Hounsgaard 1999; Perrier et al. 2000). In an isolated preparation of the embryonic mouse spinal cord, cholinergic positive feedback from motoneurons is essential for the generation of spontaneous rhythmic activity (Hanson and Landmesser 2003). In isolated neonatal rat spinal cord, ACh application can induce or facilitate the induction of rhythmic activity in motoneurons (Cowley and Schmidt 1994; Kiehn et al. 1996). In decerebrate cat preparations, combined c-fos labeling and ChAT immunohistochemistry revealed cholinergic neurons of the intermediate gray of the lumbar spinal cord that are active during fictive locomotion. Electrophysiology demonstrated that these cells are active in phase with ipsilateral extension and that they project axons to the contralateral side of the spinal cord (Huang et al. 2000).

In conclusion, the present study demonstrated that acetylcholine provides an ongoing modulation of swimming activity in the lamprey spinal cord and that these actions are mediated by both nicotinic and muscarinic ACh receptors. Acetylcholine speeds the rhythmic output of the network and may thus contribute to the regulation of rhythm generation. Acetylcholine has other locomotor network effects as well. Normally, burst duration and burst delay between segments are maintained as constant fractions of the cycle period, but ACh alters these parameters, suggesting that ACh plays a role in the regulation or in the modification of burst proportion and phase lag as an adaptive response to changes in swimming conditions.

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FOOTNOTES

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