Research Report

Metabotropic glutamate receptor agonists modify the pyloric output of the crustacean stomatogastric ganglion

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Abstract

We have studied the effects of groups I, II, and III metabotropic glutamate receptor (mGluR) agonists and antagonists on pyloric activity in the stomatogastric ganglion (STG) of the Caribbean spiny lobster Panulirus argus. We have found that agonists for all three groups of mGluRs modify the pyloric output. The group I agonist, l-quisqualic acid (l-QA), activated the pyloric central pattern generator (CPG). When the pyloric rhythm was partially suppressed by sucrose-block of input fibers in the stomatogastric nerve (stn), l-QA accelerated the rhythmic activity. In addition, the number of spike discharges was increased in pyloric motoneurons: pyloric (PY), and lateral pyloric (LP). In completely blocked preparations, a slow pyloric rhythm was initiated by l-QA. Groups II and III agonists exerted an inhibitory effect on pyloric activity. The group II agonist, (2S,1’S,2’S)-2-(Carboxycyclopropyl)glycine (L-CCG-I), decreased both the frequency of the pyloric rhythm and the number of spike discharges in the motoneurons: ventricular dilator (VD), PY, and LP. The effects of L-CCG-I were dose-dependent. The group III agonist, l-(+)-2-Amino-4-phosphonobutyric acid (l-AP4), slightly decreased the frequency of the pyloric rhythm and suppressed spike discharges in the VD neuron. All effects of mGluR agonists were reversible. The effect of l-QA was blocked by the broad spectrum mGluR antagonist (S)-Methyl-4-carboxyphenylglycine (MCPG). The inhibitory effect of L-CCG-I was prevented by MCPG and by the group II/III mGluR antagonist (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA). The inhibitory effect of l-AP4 was blocked by MPPG and partially blocked by APICA.

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1. Introduction

The crustacean stomatogastric nervous system (Fig. 1A) is a well-described circuit for studying the neural mechanisms of rhythm generation in small networks, as well as cellular and network mechanisms of neuromodulation [11,26]. The stomatogastric ganglion (STG) includes two central pattern generators (CPGs), the gastric CPG, which controls the rhythmic contractions of muscles that operate the teeth of the gastric mill, and the pyloric, which controls the rhythmic movements of the pyloric filter. The pyloric CPG (Fig. 1B) consists of 14 identified neurons (one interneuron, the anterior buster neuron (AB), and 13 motor neurons) that interact with one another through electrical and chemical synapses, which in several species are completely characterized [11,26]. When connected to the anterior commissural ganglia, the pyloric CPG oscillates at a frequency of about 1 Hz.

Chemical synaptic interactions within the STG are mediated through ionotropic receptors. Acetylcholine
(ACh) and glutamate are the two major neurotransmitters within the STG [4,11,26]. In addition, the pyloric and gastric CPGs are under the control of more than 15 neuromodulatory substances that are released by descending fibres or act hormonally through the blood stream [1,11,26,27]. Neuromodulators switch the CPGs on and off and presumably adjust their activity in relation to the bulk and consistency of ingested food. ACh, in addition to its ionotropic neurotransmitter action, is used as a neuromodulator by the anterior pyloric modulator neuron (APM) located in the oesophageal ganglion (OG in Fig. 1A). APM plays an important role in modulating the pyloric CPG. It activates the pyloric rhythm by turning on the pyloric neurons, which are in fact conditional bursters, to induce a rhythm-generating state [6,21]. The modulatory action of ACh is achieved through metabotropic, muscarinic receptors [2] and there is experimental evidence that adenosine 3',5'-cyclic monophosphate (cAMP) is involved as a second messenger [12]. By using the cAMP indicator dye (FlChR), it has been shown that pilocarpine, an ACh muscarinic receptor agonist, increases the cAMP concentration in the pyloric neuron AB. The question we address here is whether glutamate, the second neurotransmitter found within the STG, could also be used as a neuromodulator acting through metabotropic receptors. This suggestion is supported by the fact that glutamate, in addition to its fast ionotropic receptor-mediated inhibitory postsynaptic potentials in the pyloric and gastric neurons, produced several slow potentials, postsynaptic [7] or arising in response to glutamate application [17,28]. Slow depolarizing and hyperpolarizing responses to glutamate might both be mediated through metabotropic receptors.

mGluRs have been mainly described in vertebrates. Presently, eight mGluR genes have been cloned in mammals [5]. They are divided into three groups based on pharmacology, intracellular messenger coupling and gene similarity. Group I is coupled to phospholipase C, while groups II and III inhibit adenyl cyclase [5,22,23,29]. mGluRs were found to participate in the regulation of different nervous system functions in vertebrates. In particular, it has been shown that activation of mGluRs contributes to burst frequency regulation in the lamprey locomotor CPG [15,16]. Although mGluRs have been mainly studied in vertebrates, some molecular and electrophysiological evidence for the presence and functional role of mGluRs in invertebrates, including arthropods, has been reported [3,9,10,18,19,24,25]. We have found that agonists for all three groups of mGluRs have potent modulatory effects on gastric rhythm generation when superfused onto the STG [13], suggesting a metabotropic modulatory role for glutamate in the gastric CPG.

The question we address here is whether glutamate, in addition to its rapid, ionotropic inhibitory action, could also act as a neuromodulator in the pyloric circuit. We studied the effects of compounds, which are known from mammalian studies as agonists and antagonists for the three groups of mGluRs, on the activity of the pyloric system in the Caribbean spiny lobster Panulirus argus. We focused on studying the effects of the mGluR agonists on the general activity of the pyloric CPG. We demonstrate that mGluR agonists of all three groups modify the frequency of the pyloric rhythm and the spike discharges in some pyloric neurons. Some selective mGluR antagonists can block these effects. These results support the hypothesis that, in the pyloric circuit of the STG, glutamate might act not only as a conventional neurotransmitter, but could also act as a neuromodulator that realizes its effect through the mGluRs.

2. Materials and methods

Spiny lobsters, P. argus, were obtained from Pescadería Johan’s Co. in Vieques, Puerto Rico. They were kept in circulating seawater at ambient temperature (24 °C). Data are from 45 preparations. Most experiments were performed on combined preparations (Fig. 1A) as described before [20]. The stomatogastric nervous system was isolated and pinned out in a Sylgard-lined Petri dish similar to its

Fig. 1. The pyloric circuit and the stomatogastric nervous system. (A) Schematic representation of the stomatogastric system preparation (see text for details). (B) The pyloric circuit. The larger circles represent 14 pyloric neurons which are VD (ventricular dilator); LP (lateral pyloric); AB (anterior burster); PD (pyloric dilator); IC (inferior cardiac); and PY (pyloric constrictor). Small filled circles represent inhibitory synapses, while resistor symbols represent electrotonic coupling. Neurons VD and PD are cholinergic (dark gray), all others neurons are glutamatergic (light gray).
The experiments were designed to determine if the three different types of mGluR agonists, delivered by bath perfusion to the STG, could induce reversible modification of the pyloric motor output. To this end, mGluR agonists were bath-applied for 10 min and then rinsed off. To check for specificity, certain mGluR antagonists were also applied for 10 min and then an agonist was co-applied for another 10 min. For all of the mGluR compounds that were tested, an application period of 5 min was sufficient to achieve a stable effect; therefore, analysis was started 5 min after drug exposure. Two-minute segments of continuous electrophysiological recordings were analyzed with a custom made analysis program to measure frequency and spike number per burst (courtesy by Humberto Ortiz-Zuazaga). We did not include data for the IC neuron in this analysis because in *P. argus* it was not always bursting reliably as was the case for the VD neuron.

The data are presented as means ± standard deviation (SD). The results of the experiments were analyzed by a non-parametric ANOVA (Kruskal–Wallis) followed by Student–Newman–Keuls post test, or by the Wilcoxon Signed Rank test. The level of significant was set at *P* < 0.05 for all the experiments. The SigmaStat package (SPSS Science, version 2.03) was used for all analysis.

3. Results

3.1. The group I mGluR agonist, L-quisqualic acid (L-QA), accelerates the pyloric rhythm

The experiments were designed to determine if the different types of mGluR agonists, delivered by bath perfusion of the STG, could induce reversible modification of the pyloric motor pattern. In *P. argus*, it was difficult to completely suppress the pyloric rhythm by sucrose block. Therefore, most preparations are partially blocked, oscillating at lower than normal (approximately 1 Hz) frequencies. Bath application onto partially blocked preparations of 50 μM L-QA accelerated the oscillatory frequency of the pyloric CPG and increased the spike discharges in some pyloric neurons when the rhythm was slower than normal (Figs. 2A, B, and D). The average pyloric frequency increased significantly from 0.23 ± 0.06 Hz to 0.57 ± 0.14 Hz (*Fig. 2B, n = 5, P < 0.05*). In addition, L-QA increased the number of spikes per burst in motoneurons PY and LP (compare *lpn/pyn* in *Figs. 2C* and D), but did not affect PD discharges (*pdn* in *Figs. 2C* and D). In the experiment for which results are shown in *Figs. 2C–F*, L-QA did not affect the pyloric frequency since the initial frequency was close to 1 Hz.

The effects of L-QA were partially blocked by the broad spectrum mGluR antagonist, MCPG (*n* = 3), at a concentration of 1 mM (*Fig. 2F*). Preincubation of the preparation with MCPG (1 mM) for 10 min did not affect pyloric activity. The more specific group I antagonists AIDA (500
\( \mu \text{M}, n = 2 \), 4-CPG (1 mM, \( n = 3 \)), and E-4CPG (1 mM, \( n = 2 \)) did not affect the ongoing pyloric rhythm nor did they prevent the effects of \( l^-\text{QA} \) on the pyloric rhythm (data not shown). The effects of \( l^-\text{QA} \) were also not blocked by 50 \( \mu \text{M} \) CNQX, a mammalian ionotropic glutamate receptor antagonist (\( n = 3 \), data not shown).

### 3.2. The group II mGluR agonist, \( L^-\text{CCG-I} \), inhibits the pyloric rhythm

\( L^-\text{CCG-I} \) exerted a significant inhibitory effect on the pyloric rhythm when applied at a concentration of 50 \( \mu \text{M} \) on combined preparations (\( n = 15 \)). In 10 experiments, bath application of 50 \( \mu \text{M} \) \( L^-\text{CCG-I} \) decreased the frequency of the pyloric rhythm (Fig. 3B), whereas in five other experiments the pyloric activity was almost completely suppressed (as in Fig. 3C at 100 \( \mu \text{M} \)). The frequency of the pyloric rhythm decreased significantly from 0.91 ± 0.24 Hz to 0.48 ± 0.4 Hz (\( n = 15 \), \( P \leq 0.05 \)) after application of 50 \( \mu \text{M} \) \( L^-\text{CCG-I} \) (Fig. 3E). In addition to its influence on the frequency of the pyloric rhythm, \( L^-\text{CCG-I} \) appeared to specifically affect the activity of individual pyloric neurons, although we cannot rule out that these observations reflect indirect effects. The most prominent inhibitory effect was observed on neuron VD (\( mnv \) and VD neuron in Figs. 3B and C). Spike discharges in the LP neuron were also affected (\( lvn \) and \( lpn \) in Figs. 3B and C). On the other hand, \( L^-\text{CCG-I} \) exerted only a small effect, if any, on spike discharges in the PDs at 50 \( \mu \text{M} \) (Fig. 3B) but a much more pronounced effect at 100 \( \mu \text{M} \) (Fig. 3C). In experiments in which \( L^-\text{CCG-I} \) almost completely inhibited the pyloric rhythm, activity of most neurons, except PD, was suppressed (Fig. 3C). All effects of \( L^-\text{CCG-I} \) were reversible (Fig. 3D) and dose-dependent (\( n = 5 \)).

The effects of 50 \( \mu \text{M} \) \( L^-\text{CCG-I} \) could be completely or partially blocked by application of the group II antagonist APICA at a concentration of 1 mM (Fig. 3E). In the experiment illustrated in Figs. 3A–C, \( L^-\text{CCG-I} \) clearly decreased the pyloric activity (Fig. 3B), but not significantly different from the frequency in APICA alone (Fig. 3E). When LCCG-I was co-applied with 1 mM APICA, it did not significantly reduce the pyloric frequency (Fig. 3E). The initial frequency of the pyloric rhythm of 0.77 ± 0.09 Hz was reduced to 0.34 ± 0.13 Hz (\( n = 3 \), \( P \leq 0.05 \)) by 50 \( \mu \text{M} \) L-CCG-I. Application of 1 mM of the antagonist APICA alone reduced the pyloric frequency to 0.51 ± 0.14 Hz (Fig. 3D). When 50 \( \mu \text{M} \) of L-CCG-I was co-applied with 1 mM APICA, the frequency changed to 0.59 ± 0.29 Hz (Fig. 3E). This value is significantly different from that observed after application of L-CCG-I alone (\( n = 4 \), \( P \leq 0.05 \)). The effect
of L-CCG-I was also blocked by the broad spectrum mGluR antagonist MCPG at 1 mM ($n = 4$, $P < 0.05$, data not shown) and by the group II/III antagonist MPPG at 1 mM ($n = 5$, $P < 0.05$, Figs. 3E, F, and G). PTX, which at 10 μM blocks fast inhibitory glutamatergic interactions within the pyloric network, did not prevent the inhibitory effect of L-CCG-I ($n = 3$, data not shown).

3.3. The group III mGluR agonist, L-AP4 inhibits the pyloric rhythm

L-AP4, an agonist for group III mGluRs, also inhibited pyloric activity (Fig. 4). However, higher concentrations than were used for other agonists were needed. Under the influence of L-AP4, at a concentration of 200 μM, the frequency of the pyloric rhythm decreased significantly from $0.96 \pm 0.26$ Hz to $0.82 \pm 0.20$ Hz ($n = 11$, $P < 0.05$; Fig. 4E). Like L-CCG-I, 200 μM of L-AP4 suppressed spike discharges in the VD neuron (mvn and VD in Figs. 4B and F). In the VD neuron, the number of spikes per burst decreased significantly from $8.0 \pm 1$ to $4.0 \pm 0.4$ ($n = 7$, $P < 0.05$; Figs. 4A, B, and F). The effects of L-AP4 were reversible (Figs. 4C and E). MPPG (1 mM), an antagonist for group II/III mGluRs, did not influence the ongoing pyloric activity in combined preparations but blocked the inhibitory effects of 200 μM L-AP4 ($n = 5$, Figs. 4D and G). APICA, a mammalian group II mGluR antagonist, partially blocked the effect of 200 μM L-AP4 when applied at a concentration of 1 mM ($n = 4$, data not shown).

4. Discussion

The data presented here show that mGluR agonists for all three mammalian-defined mGluR groups affect the pyloric network output, and that some mGluR antagonists block these effects at least partially. Since glutamate is one of the two major inhibitory transmitters at fast synapses in the pyloric circuit of the STG, this result is of considerable interest. It suggests (i) that in this CPG circuit glutamate acts not only as a fast conventional neurotransmitter, but also as a neuromodulator, and (ii) that the modulatory effects of glutamate may be mediated via mGluRs, similar to those described in mammals. We have shown earlier [13] that groups I, II, and III mGluR compounds have strong and distinct effects on gastric rhythm generation. Comparison of the data obtained in the present study with those obtained in the gastric system shows that the effects of mGluR compounds on the gastric CPG are more potent than on the pyloric CPG.

The group I mGluR agonists, L-QA, exerted an accelerating effect on the pyloric CPG, but this observation was
robust only when the normal firing frequency was reduced by decreasing modulatory drive from the commissural ganglia. In addition, there were increased spike discharges in LP and PY. This generally excitatory effect is similar to the effects of mGluR group I reagents we described in the gastric circuit [13] and also to those described in many vertebrate systems [5]. These effects were blocked by the broad spectrum mGluR antagonist, MCPG. Antagonists that have been shown in mammalian studies to block group I mGluRs (AIDA, 4-CPG, and E-4CPG) neither interfered with the action of group I agonists, nor induced detectable changes in native pyloric rhythms. Therefore, we are careful to note that the effects of group I agonists on pyloric rhythm generation do not prove the presence and functional importance of crustacean homologues of mammalian group I mGluRs in this circuit. As in most mammalian preparations, agonists for group II and III mGluRs exerted inhibitory effects on the pyloric network. They inhibited both pyloric rhythm frequency and the action potential firing activity of individual neurons (particularly in motoneurons VD, PY, and LP). The group II agonist, L-CCG-I, had a stronger effect than the group III agonist, l-AP4. These findings are again similar to results that were obtained in the gastric circuit where groups II and III agonists always inhibited all of the motor neurons, and where L-CCG-I was also more potent than l-AP4 [13]. The effects of the group II agonist L-CCG-I were completely or partially blocked by the specific group II mGluR antagonist, APICA, by the broad spectrum mGluR antagonists, MCPG and by the specific group II/III antagonist MPPG. Unlike in the gastric circuit, however, APICA did not have a significant effect on pyloric rhythm generation when applied alone. A slight reduction in pyloric frequency seen in some experiments was not statistically significant. The specific group II/III mGluR antagonist, MPPG, blocked the effects of the group III agonist, l-AP4.

In the absence of clear effects of group II and III antagonists on the native pyloric rhythm, the experimental evidence is not strong enough to prove the presence of both groups II and III mGluRs in STG neurons. However, the inhibitory effects of both l-AP4 and especially L-CCG-I on pyloric rhythm generation, together with the complete or

Fig. 4. Effect of the group III mGluR agonist, l-AP4 on pyloric rhythm and its inhibition by the mGluR antagonist MPPG. (A) Extracellular recordings of the initial pyloric rhythm in motor nerves mnv, pyn, and lnv and an intracellular recording from the VD neuron. (B) Recordings 5 min after bath application of 200 μM l-AP4. (C) Recordings after 45 min of washing showing recovery of the rhythm. (D) 200 μM l-AP4 was bath applied together with the group III mGluR antagonist MPPG (1 mM). (E) Effect of 200 μM l-AP4 on pyloric frequency (n = 11, P ≤ 0.05). (F) Effect of 200 μM l-AP4 on spikes per burst in the VD neuron (n = 7, P ≤ 0.05). (G) The group II/III mGluR antagonist, MPPG (1 mM) blocked the reduction in spikes per burst in neuron VD induced by 200 μM l-AP4. Asterisks represent statistically significant results (P ≤ 0.05).
partial blockage of these effects by mGluR-specific antagonists, allow us to hypothesize that mGluRs related to mammalian group II and III are expressed in pyloric neurons. The apparent cross-reactivity between group II and III compounds may be interpreted in different ways. Both groups II and III mGluRs may be present in the STG, with a pharmacology that is less distinct when the mammalian-defined pharmacological tools are used. Alternatively, a single type of mGluR with mixed pharmacology may be present in STG neurons. In addition, in a study on the intact network, as presented here, mGluR compounds may target different types of mGluRs expressed differentially in neurons of the STG circuits, leading to the masking of effects of individual drugs and thus give erroneous negative results.

In this work, we confined ourselves to studying the effects of pharmacological agents known to activate mGluRs and did not study the effect of glutamate itself. The reason for this approach was that glutamate applied to the STG might activate both ionotropic and different groups of metabotropic receptors simultaneously. In this case, the effect of glutamate would be very complex and difficult to interpret. However, preliminary results have shown that glutamate at low concentration (10 μM) affects the activity of isolated pyloric neurons when the ionotropic receptors are blocked by picrotoxin (R. Levi, personal communication).

Previous work has shown that an intracellular cAMP elevation stimulates activity of pyloric neurons [8]. By using the cAMP indicator dye (FChR), it has been shown that some neuromodulators that stimulate pyloric activity (pilocarpine, octopamine, dopamine, and serotonin) increase cAMP concentrations in pyloric neurons [12]. Groups II and III mGluRs inhibit adenylyl cyclase in mammalian cells and thus decrease cAMP formation in vertebrate neurons [5,22,23,28]. Therefore, we suggest that the effects of groups II and III agonists on pyloric activity could be brought about by decreasing cAMP levels. An important functional implication follows from this suggestion, namely that compounds that activate groups II and III mGluRs, may act as antagonists for neuromodulators which stimulate the pyloric activity via an increase of the intracellular cAMP concentration. Indeed, preliminary results on the pyloric system suggest that cyclic nucleotides are involved in group II mGluR signaling [14].

Overall, our data suggest that, as in the gastric circuit, mGluRs similar to mammalian groups II and III are involved in inhibiting the pyloric system, while group I mGluRs are involved in activating the pyloric system. However, the functional role of mGluRs in the regulation of the pyloric system in vivo remains to be determined. In this regard, it will be interesting to determine whether glutamate acting on mGluRs is released from descending fibers coming from higher centers or whether it is the glutamate released by pyloric and gastric neurons within the ganglion. Experiments on functionally isolated STG neurons will clarify this point.

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