

RESEARCH ARTICLE

Nitric oxide modulates a swimmeret beating rhythm in the crayfish

Atsuki Mita¹, Misaki Yoshida¹ and Toshiki Nagayama^{2,*}

ABSTRACT

The modulatory effects of nitric oxide (NO) and cAMP on the rhythmic beating activity of the swimmeret motor neurones in the crayfish were examined. Swimmerets are paired appendages located on the ventral side of each abdominal segment that show rhythmic beating activity during forward swimming, postural righting behaviour and egg ventilation in gravid females. In isolated abdominal nerve cord preparations, swimmeret motor neurones are usually silent or show a continuous low-frequency spiking activity. Application of carbachol, a cholinergic agonist, elicited rhythmic bursts of motor neurone spikes. The co-application of L-arginine, the substrate for NO synthesis with carbachol increased the burst frequency of the motor neurones. The co-application of the NO donor SNAP with carbachol also increased the burst frequency of the motor neurones. By contrast, co-application of a NOS inhibitor, L-NAME, with carbachol decreased beating frequency of the motor neurones. These results indicate that NO may act as a neuromodulator to facilitate swimmeret beating activity. The facilitatory effect of L-arginine was cancelled by co-application of the soluble guanylate cyclase (sGC) inhibitor ODQ suggesting that NO acts by activating sGC to promote the production of cGMP. Application of L-arginine alone or membrane-permeable cGMP analogue 8-Br-cGMP alone did not elicit rhythmic activity of motor neurones, but co-application of 8-Br-cGMP with carbachol increased bursting frequency of the motor neurones. Furthermore, application of the membrane-permeable cAMP analogue CPT-cAMP alone produced rhythmic bursting of swimmeret motor neurones, and the bursting frequency elicited by CPT-cAMP was increased by co-application with L-arginine. Co-application of the adenylate cyclase inhibitor SQ22536 ceased rhythmic bursts of motor neurone spikes elicited by carbachol. These results suggest that a cAMP system enables the rhythmic bursts of motor neurone spikes and that a NO–cGMP signaling pathway increases cAMP activity to facilitate swimmeret beating.

KEY WORDS: Neuromodulation, Central pattern generator, cGMP signaling, cAMP signaling, L-arginine

INTRODUCTION

The free radical nitric oxide (NO) is now well known to function as a neuromodulator that affects neural circuits and neuronal activity in both vertebrate and invertebrate animals (Garthwaite et al., 1988; Wood and Garthwaite, 1994). For example, the synaptic responses of intersegmental ascending interneurons or spiking local

interneurons in the terminal abdominal ganglion of the crayfish to mechanosensory stimulation of the tail fan are enhanced or depressed by NO (Aonuma and Newland, 2001; Aonuma and Newland, 2002). Local reflexes of the tail fan motor neurones of the crayfish are also enhanced by NO (Araki et al., 2004). Furthermore, NO affects rhythmic motor activity induced by central pattern generators (CPGs). NO inhibits the swimming rhythm of *Xenopus laevis* tadpoles (McLean and Sillar, 2002), suppresses a feeding response of the pond snail (Kobayashi et al., 2000) and decreases the heartbeat frequency in the lobster (Mahadevan et al., 2004). By contrast, NO is also reported to increase respiratory rhythm of the bullfrog (Hedrick and Morales, 1999), the swimming frequency of jellyfish (Moroz et al., 2004) and the oviposition digging rhythm of locusts (Newland and Yates, 2007).

The swimmerets are paired appendages located on the ventral side of each segment of the crayfish abdomen. Four pairs of swimmerets on the 2nd to 5th abdominal segments in female crayfish and three pairs of swimmerets from the 3rd to 5th abdominal segment of male crayfish beat rhythmically to generate forward thrust through cycles of power-stroke and return-stroke movements. They show a rhythmic beating activity during forward swimming, postural-righting behaviour and egg ventilation in gravid females (e.g. Davis, 1968). The stroke of each swimmeret is controlled by antagonistic power-stroke and return-stroke motor neurones that fire in strict anti-phase (Hughes and Wiersma, 1960). Rhythmic bursts of motor neurone spikes are generated by chains of serially repeated pairs of CPGs, one in each hemiganglion, that are interconnected both bilaterally across the midline and across abdominal segments (Ikeda and Wiersma, 1964; Wiersma and Ikeda, 1964). The local pattern-generating circuits, including nonspiking local interneurons, and the pathways for intersegmental coordination have been well characterized by the detailed studies of Mulloney and colleagues (Mulloney and Hall, 2000; Mulloney and Hall, 2003; Mulloney and Hall, 2007; Mulloney et al., 2006; Paul and Mulloney, 1985; Smarandache et al., 2009; Smarandache-Wellmann et al., 2013; Smarandache-Wellmann et al., 2014; for a review, see Mulloney and Smarandache, 2010; Mulloney and Smarandache-Wellmann, 2012). There is, however, almost no evidence that the rhythmic beating activity of the swimmeret motor neurones is affected by NO. NO is synthesized from its substrate, L-arginine by the enzyme nitric oxide synthase (NOS) (Moncada et al., 1991). The most common target of NO is the enzyme-soluble guanylate cyclase (sGC) that promotes the production of cGMP. In turn, cGMP acts upon cGMP-dependent protein kinase (PKC) (Bredt and Snyder, 1989). Furthermore, the NO–cGMP signaling pathway can affect the cAMP system to form long-term memory in crickets (Matsumoto et al., 2013). By applying pharmacological agents related to NO–cGMP signaling, we show in this paper that NO modulates the rhythmic beating activity of the swimmeret motor neurones in the crayfish via an sGC–cGMP signaling pathway to affect cAMP activity.

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RESULTS

Effect of increasing NO levels on the rhythmic beating activity of the swimmeret motor neurones

The swimmeret beating rhythm is generated by chains of serially repeated pairs of CPGs, one in each hemiganglion, which are interconnected both bilaterally across the midline and across body segments. In more than 95% of the isolated abdominal nerve cord preparations, swimmeret motor neurones showed no spontaneous motor neurone activity or a continuous burst of spikes at a low frequency. When carbachol, a cholinergic agonist, of more than $5 \mu\text{mol l}^{-1}$ in concentration was applied, swimmeret motor neurones showed rhythmic bursts of motor neurone spikes immediately following carbachol application (see supplementary material Fig. S1). The rhythmic bursts of motor neurone spikes were not observed after a wash with normal saline.

To determine whether NO modulates the rhythmic beating activity of the swimmeret motor neurones, endogenous NO levels were elevated by applying L-arginine, the substrate for NO synthesis. During the first carbachol session, application of $8 \mu\text{mol l}^{-1}$ carbachol alone elicited reciprocal and anti-phase rhythmic bursts of motor neurone spikes in both the power-stroke (PS) and return-stroke (RS) motor neurones on the 5th abdominal ganglion (Fig. 1A). The cycle of the power-stroke motor neurones was about 600 ms (590.7 ± 21.5 ms, mean \pm s.e.) in this preparation. The mean cycle period for all experiments was 1089 ± 117.4 ms when $8 \mu\text{mol l}^{-1}$ carbachol was applied. During the test session with co-application of 5 mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol, the beating frequency of both the power-stroke and return-stroke motor neurones increased significantly. The cycle period shortened to 314.4 ± 20.9 ms (Fig. 1B). Following a wash in normal saline, the cycle period of swimmeret beating activity induced solely by application of $8 \mu\text{mol l}^{-1}$ carbachol during the second carbachol session returned to 425.6 ± 19.6 ms (Fig. 1C). Relative changes of cycle period of the power-stroke motor neurones from the first carbachol session are plotted in Fig. 1D from 12 crayfish. Co-application of L-arginine with carbachol significantly decreased the cycle period to 0.59 ± 0.01 that of the first and second carbachol

sessions ($P < 0.001$; paired *t*-test). When 5 mmol l^{-1} D-arginine, the isomer of L-arginine, was applied with $8 \mu\text{mol l}^{-1}$ carbachol, the cycle period of the power-stroke motor neurones was unchanged compared with the first and second carbachol sessions (Fig. 1E) (1.00 ± 0.01 in the first carbachol session, 1.00 ± 0.01 in the test session of carbachol with D-arginine, and 1.01 ± 0.01 in the second carbachol session; $n=5$). These results indicated that an elevation of the endogenous NO levels increased rhythmic beating activity of the swimmeret motor neurones. The sole application of 5 mmol l^{-1} L-arginine dissolved in physiological saline, however, elicited no rhythmic bursts of motor neurone spikes (not shown), which suggests L-arginine itself could not elicit rhythmic bursts of motor neurone spikes.

The NO donor, SNAP, increased exogenous levels of NO within the abdominal ganglia. During the first carbachol session without co-application of SNAP, the cycle period of swimmeret beating activity was 1545.4 ± 82.1 ms (Fig. 2A). During the test session, co-application of $400 \mu\text{mol l}^{-1}$ SNAP with carbachol shortened the cycle period to 1061.8 ± 39.5 ms (Fig. 2B). After washing, the cycle period at the second carbachol session was 1282.3 ± 52.9 ms (Fig. 2C). Relative changes in the cycle period from the first carbachol session were plotted in Fig. 2D from 12 crayfish. Application of SNAP significantly decreased the cycle period to 0.67 ± 0.05 that was significantly shorter than that of the first (1.00 ± 0.00) and the second (0.89 ± 0.06) carbachol sessions without co-application of SNAP ($P < 0.001$ from the first session and $P = 0.01$ from the second session; paired *t*-test).

Effects of reduction of NO levels on the rhythmic beating activity of the swimmeret motor neurones

To reduce the availability of endogenous NO, a NOS inhibitor, L-NAME (Schuppe et al., 2002) was applied with carbachol. Before application of L-NAME during the first carbachol session, the cycle period of swimmeret beating activity was 867.2 ± 23.7 ms (Fig. 3A). When carbachol was applied with 5 mmol l^{-1} L-NAME during the test session, the cycle period increased to 1026.7 ± 23.7 ms (Fig. 3B). After washing, the cycle period during the second carbachol session

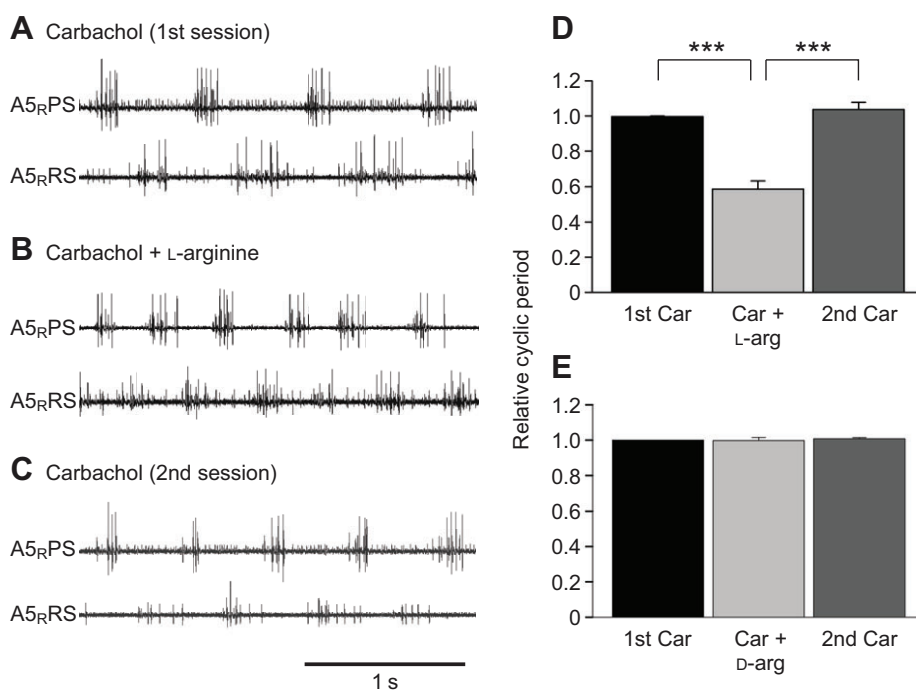


Fig. 1. Effect of L-arginine on the rhythmic beating activity of swimmeret motor neurones. (A) The first carbachol session. Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited the anti-phase rhythmic beating activity between the power-stroke (PS) and the return-stroke (RS) motor neurones in the 4th abdominal ganglion. (B) The test session. The burst frequency of the motor neurones increased by co-application of 5 mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol. (C) The second carbachol session. The burst frequency of the motor neurones returned to control level during the second application of $8 \mu\text{mol l}^{-1}$ carbachol alone. (D) Relative change in cycle period of rhythmic bursts of PS motor neurone spikes before and after L-arginine application. The cycle period was expressed as the rate of the initial averaged cycle period of the first carbachol session. Application of L-arginine caused a statistically significant shortening of the cycle period ($***P < 0.001$; paired *t*-test, from 12 crayfish). (E) Relative change in cycle period of rhythmic bursts of power-stroke motor neurone spikes before and after D-arginine application. Application of 5 mmol l^{-1} D-arginine ($n=5$) had no effect on cycle period. Results are means \pm s.e.m. Car, carbachol.

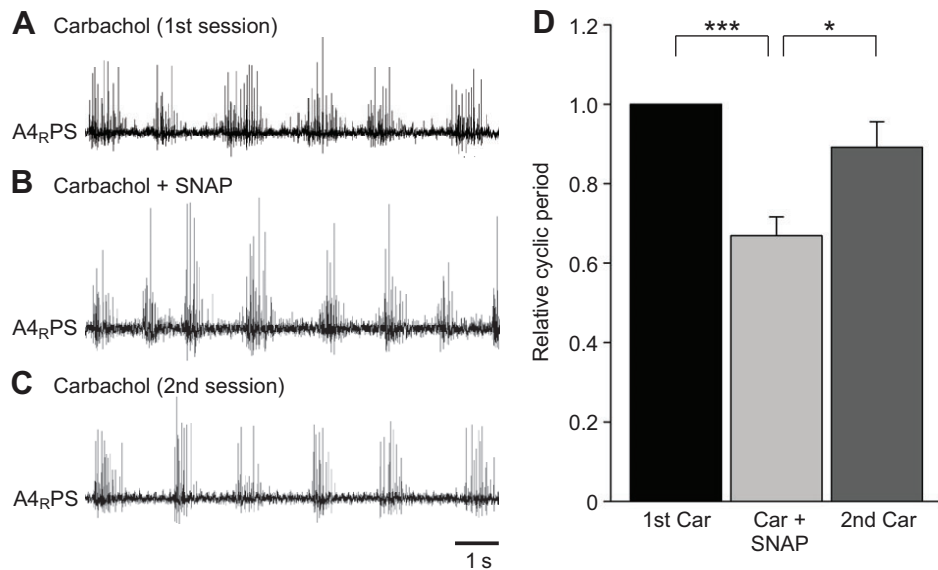


Fig. 2. Effect of SNAP on the rhythmic beating activity of swimmeret motor neurones. (A) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited the rhythmic beating activity of the PS motor neurones in the 4th abdominal ganglion. (B) The burst frequency of the motor neurones increased during co-application of $400 \mu\text{mol l}^{-1}$ SNAP with $8 \mu\text{mol l}^{-1}$ carbachol. (C) The burst frequency of the motor neurones returned to control level during the second application of $8 \mu\text{mol l}^{-1}$ carbachol alone. (D) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after SNAP application. The cycle period was expressed as the rate of the initial averaged cycle period of the first carbachol session. Application of SNAP caused a statistically significant shortening of the cycle period ($***P<0.001$ from the first carbachol session and $**P=0.01$ from the second carbachol session; paired *t*-test, from 12 crayfish). Results are means \pm s.e.m. Car, carbachol.

was 829.0 ± 8.5 ms (Fig. 3C). Relative changes in cycle period from the first carbachol session are plotted in Fig. 3D from 10 crayfish. Application of L-NAME increased the cycle period to 1.33 ± 0.04 so that it was significantly longer than that of the first two carbachol sessions without co-application of L-NAME ($P<0.001$ from the first session; paired *t*-test, and $P<0.05$ from the second session; Wilcoxon signed rank test). By contrast, when 5 mmol l^{-1} D-NAME, an isomer of L-NAME, was applied with $8 \mu\text{mol l}^{-1}$ carbachol ($n=5$), the cycle period (1.00 ± 0.01) of the power-stroke motor neurones was similar to that of the first two carbachol sessions (Fig. 3E). These results suggested that NO affects rhythmic beating activity of swimmeret motor neurones under normal physiological conditions.

L-arginine increases NO levels

Schuppe et al. (Schuppe et al., 2002) show using 4,5-diaminofluorescein imaging that application of L-arginine increases the synthesis of NO in the terminal abdominal ganglion of the

crayfish. To confirm physiologically that L-arginine generated NO, the following two experiments were done (Fig. 4). As shown in Fig. 1, co-application of 5 mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol increased the burst frequency of the power-stroke motor neurones (cf. Fig. 4A,B). The cycle period was 625.6 ± 19.3 ms for the sole application of carbachol (Fig. 4A) and shortened to 266.0 ± 13.8 ms for the co-application of L-arginine with carbachol (Fig. 4B). The cycle period returned to control levels (520.3 ± 21.3 ms) when carbachol was applied with both 5 mmol l^{-1} L-arginine and $400 \mu\text{mol l}^{-1}$ SNAP (Fig. 4C). The facilitatory effect of L-arginine also decreased when NO scavenger, PTIO, was applied simultaneously with L-arginine (Fig. 4D–G). The cycle period was about 1.4 s for the sole application of $8 \mu\text{mol l}^{-1}$ carbachol (Fig. 4D) and shortened to 1.1 s for co-application of 5 mmol l^{-1} L-arginine with carbachol (Fig. 4E). The cycle period returned to control levels when $50 \mu\text{mol l}^{-1}$ PTIO was applied with L-arginine and carbachol (Fig. 4F). Relative changes in cycle period from the carbachol

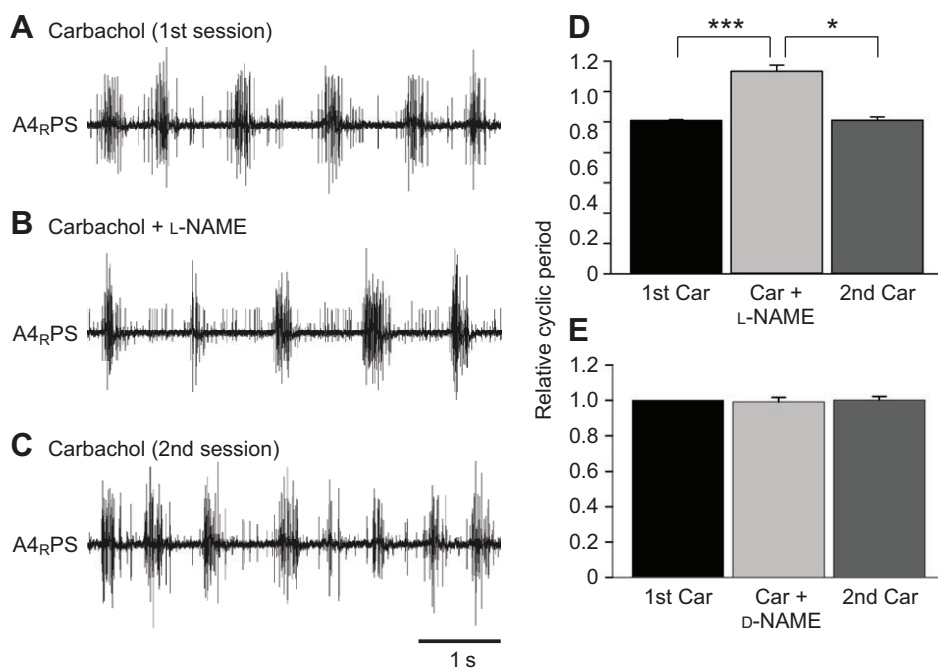


Fig. 3. Effect of L-NAME on the rhythmic beating activity of swimmeret motor neurones. (A) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited rhythmic beating activity in the PS motor neurones in the 4th abdominal ganglion. (B) The burst frequency of the motor neurones decreased by co-application of 5 mmol l^{-1} L-NAME with $8 \mu\text{mol l}^{-1}$ carbachol. (C) The burst frequency of the motor neurones returned to control level during the second application of $8 \mu\text{mol l}^{-1}$ carbachol alone. (D) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after L-NAME application. The cycle period was expressed as the rate of the initial averaged cycle period of the first carbachol session. Application of L-NAME caused a statistically significant lengthening of the cycle period ($***P<0.001$ from the first carbachol session; paired *t*-test and $*P<0.05$ from the second carbachol session; Wilcoxon signed rank test, from 10 crayfish). (E) Relative change in cycle period of rhythmic bursts of PS motor neurone spikes before and after D-NAME application. Application of 5 mmol l^{-1} D-NAME ($n=5$) had no effect on cycle period. Results are means \pm s.e.m. Car, carbachol.

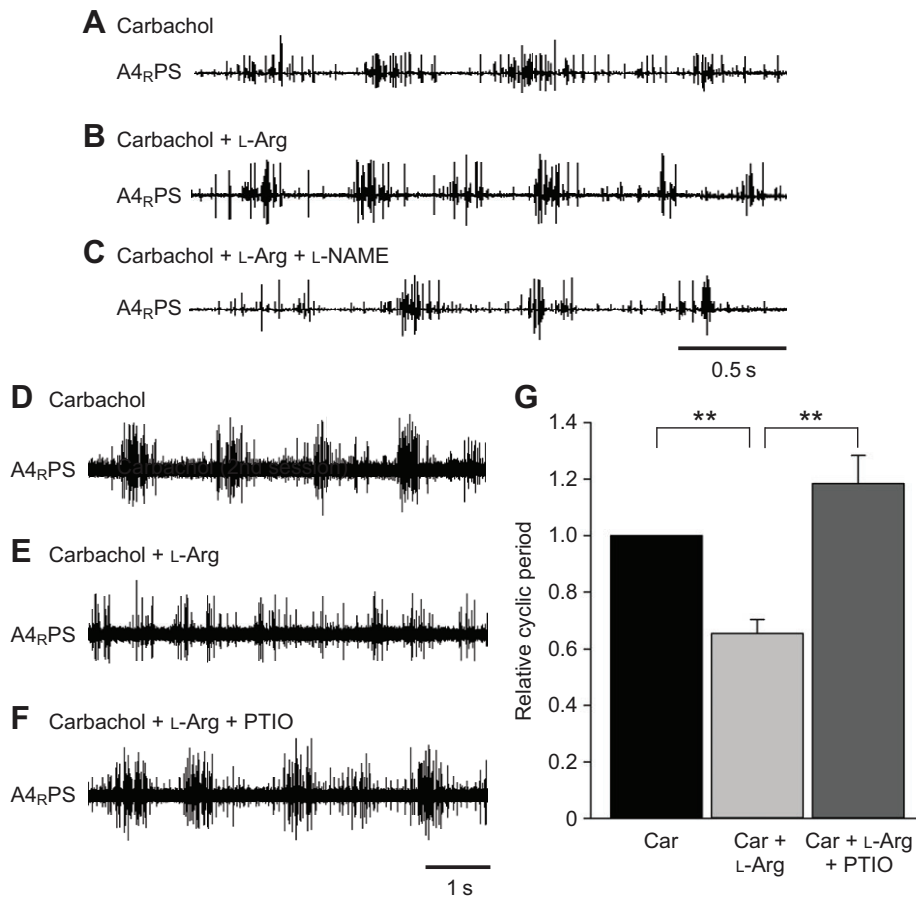


Fig. 4. Effect of L-arginine is blocked by co-application of L-NAME and PTIO.

(A) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited rhythmic beating activity in the PS motor neurones in the 4th abdominal ganglion. (B) The burst frequency of the motor neurones increased by co-application of 5mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol. (C) Increase in the burst frequency of the motor neurones was blocked under co-application of 5mmol l^{-1} L-NAME with 5mmol l^{-1} L-arginine. (D) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited rhythmic beating activity in the PS motor neurones in the 4th abdominal ganglion. (E) The burst frequency of the motor neurones increased by co-application of 5mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol. (F) Increase in the burst frequency of the motor neurones was blocked upon co-application of $50 \mu\text{mol l}^{-1}$ PTIO with 5mmol l^{-1} L-arginine. (G) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after PTIO application. The cycle period was expressed as the rate of the initial averaged cycle period of the carbachol session. Co-application of L-arginine without PTIO caused a statistically significant shortening of the cycle period (** $P < 0.01$; paired *t*-test, means \pm s.e.m. from five crayfish). Co-application of L-arginine with PTIO caused a statistically significant lengthening of the cycle period compared with that of the carbachol and L-arginine session (* $P < 0.01$; paired *t*-test, from five crayfish) as in the carbachol session ($P = 0.138$; paired *t*-test).

session were plotted in Fig. 4G from five crayfish. Application of L-arginine without PTIO significantly decreased the cycle period to 0.65 ± 0.05 compared with the sole application of carbachol. The cycle period returned to control levels, 1.18 ± 0.10 , when PTIO was applied with L-arginine and carbachol ($P = 0.138$ against the carbachol session; paired *t*-test). There was a significant difference between L-arginine without PTIO and L-arginine with PTIO ($P = 0.007$; paired *t*-test). These results strongly suggest that application of L-arginine generates NO.

NO affects sGC–cGMP signaling pathway

Soluble guanylate cyclase (sGC) is reported to be the main molecular target of NO (Bredt and Snyder, 1989) and promotes the synthesis of the second messenger cGMP. Thus, we examined the effect of cGMP on the rhythmic beating activity of the swimmeret motor neurones by application of a membrane-permeable cGMP analogue, 8-Br-cGMP at $100 \mu\text{mol l}^{-1}$ concentration. During the first carbachol session, before co-application with 8-Br-cGMP, the cycle period was 989.2 ± 6.9 ms (Fig. 5A). Co-application of $100 \mu\text{mol l}^{-1}$ 8-Br-cGMP with carbachol shortened the cycle period to 764.2 ± 5.0 ms (Fig. 5B). After washing, the cycle period of swimmeret beating during the second carbachol session was 776.0 ± 5.7 ms, partially returning to the initial control level of the first session (Fig. 5C). Relative changes in cycle period from the first session are plotted in Fig. 5D from six crayfish. Application of 8-Br-cGMP significantly decreased the cycle period to 0.63 ± 0.11 from the first carbachol session ($P < 0.05$; paired *t*-test). These results suggest that NO mediates its effect via cGMP. After washing, however, the effect of 8-Br-cGMP still partly remained and the cyclic period was 0.80 ± 0.08 . There was no statistical difference in

relative cycle period between the test session with 8-Br-cGMP application and the second carbachol session without 8-Br-cGMP ($P = 0.063$; Wilcoxon signed rank test).

ODQ is a specific inhibitor of sGC. To examine whether NO affected the cyclic period of the rhythmic bursts of motor neurone spikes via a sGC–cGMP pathway, a series of experiments in which 5mmol l^{-1} L-arginine was applied with and without $1 \mu\text{mol l}^{-1}$ ODQ was carried out. During the first control session, application of $8 \mu\text{mol l}^{-1}$ carbachol alone elicited rhythmic beating activity of the power-stroke motor neurones in which the cycle period was 968.2 ± 15.8 ms (Fig. 6A). After co-application of carbachol with 5mmol l^{-1} L-arginine, the cycle period shortened to 699.5 ± 11.1 ms (Fig. 6B). Following a wash with normal saline, carbachol was applied with both 5mmol l^{-1} L-arginine and $1 \mu\text{mol l}^{-1}$ ODQ. In this session, the cycle period increased to 726.7 ± 9.5 ms (Fig. 6C). During the next session, co-application of carbachol and 5mmol l^{-1} L-arginine without ODQ caused rhythmic bursts of spikes in which the cycle period shortened to 616.8 ± 13.2 ms (Fig. 6D). During the last session, application of $8 \mu\text{mol l}^{-1}$ carbachol alone lengthened the cycle period to 816.2 ± 9.6 ms (not shown). Relative changes in cycle period of L-arginine application without and with $1 \mu\text{mol l}^{-1}$ ODQ are plotted in Fig. 6E from six crayfish. Application of L-arginine without ODQ significantly decreased the cycle period to 0.81 ± 0.04 from the first carbachol session ($P < 0.01$; paired *t*-test). The cycle period returned to control levels, 1.04 ± 0.17 , when ODQ was applied with L-arginine and carbachol ($P = 0.688$ against the first carbachol session; paired *t*-test). There was a significant difference between L-arginine without ODQ and L-arginine with ODQ ($P = 0.031$; paired *t*-test). These results suggest that NO activates sGC to promote the production of cGMP.

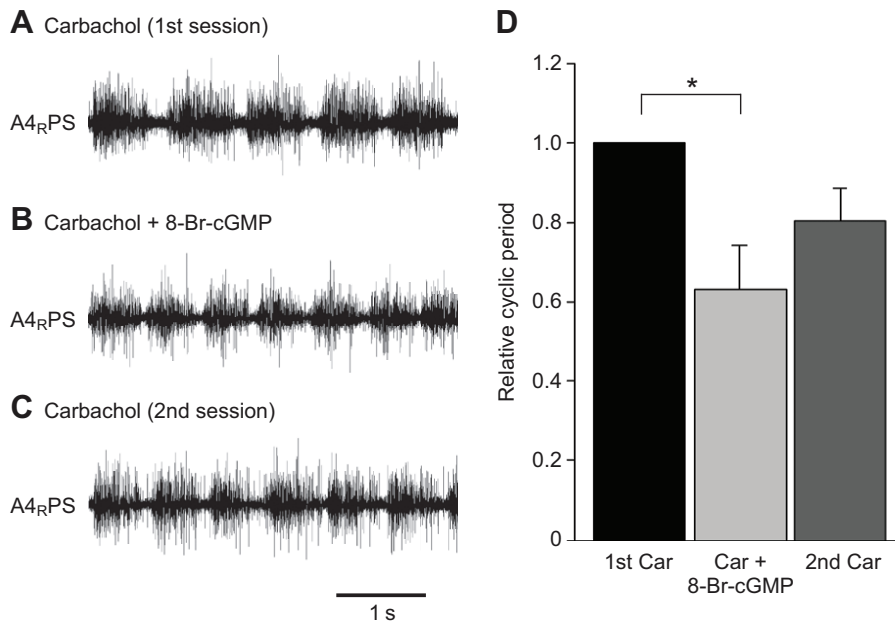


Fig. 5. Effect of 8-Br-cGMP on the rhythmic beating activity of swimmeret motor neurones. (A) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited rhythmic beating activity in the PS motor neurones in the 4th abdominal ganglion. (B) The burst frequency of the motor neurones increased during co-application of $100 \mu\text{mol l}^{-1}$ 8-Br-cGMP with $8 \mu\text{mol l}^{-1}$ carbachol. (C) The burst frequency of the motor neurones gradually returned to the control level during the second application of $8 \mu\text{mol l}^{-1}$ carbachol alone. (D) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after 8-Br-cGMP application. The cycle period was expressed as the rate of the initial averaged cycle period of the first carbachol session. Application of 8-Br-cGMP caused a statistically significant shortening of the cycle period ($***P < 0.05$ from the first carbachol session; paired t -test, from six crayfish). Results are means \pm s.e.m. Car, carbachol.

sGC-cGMP signaling pathway affected cAMP activation

Although carbachol is a cholinergic agonist that activates both nicotinic ion channels and muscarinic receptors that activate a second messenger signaling pathway, the sole application of $100 \mu\text{mol l}^{-1}$ 8-Br-cGMP dissolved in physiological saline elicited no rhythmic bursting activity of the swimmeret motor neurones (not shown). The effect of cAMP, a possible second messenger, on rhythmic bursting of the motor neurones was, therefore, examined. Before application of the mixture of $100 \mu\text{mol l}^{-1}$ pCPT-cAMP and $100 \mu\text{mol l}^{-1}$ IBMX, no rhythmic bursts of spikes were observed in

the motor neurones (Fig. 7A). Application of pCPT-cAMP and IBMX elicited rhythmic bursts of motor neurone spikes in which the cycle period was 907.7 ± 14.8 ms (Fig. 7B). Sole application of IBMX did not generate any rhythmic bursts (not shown). Co-application of 5 mmol l^{-1} L-arginine with pCPT-cAMP and IBMX decreased the cycle period to 567.0 ± 3.1 ms (Fig. 7C). Relative changes of cycle period of the power-stroke motor neurones are plotted in Fig. 6D from three crayfish. Co-application of pCPT-cAMP and IBMX with L-arginine significantly decreased the cycle period to 0.46 ± 0.09 , significantly shorter than the period with pCPT-

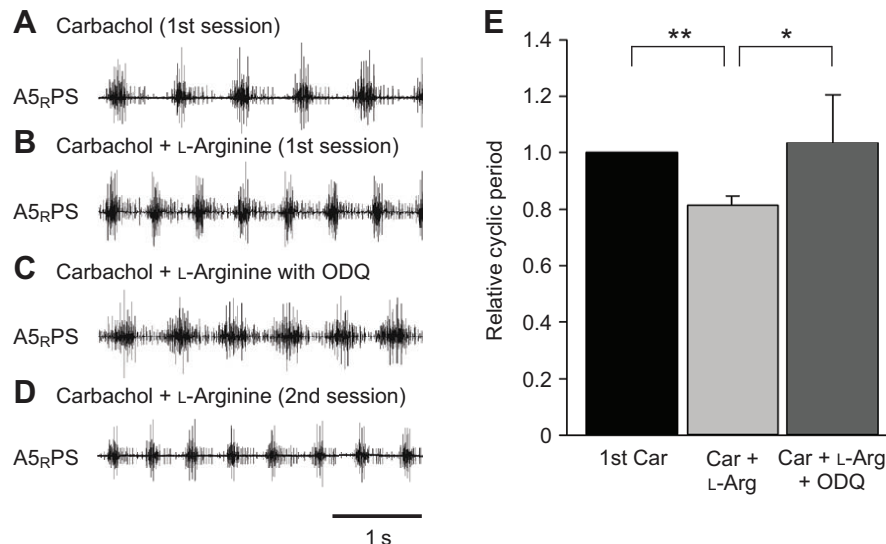


Fig. 6. Effect of ODQ on the rhythmic beating activity of swimmeret motor neurones. (A) Application of $8 \mu\text{mol l}^{-1}$ carbachol elicited rhythmic beating activity in the PS motor neurones in the 5th abdominal ganglion. (B) The burst frequency of the motor neurones increased during co-application of 5 mmol l^{-1} L-arginine with $8 \mu\text{mol l}^{-1}$ carbachol. (C) The burst frequency of the motor neurones returned to that of the first carbachol session during the application of $8 \mu\text{mol l}^{-1}$ carbachol with both 5 mmol l^{-1} L-arginine and $1 \mu\text{mol l}^{-1}$ ODQ. (D) The second carbachol and L-arginine session. The burst frequency of the motor neurones increased again during co-application of $8 \mu\text{mol l}^{-1}$ carbachol and 5 mmol l^{-1} L-arginine without $1 \mu\text{mol l}^{-1}$ ODQ. (E) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after ODQ application. The cycle period was expressed as the rate of the initial averaged cycle period of the carbachol session. Co-application of L-arginine without ODQ (first carbachol and L-arginine session) caused a statistically significant shortening of the cycle period ($**P < 0.01$; paired t -test, means \pm s.e.m. from six crayfish). Co-application of L-arginine with ODQ caused a statistically significant lengthening of the cycle period from that of the first carbachol and L-arginine session ($*P < 0.05$; paired t -test, from six crayfish) as in the carbachol session ($P = 0.688$; paired t -test).

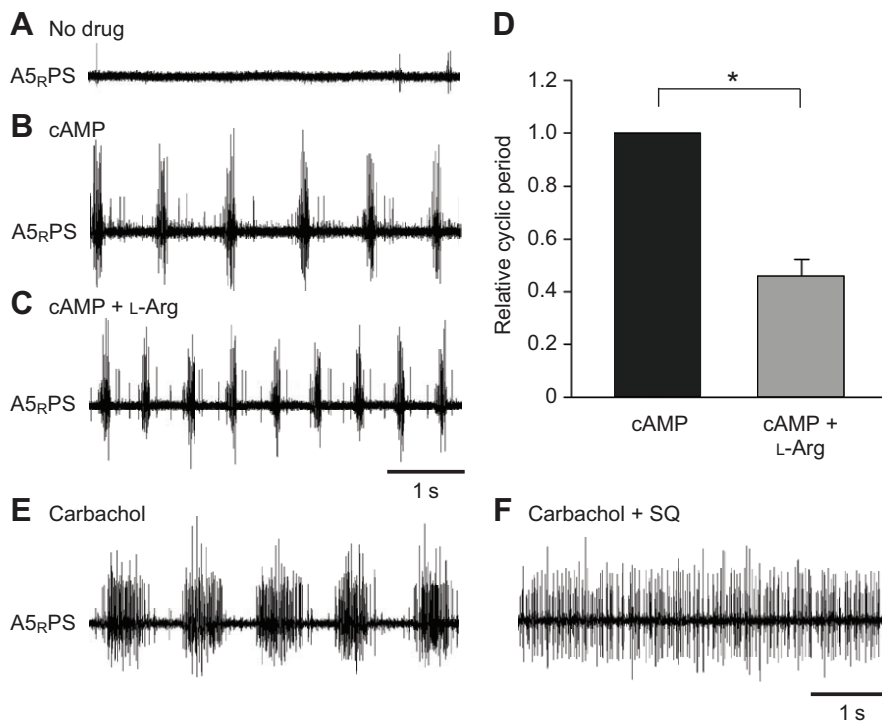


Fig. 7. cAMP affects rhythmic beating activity of the swimmeret motor neurones. (A–C) Effect of cAMP on the rhythmic beating activity of swimmeret motor neurones. (A) No rhythmic beating activity was observed in the PS motor neurones in the 5th abdominal ganglion before pCPT-cAMP application. (B) Application of the mixture of 100 $\mu\text{mol l}^{-1}$ pCPT-cAMP and 100 $\mu\text{mol l}^{-1}$ IBMX elicited rhythmic beating activity of the motor neurones. (C) The burst frequency of the motor neurones increased during co-application of 5 mmol l^{-1} L-arginine with pCPT-cAMP and IBMX. (D) Relative change in cycle period of rhythmic bursts of motor neurone spikes before and after L-arginine application. The cycle period was expressed as the rate of the initial averaged cycle period of the pCPT-cAMP session. Application of L-arginine caused a statistically significant shortening of the cycle period ($*P < 0.05$; paired *t*-test, from three crayfish). Results are means \pm s.e.m. (E, F) Effect of adenylate cyclase inhibitor on rhythmic beating activity of the swimmeret motor neurones. Carbachol-induced rhythmic beating activity of spikes (E) was stopped during co-application of 100 $\mu\text{mol l}^{-1}$ SQ22536 with 8 $\mu\text{mol l}^{-1}$ carbachol (F). Recordings of A–C were from one crayfish and those of E–F were from another crayfish.

cAMP and IBMX alone ($P < 0.05$; paired *t*-test). Thus, NO facilitates the cAMP-elicited beating of the motor neurones. Furthermore, rhythmic bursts of motor neurone spikes elicited by 8 $\mu\text{mol l}^{-1}$ carbachol (Fig. 7E) became unclear and the motor neurones fired continuously during the co-application of 100 $\mu\text{mol l}^{-1}$ SQ22536, an adenylate cyclase inhibitor, with carbachol (Fig. 7F). We examined this series of experiments in three crayfish and found similar effects of SQ22536 that ceased rhythmic bursts of motor neurone spikes elicited by carbachol. These results suggest that cAMP contributes to generation of the rhythmic bursting of the swimmeret motor neurones and that NO affects cAMP activity to facilitate bursting via the sGC–cGMP signaling pathway.

DISCUSSION

Endogenous NO levels contribute to swimmeret beating rhythm

Upregulating exogenous NO levels by applying the NO donor SNAP led to an increase in rhythmic beating activity of the swimmeret motor neurones. Furthermore, upregulating endogenous NO levels by applying the substrate for NO synthesis, L-arginine had a similar facilitatory effect on beating frequency. In contrast, the inactive isomer, D-arginine had no effect. The duration of the cycle period elicited by application of carbachol was significantly shortened to less than 70% by co-application with SNAP and/or L-arginine. In CPGs of some arthropods and molluscs, NO elicited a rhythmic motor pattern from silent preparations (Elphick et al., 1995; Rast, 2001). For example, application of L-arginine or the NO donors hydroxylamine and SNP to preparations without any spontaneous gastric mill neurone activity in the stomatogastric ganglion generates gastric mill rhythms in the crab (Stein et al., 2005). Although we did not examine NO donor application in this study, the NO level would certainly increase upon application of L-arginine because the increase in the burst frequency of the swimmeret motor neurones was blocked with co-application of the NOS inhibitor L-NAME or the NO scavenger PTIO, with L-arginine. Furthermore, Schuppe et

al. (Schuppe et al., 2002) show that levels of NO increase with application of L-arginine in the abdominal ganglia of the crayfish using 4,5-diaminofluorescein imaging. Because application of L-arginine alone failed to generate any rhythmic beating activity of the swimmeret motor neurones in this study, NO does not appear to act as neurotransmitter to trigger rhythmic bursts of motor neurone spikes, but instead contributes as neuromodulator to regulate swimmeret beating activity.

Decreasing NO levels with the NOS inhibitor, L-NAME reduced rhythmic beating activity of the swimmeret motor neurones. The cycle period elicited by carbachol was significantly extended by co-application with L-NAME. Furthermore, the cycle period elicited by carbachol was not affected by co-application with D-NAME. These results strongly suggest that the application of carbachol, in part, increased endogenous NO levels that contributed to rhythmic beating activity of the swimmeret motor neurones under normal physiological conditions. NO is synthesized from L-arginine in a Ca^{2+} /calmodulin-dependent process by the enzyme NOS, in a reaction requiring oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) (Moncada et al., 1991). NADPH-diaphorase (NADPH-d) histochemistry has been widely adopted to detect NOS-containing neurones because neural NOS and NADPH-d activity reside in the same molecule (Dawson et al., 1991; Norris et al., 1995). In crustaceans, including crayfish, NOS-containing neurones have been revealed throughout the central nervous system (Johansson and Carlberg, 1994; Talavera et al., 1995; Schuppe et al., 2001a; Schuppe et al., 2001b; Ott et al., 2007). These findings suggest that NO is released from certain neurones that contribute to the swimmeret CPG network and modulates rhythmic beating activity of swimmeret motor neurones. NADPH-d-positive branches are extended within the lateral neuropils from the 2nd to 5th abdominal ganglion where dendrites of swimmeret motor neurones and relevant interneurones also projected (Mulloney and Hall, 2000) (H. Aonuma, personal communication). Further histochemical and immunocytochemical studies are needed to identify NOS-containing neurones within swimmeret CPGs.

cGMP is a target of NO

In this study, application of the membrane-permeable cGMP analogue 8-Br-cGMP increased the frequency of beating in the swimmeret motor neurones. The sGC inhibitor ODQ reversed the facilitatory effects of L-arginine. These results indicate that NO activates sGC and promotes the production of cGMP. Aonuma (Aonuma, 2002) showed the distribution of NO-induced cGMP-like immunoreactive neurones in the abdominal nervous system. Some axons projecting from nerve 1 of the abdominal ganglia are immunopositive (H. Aonuma, personal communication). An NO–cGMP pathway has been widely reported in many sensory and motor systems in both vertebrates and invertebrates (Bawin et al., 1994; Bicker and Schmachtenberg, 1997; Araki et al., 2004; Moroz et al., 2004; Newland and Yates, 2007). Newland and Yates (Newland and Yates, 2007) show that bath application of the generic protein kinase inhibitor H-7 and a selective cGMP-dependent protein kinase (PKG) inhibitor KT-5823 reduced the frequency of the oviposition digging rhythm of the locust. NO plays a key role in regulating the frequency of the CPG by acting via sGC/cGMP-PKG. PKG affects the opening and closing of potassium channels (Bredt and Snyder, 1989), which might regulate the beating frequency of the swimmeret motor neurones.

Our results indicate that cGMP does not itself generate rhythmic activity in motor neurones, but increased cAMP alone does generate rhythmic bursting activity of motor neurones in otherwise silent preparations. Furthermore, the rhythmic bursts of motor neurone spikes elicited by carbachol ceased during co-application of the adenylate cyclase inhibitor, SQ22536. These results suggest that activation of cAMP can generate rhythmic beating activity of the swimmeret motor neurones. Braun and Mulloney (Braun and Mulloney, 1993) show that muscarinic agonists of acetylcholine induce the swimmeret motor pattern but nicotinic agonists do not. Nicotine increases the burst frequency in active preparations that show rhythmic bursts of motor neurone spikes. Because muscarinic receptors belong to a superfamily of G-protein-coupled receptors (Collin et al., 2013), carbachol could induce rhythmic bursts of motor neurone spikes via the activation of cAMP. The finding in this study that the burst frequency of the swimmeret motor neurones elicited by cAMP was facilitated by co-application of L-arginine suggests that the NO–cGMP signaling pathway affects cAMP activity. In long-term memory formation of cricket olfactory learning, the NO–cGMP system activates the cAMP system through activation of the cyclic nucleotide-gated channel and the calcium–calmodulin system (Matsumoto et al., 2006; Matsumoto et al., 2009). In honeybees, cGMP and cAMP converge on protein kinase A to form long-term memory (Lebouille and Müller, 2004). Further studies to clarify the relationship between cGMP and cAMP systems could provide a detailed understanding of the cellular and molecular modulatory mechanisms of the NO–cGMP signaling pathway.

Possible function of NO in swimmeret beating

Swimmerets show active rhythmic beating for forward swimming, righting postural control and egg ventilation in gravid females. Activity in the swimmeret system must coordinate with other motor systems generating abdominal posture and walking (Mulloney and Smarandache-Wellmann, 2012). For example, if the abdomen of a crayfish is flexed, swimmeret beating would be prevented because a curled abdomen blocks the power strokes. The walking and swimmeret systems are usually active simultaneously. NO and other neuromodulators would, therefore, contribute to the coordination of multiple motor systems depending on the behavioural context.

Biogenic amines are known to modulate motor outputs of crayfish (e.g. Momohara et al., 2013). For example, octopamine effectively inhibits the expression of swimmeret motor activity (Mulloney et al., 1987). Bath application of serotonin also terminates rhythmic beating activity of the swimmeret motor neurones induced by carbachol (T.N., unpublished data). By contrast, dopamine is known to generate the expression of the swimmeret motor pattern from silent preparations in the lobster (Barthe et al., 1989).

Thus, we characterize the modulatory effect of NO in facilitating the rhythmic beating of swimmeret motor neurones. At present, the location of NO-containing neurones is unclear, as are the targets of NO in the swimmeret central pattern-generating circuits. Further physiological and morphological analyses are needed to clarify the functional role of NO in the crayfish swimmeret system.

MATERIALS AND METHODS

Specimen preparation

Experiments were performed on female crayfish *Procambarus clarkii* Girard 1852 of 6–9 cm body length from rostrum to telson, at room temperature (24–25°C). Crayfish were obtained locally from a commercial supplier (Okayama, Japan), maintained in freshwater laboratory tanks, and fed weekly on a diet of chopped potato and liver.

The abdominal nerve chain from the 2nd to 6th (terminal) abdominal ganglion with relevant nerve roots was isolated from the abdomen and pinned, dorsal side up, to the base of a 4 ml Sylgard-lined chamber containing physiological saline (van Harreveld, 1936). The dorsal ganglionic sheaths from the 2nd to the 5th abdominal ganglion were surgically removed with fine forceps to facilitate drug perfusion.

The spike activity of swimmeret motor neurones was recorded extracellularly using pin or suction electrodes from the 1st motor root in either the 3rd, 4th or 5th abdominal ganglia. The pin electrode was placed in contact with the anterior branch of the 1st motor root to record the activity of RS motor neurones and with the posterior branch to record the activity of PS motor neurones. The pin electrodes were insulated with petroleum jelly (Vaseline: liquid paraffin, 3:1). The suction electrode was placed on the cut motor branches of power-stroke motor neurones. All procedures were carried out following institutional guidelines for animal care.

Preparation of pharmacological agents

The following pharmacological agents were obtained from Sigma (St Louis, MO, USA): carbamoylcholine chloride (carbachol) as cholinergic agonist, L-arginine for endogenous NO synthesis, D-arginine as an isomer of L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME) as NOS inhibitor, N^G-nitro-D-arginine methyl ester (D-NAME) as an isomer of L-NAME, S-nitroso-N-acetyl-penicillamine (SNAP) as a NO donor, 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide (PTIO), 8-bromo-guanosine 3',5'-cyclic monophosphate (8-Br-cGMP) as a membrane permeable cGMP analogue, ¹H-[1,2,4]oxadiazolo-[4,3-a]wuinoxaline-1-one (ODQ) as a sGC inhibitor, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (pCPT-cAMP) as a membrane-permeable cAMP analogue, IBMX as a phosphodiesterase inhibitor and SQ22536 as an adenylate cyclase inhibitor. Carbachol was dissolved in physiological saline to 8 μmol l⁻¹ concentration. SNAP, PTIO and 8-Br-cGMP were dissolved in 8 μmol l⁻¹ carbachol-containing solution and made up to 400 μmol l⁻¹, 50 μmol l⁻¹ and 100 μmol l⁻¹ in concentration, respectively. L-arginine, D-arginine, L-NAME and D-NAME were dissolved in physiological saline to 100 mmol l⁻¹ first and then dissolved in 8 μmol l⁻¹ carbachol-containing solution to make up to 5 mmol l⁻¹ concentration. ODQ was dissolved in DMSO to 25 mmol l⁻¹ first, and then dissolved in 8 μmol l⁻¹ carbachol-containing solution to make up to 1 μmol l⁻¹ concentration. A mixture of 100 μmol l⁻¹ pCPT-cAMP and 100 μmol l⁻¹ IBMX was prepared by dissolving it in physiological saline or 5 mmol l⁻¹ L-arginine-containing solution. SQ22536 was dissolved in 8 μmol l⁻¹ carbachol-containing solution to make up to 100 μmol l⁻¹ concentration. These drugs were prepared prior to application and used within 5 min. The concentration of drugs used in this study was based on Araki et al. (Araki et al., 2004; Araki et al., 2005).

Spike-activity recording

In the isolated preparations, swimmeret motor neurones were usually silent or showed tonic spiking at a low frequency. To elicit rhythmic activity of swimmeret motor neurones, physiological saline in the chamber was changed to carbachol-containing solution (Braun and Mulloney, 1993; Mulloney, 1997). The bathing solution was washed out three times with $8 \mu\text{mol l}^{-1}$ carbachol-containing solution and then spike activity of the motor neurones was usually recorded 3 min after the third replacement of carbachol-containing solution for at least 1 min (first carbachol session). Then, each preparation was washed with normal saline several times until rhythmic bursts of motor neurone spikes disappeared. Then, each preparation was replaced with one of 5 mmol l^{-1} L-arginine, 5 mmol l^{-1} D-arginine, 5 mmol l^{-1} L-NAME, 5 mmol l^{-1} D-NAME, $400 \mu\text{mol l}^{-1}$ SNAP, $100 \mu\text{mol l}^{-1}$ 8-Br-cGMP, $1 \mu\text{mol l}^{-1}$ ODQ or $100 \mu\text{mol l}^{-1}$ SQ22536 with $8 \mu\text{mol l}^{-1}$ carbachol-containing solution. The bathing solution was washed out three times and the spike activity of the motor neurones recorded 3 min after the third replacement of the test solution for at least 1 min (test session). Each preparation was washed again with normal saline several times until the motor neurones became silent. The bathing solution was washed out three times with $8 \mu\text{mol l}^{-1}$ carbachol-containing solution and the spike activity of the motor neurones recorded 3 min after the third replacement of carbachol-containing solution for at least 1 min (second carbachol session). Each preparation was tested only once with a particular drug, with the exception of the series of L-arginine and PTIO applications and L-arginine and ODQ applications. In the experiments using pCPT-cAMP, the mixture of $100 \mu\text{mol l}^{-1}$ pCPT-cAMP and $100 \mu\text{mol l}^{-1}$ IBMX was applied first and then the effect of L-arginine was examined by co-application of L-arginine with pCPT-cAMP and IBMX after wash with normal saline several times.

Analysis

All recordings were stored, displayed and analyzed using PowerLab. Data were based on recordings from 62 crayfish. The cycle period of rhythmic bursts of motor neurone spikes was defined as the time from the first spike of one burst to the first spike of next burst and was analyzed using a paired *t*-test if data were normally distributed, or a Wilcoxon signed rank test if not. Statistical analyses were carried out using SigmaPlot v12.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.M., M.Y. and T.N. conceived and designed the experiments, analyzed the data and wrote the paper; A.M. and M.Y. performed the experiments.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.110551/-DC1>

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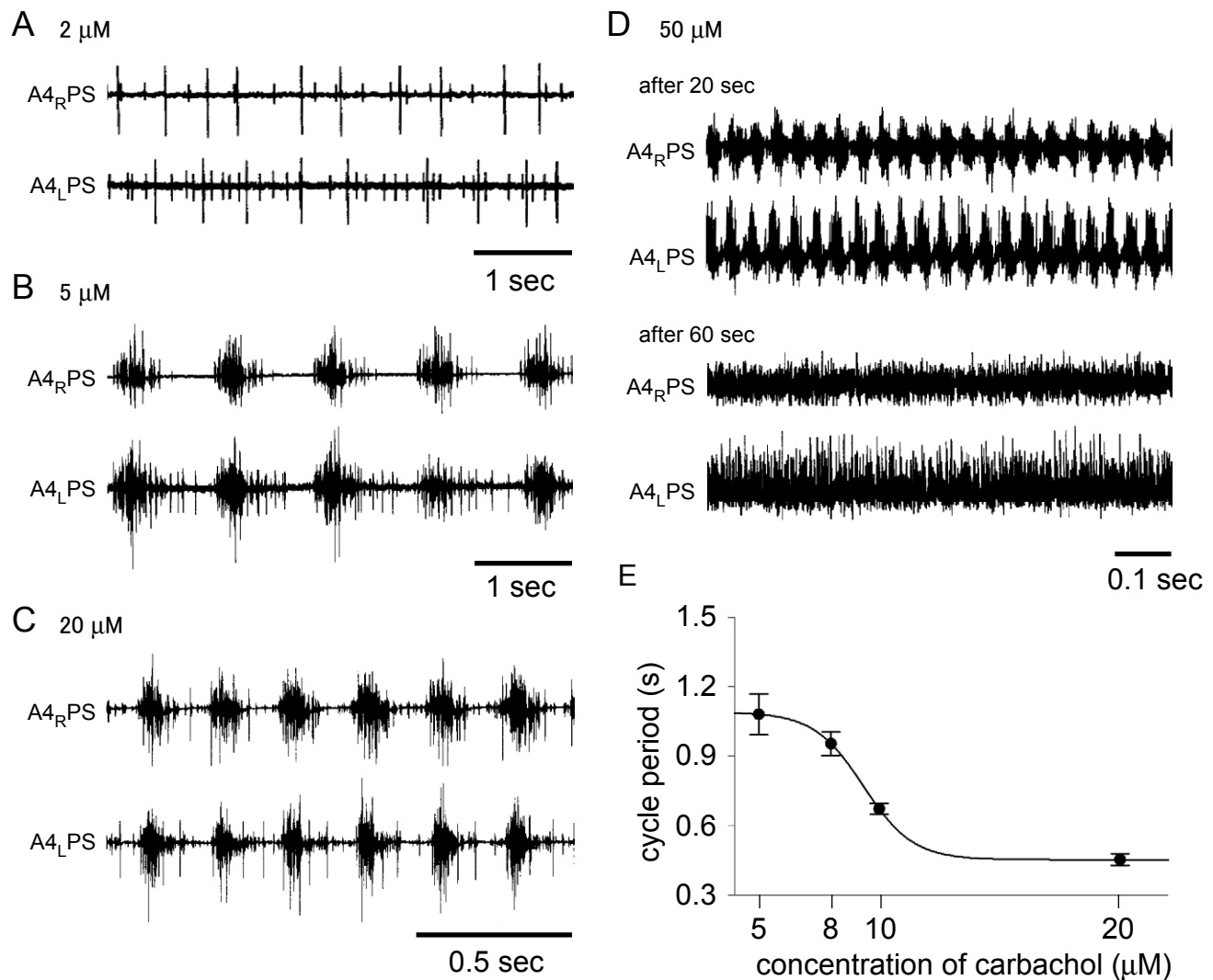


Fig. S1. Dose-dependent effect of carbachol upon rhythmic beating activity of the swimmeret motor neurones. (A) Application of 2 μM carbachol elicited spikes of right (upper trace) and left (lower trace) power-stroke (PS) motor neurones in the 4th abdominal ganglion from silent preparation with no rhythmic beating activity. (B) Application of 5 μM carbachol elicited synchronous rhythmic bursts of spikes in the motor neurones on both sides. (C) Burst frequency of both motor neurones was increased when 20 μM carbachol was applied. (D) Application of 50 μM carbachol elicited high frequency of rhythmic bursts of motor neurone spikes first (upper traces: after 20 s). Then, spike trains of the motor neurones showed continuous spiking activity without any rhythms (lower traces: after 60 s). (E) Semilogarithmic dose-response relationship between the concentration of applied carbachol and the cycle period of the power-stroke swimmeret motor neurones (mean \pm s.e.m.). The cycle period was defined as a time from the first spike of the burst to the first spike of next burst.