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Modulation of a Neural Network by Physiological Levels of Oxygen in Lobster Stomatogastric Ganglion

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Although a large body of literature has been devoted to the role of O_2 in the CNS, how neural networks function during long-term exposures to low but physiological O_2 partial pressure (Po_2) has never been studied. We addressed this issue in crustaceans, where arterial blood Po_2 is set in the 1–3 kPa range, a level that is similar to the most frequently measured tissue Po_2 in the vertebrate CNS. We demonstrate that over its physiological range, O_2 can reversibly modify the activity of the pyloric network in the lobster *Homarus gammarus*. This network is composed of 12 identified neurons that spontaneously generate a triphasic rhythmic motor output *in vitro* as well as *in vivo*. When Po_2 decreased from 20 to 1 kPa, the pyloric cycle period increased by 30–40%, and the neuronal pattern was modified. These effects were all dose- and state-dependent. Specifically, we found that the single lateral pyloric (LP) neuron was responsible for the O_2-mediated changes. At low Po_2, the LP burst duration increased without change in its intraburst firing frequency. Because LP inhibits the pyloric pacemaker neurons, the increased LP burst duration delayed the onset of each rhythmic pacemaker burst, thereby reducing significantly the cycling frequency. When we deleted LP, the network was no longer O_2-sensitive.

In conclusion, we propose that (1) O_2 has specific neuromodulator-like actions in the CNS and that (2) the physiological role of this reduction of activity and energy expenditure could be a key adaptation for tolerating low but physiological Po_2 in sensitive neural networks.

Key words: respiration; oxygen; hypoxia; neural network; stomatogastric nervous system; crustaceans

In the vertebrate brain, O_2 partial pressure (Po_2) *in vivo* remains mainly in a low and narrow range between 1 and 3 kPa (Lubbers, 1968; Siesjo, 1978), whereas numerous metabolic processes and enzyme reactions exhibit a K_m that is higher than the corresponding mean O_2 concentrations (Jones et al., 1985; Connett et al., 1990; Vanderkooi et al., 1991). When studied *in vitro*, however, CNS tissue is superfused with salines equilibrated with air ($Po_2 \approx$ 21 kPa), carbogen (Po_2 = 95 kPa), or a N_2/CO_2 mixture (Po_2 = 0 kPa). For reference, 1 kPa = 7.5 mm Hg or torr; in a saline solution, 1 kPa corresponds to an O_2 fraction of $\approx$ 1%. This raises the question as to how neural networks operate at low but physiological Po_2.

In vertebrates, no information is available regarding how changes in physiological O_2 levels influence network activity. Furthermore, such studies have the added problems resulting from artificial perfusion, including altered O_2 and glucose supply and clearance of metabolic end products. When these animals are studied *in vivo*, tissues are usually perfused. *In vitro*, however, which is more amenable to a cellular analysis of network activity, these tissues can only be superfused, which circumvents the normal circulatory pathways. In contrast, insights regarding the influence of O_2 levels can be gained from cellular studies using the crustacean stomatogastric nervous system (STNS). This system includes the pyloric neural circuit, which constitutes one of the best understood neural networks (Harris-Warrick et al., 1992). This network is composed of 12 identified neurons that spontaneously generate a rhythmic motor pattern both *in vivo* and *in vitro*. It is located in the stomatogastric ganglion (STG), which *in situ* resides in the lumen of an artery where Po_2 remains primarily in the 1–3 kPa range (Forgue et al., 1992b; Fig. 1*B*), i.e., just above Po_2 at the anaerobic threshold in crustaceans (Forgue et al., 1992a). A unique feature, at least in the European lobster *Homarus gammarus*, is that the STG is simply bathed in arterial blood without any microcirculation. This removes the need for artificial perfusion during *in vitro* experiments and allows us to study neural network operation at low but physiological Po_2 by simply superfusing the STG in artificial glass vessels. A second fundamental point, which eliminates the problem of an adequate continuous supply of glucose by perfusion, is the existence of exceptionally large glycogen stores in crustacean nervous tissue. The crustacean nervous system contains ~80 μmol of glycogen per gram of tissue, in contrast to only 2 $\mu mol \cdot gm^{-1}$ in the mouse brain (Treherne, 1966; Wegener, 1981).

We demonstrate here that over its low but physiological range, O_2 can influence neural network activity in a manner equivalent to that of a neuromodulatory transmitter. Rather than having global effects, changes in O_2 levels selectively influenced the network output via a single pyloric network neuron, the LP neuron. Specifically, at low Po_2, the duration of the rhythmic LP burst increased, thereby delaying each subsequent burst of the pyloric pacemaker neurons and significantly reducing the cycling activity of the pyloric network.

MATERIALS AND METHODS

All experiments were performed on the European lobster *H. gammarus*, weighing between 300 and 700 gm (n = 46), in the intermolt stage. Lobsters were obtained from local suppliers and acclimated in the laboratory for at least 1 week. They were maintained in aerated seawater

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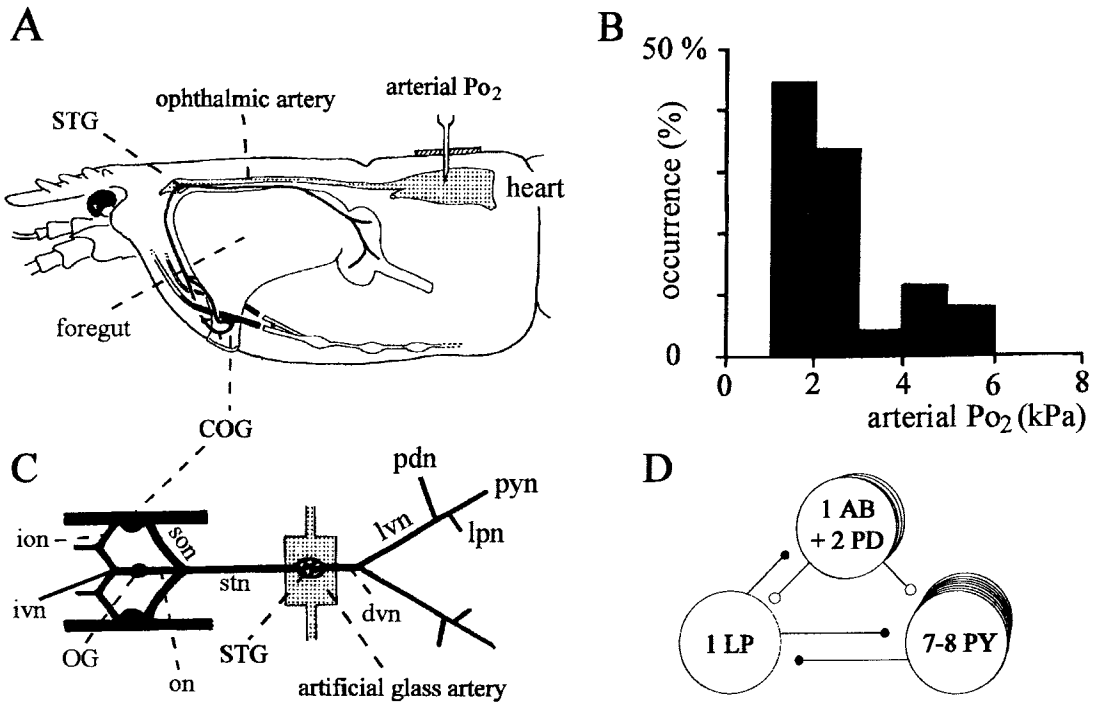

Figure 1. Experimental preparation. *A*, Lateral (*left*) view of the anterior region of a lobster showing the positions of the foregut, the STNS, the heart, and the ophthalmic artery, where the stomatogastric ganglion (*STG*) is located. A needle is shown inserted into the heart to illustrate how arterial Po_2 can be measured by heart puncture and where India ink was injected. *B*, Frequency distribution of arterial O_2 partial pressure (arterial Po_2) in 27 resting and unfed *H. gammarus* lobsters. The most frequently measured arterial Po_2 was 1–3 kPa, with a mode of 1–2 kPa. Composite figure from Forgue et al., 1992b, plus unpublished data. *C*, Diagram of the *in vitro* STNS. The *gray box* represents the glass box used as an artificial artery. The STG, pinned on a small piece of Sylgard, was placed in this box and superfused with saline equilibrated at various Po_2 levels while the remainder of the system was superfused with a standard aerated saline (Po_2 = 20 kPa, $[O_2]$ = 250 μM). *D*, The pyloric wiring diagram showing the pyloric neurons and their synaptic interactions. *Open symbols* represent cholinergic inhibitory synapses; *closed symbols* represent glutamatergic inhibitory synapses. *AB*, anterior burster neuron; *COG*, commissural ganglion; *dvn*, dorsal ventricular nerve; *ion*, inferior esophageal nerve; *ivn*, inferior ventricular nerve; *LP*, lateral pyloric constrictor neuron; *lpn*, LP nerve; *lvn*, lateral ventricular nerve; *OG*, esophageal ganglion; *on*, esophageal nerve; *PD*, pyloric dilatator neuron; *pdn*, PD nerve; *PY*, pyloric constrictor neuron; *pyn*, PY nerve; *son*, superior esophageal nerve; *STG*, stomatogastric ganglion, *stn*, stomatogastric nerve.

tanks (salinity = 30–32‰) at temperatures ranging from 8–14°C and fed weekly with fish meat. Lobsters were anesthetized by chilling in ice for 30–45 min before dissection.

Vascular anatomy. Experiments were performed on five animals. The STNS consists of four interconnected ganglia, including the STG (contains ~30 neurons), two commissural ganglia (CoGs) (containing ~500 neurons each), and the esophageal ganglion (OG) (contains ~15 neurons). The microcirculation in the STNS ganglia was traced by 1 ml injections of India ink into the heart (Fig. 1*A*). Using this technique, the black-labeled arterial blood first reaches the STG, which is located in the lumen of the ophthalmic artery at a distance of 3–5 cm from the heart of a 300–500 gm lobster. The arterial blood then flows to the CoGs and OG via secondary arteries. Five minutes after injection of ink, the anterior part of the STNS was dissected according to standard dissection procedures (Selverston and Moulins, 1987). The dissected STNS tissue was then cleared in methyl salicylate to reveal the finely stained microvascularization plexus. Such preparations were observed using light microscopy.

Electrophysiology. Experiments were performed on 41 preparations. The complete STNS was dissected and isolated from the foregut (Fig. 1*C*). The STG, CoGs, and commissural connectives were desheathed to allow access in the STG for intracellular recordings and to allow a better oxygenation in the CoGs. In terms of O_2 supply, desheathing the STG corresponds to eliminating a diffusion barrier. In *H. gammarus,* the sheath thickness is 3–5 μm (Cournil et al., 1990). Consequently, there should be improved intracellular oxygenation in both STG and CoGs, and the main experimental bias could be that all reported observations might occur *in vivo* at slightly higher blood Po_2. When required, selected neurons were deleted from the network by photoinactivation (Miller and Selverston, 1979). In short, this involves filling a neuron iontophoretically with Lucifer yellow dye and then illuminating the ganglion at 450–490 nm until the membrane potential reaches 0 mV and activity vanishes on the corresponding nerve.

The STNS was pinned down in a Sylgard-lined petri dish by small pins inserted through the cut ends of various nerve roots. The STG was pinned onto a separate Sylgard plate (10 × 6 × 2 mm) and enclosed in a 300 μl glass chamber (internal size, 10 × 6 × 5 mm). The chamber was supplied via gravity with saline at constant flow (3–4 ml · min^{-1}), and three holes in the upper part of the chamber (inner diameter, 1.1–1.2 mm each) allowed access for simultaneous intracellular recordings from the somata of three identified neurons with standard glass micropipettes filled with 3 M KCl (resistance, 10–20 MW). World Precision Instruments amplifiers were used for intracellular recordings and current injections. Extracellular activity was monitored with monopolar platinum electrodes and laboratory-constructed extracellular amplifiers. Signals were displayed on a Tektronix 5113 oscilloscope, stored on videotape coupled to a NeuroCorder DR 890, and recorded directly with a Gould ES 1000 electrostatic chart recorder.

Physiological saline used for STNS superfusion included NaCl, 479 mM; KCl, 13.2 mM; $CaCl_2$, 13.7 mM; $MgSO_4$, 10 mM; Na_2SO_4, 3.9 mM; HEPES, 5 mM. The pH was adjusted to 7.45 with HCl. The entire preparation was superfused continuously with saline held at a constant temperature of 14.0 ± 0.2°C by means of a laboratory-constructed thermoelectric device.

Gas mixtures. The $N_2/O_2/CO_2$ gas mixture was obtained via mass flow controllers (Tylan General, model FC-260) driven by a laboratory-constructed programmable control unit. During experiments, Po_2 was varied in the 1–20 kPa range. The CO_2 partial pressure (Pco_2) was maintained at 0.4 kPa, a value typical of blood Pco_2 in water-breathing animals (Rahn, 1966). The gas mixtures bubbled through the reservoir of saline feeding the STG. Between experiments, the gas-phase composition was analyzed using a paramagnetic O_2 analyzer (Servomex 1100A) and an infrared CO_2 analyzer (Servomex 1410B) calibrated with high grade N_2 and precision gas mixtures (Fo_2 = 3.99 ± 0.04%; Fco_2 = 1.01 ± 0.01%). In the STG chamber, the absolute Po_2 value was also measured periodically at the ganglion level by sampling saline through a system consisting

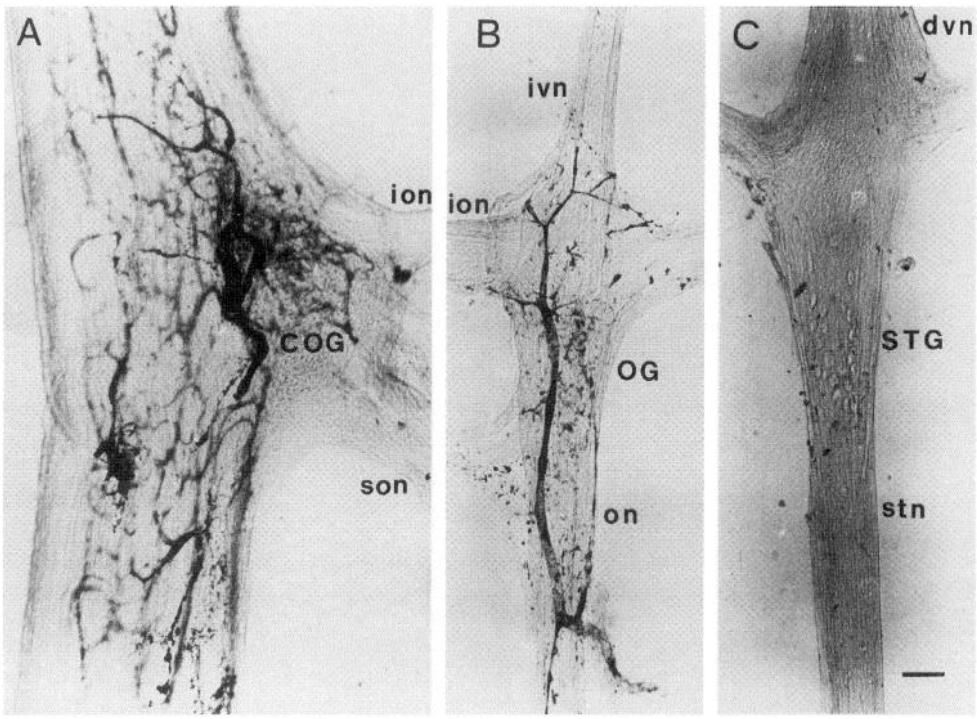

Figure 2. Microcirculation in the STNS including the (*A*) left CoG (*COG*), (*B*) OG, and (*C*) STG. The arterial microcirculation is visualized with India ink. The CoG is desheathed, whereas the OG and STG are ensheathed (tissues clarified with methyl salicylate). Notice the rich vascularization in the CoG, a few microvessels in the OG, and no microvessels in the STG. STG neuron somata are visible on the *left* in C. Same symbols and abbreviations as defined in legend to Figure 1. Scale bar, 200 μm.

of a broken-tipped glass micropipette, an O_2 polarographic electrode (Radiometer, type E 5046) set at 14°C, and a Gilson pump placed in serial order. After a change of gas composition in the bottle supplying the STG chamber, 95% of the Po_2 value in the STG was changed in <3–4 min. At the lowest Po_2 levels, the actual value was ± 0.1 kPa of the nominal value. The O_2 concentration in the saline was calculated according to Henry's law ($Co_2 = \alpha o_2 \cdot Po_2$) with $\alpha o_2 = 12.4\ \mu mol \cdot L^{-1} \cdot kPa^{-1}$ at 14°C.

Data are reported as means ± 1 SE, except where stated otherwise. Differences were evaluated using the paired Student's *t*-test, and $p < 0.01$ was taken as the fiducial limit of significance.

RESULTS

The STNS in *H. gammarus*

The STNS of decapod crustaceans generates the motor pattern that drives the rhythmic activity of the striated muscles that produce the movements of the foregut (Fig. 1*A*; also see Maynard and Dando, 1974). *In vitro*, the STNS of *H. gammarus* produces three motor outputs spontaneously and continuously. These motor programs are generated by three discrete neural networks (Meyrand et al., 1994). Among them, the pyloric network presents the most active rhythm with the highest bursting frequency. It is composed of 12 neurons, all located in the STG, which are responsible for the peristaltic-like dilation and constriction of the pyloric chamber. Their rhythmic output is a triphasic recurrent activity involving the sequential activation of pyloric dilator (PD), anterior constrictor (LP), and posterior constrictor (PY) motoneurons. The *in vitro* pyloric rhythm is similar to the rhythm expressed *in vivo* (Rezer and Moulins, 1983). This rhythmic motor pattern has been studied extensively during the past 20 years (Miller, 1987; Harris-Warrick et al., 1992), and a wiring diagram has been developed that results from experiments that combine electrophysiological, pharmacological, and single neuron photoinactivation techniques (Miller and Selverston, 1979; Cazalets et al., 1990b). The three functional subsets of neurons that compose the pyloric network are illustrated in Figure 1*D*. The dilator group consists of two PD neurons and one pyloric interneuron, the anterior burster neuron. These three neurons are strongly electrically coupled and consequently exhibit synchronous activity. They express endogenous oscillatory properties and are considered the pacemaker unit of this network (Cazalets et al., 1987; Miller, 1987). They rhythmically inhibit the constrictor neurons, which consist of two subsets. The first one is composed of the single LP constrictor neuron, and the second one consists of seven to eight electrically coupled pyloric (PY) constrictor neurons. Although the two sets of constrictor neurons make reciprocal inhibitory connections, the LP neuron is the only cell in the pyloric network that makes an inhibitory synapse onto the pacemaker group (Fig. 1*D*). Consequently, within the pyloric network, LP is the only neuron able to modify directly the rhythmic activity of the pacemaker group.

All neurons of the pyloric network express intrinsic oscillatory and/or plateau properties (Cazalets et al., 1990b), which play a critical role in the expression of the network activity (Bal et al., 1988). Moreover, these properties as well as the synaptic properties are under the control of descending modulatory inputs from the CoG and the OG (Nagy et al., 1988; Cazalets et al., 1990a; Cournil et al., 1990; Meyrand et al., 1991).

Vascular anatomy of the STNS in *H. gammarus*

One of the major problems in studying O_2 supply mechanisms in the *in vitro* CNS is the presence of the microcirculation. Although King (1976) reported the presence of small blood vessels within the STG neuropil of the spiny lobster *Panulirus interruptus*, Moulins and coworkers (unpublished data) never observed such vessels in the *H. gammarus* STG, at either the light or the ultrastructural level. We reexamined this issue in *H. gammarus* by using India ink as a tracer. After ink injections into the heart, we observed no microvascularization in the ensheathed STG, in contrast to the vessels revealed in the desheathed CoG and ensheathed OG (Fig. 2). The dye reliably stained ($n = 5$) a complex and rich vascularized system in both CoGs (Fig. 2*A*) and OG (Fig. 2*B*), but we never observed stained vessels in the STG (Fig. 2*C*). Thus, *in situ* the STG is simply bathed in the arterial blood, which has a Po_2 mainly in the 1–3 kPa range (Fig. 1*B*). This unique

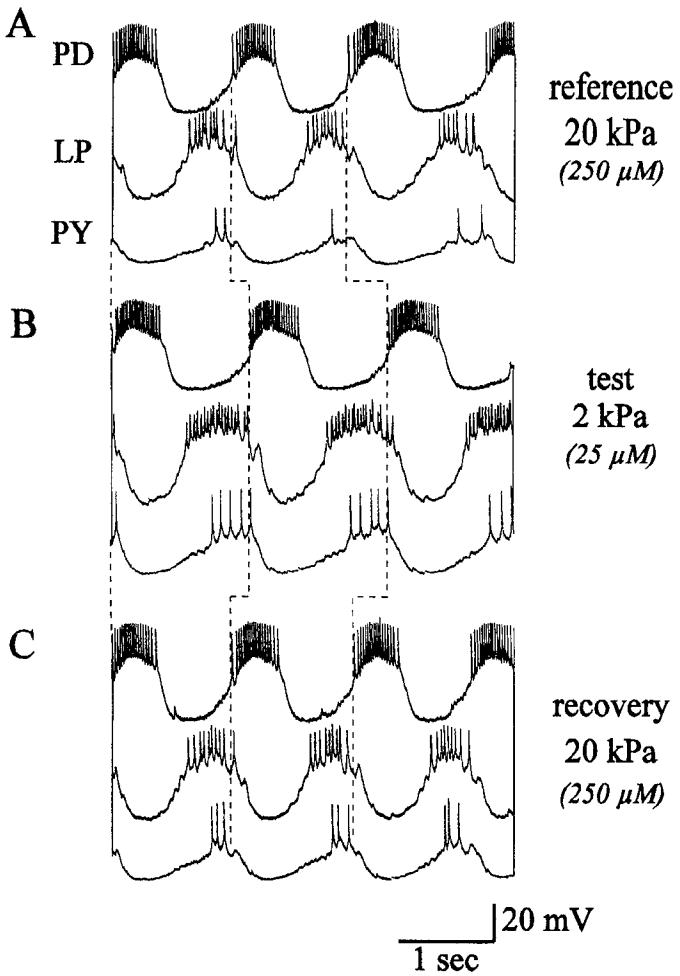

Figure 3. Effect of low but physiological Po_2 on the pyloric rhythm. The pyloric rhythm is monitored by simultaneous intracellular recordings from one pacemaker neuron (*PD*) plus the constrictor neurons *LP* and *PY*. *A*, Spontaneous pyloric rhythm at Po_2 = 20 kPa. *B*, At the low end of the physiological Po_2 range (Po_2 = 2 kPa), LP burst duration increases. This delays the onset of each subsequent burst in the pyloric pacemaker neurons and increases the pyloric cycle period. *C*, recovery at Po_2 = 20 kPa. Pyloric rhythms were recorded after 60 min superfusion with each solution. The O_2 concentrations that correspond to each applied Po_2 are shown in *parentheses*.

anatomical feature removes the need for artificial perfusion. It allowed us to study the functioning of the pyloric network by performing *in vitro* experiments in which the STG was superfused via artificial glass arteries that closely mimicked the *in vivo* situation. Because of their rich vascularization, however, the *in vitro* situation for the CoGs and OG was very different from *in vivo*. Consequently, to preserve the activity of the modulatory inputs to the STG, these ganglia were always superfused with aerated saline at Po_2 = 20 kPa and Pco_2 = 0.4 kPa.

Effects of physiological Po_2 on the pyloric rhythm

When the STG was superfused with a reference aerated saline (i.e., Po_2 = 20 kPa corresponding to an O_2 concentration of 250 μM), the pyloric network generated its rhythmic triphasic output, and the pyloric neurons showed their well described bursting activity (Fig. 3*A*). Decreasing Po_2 toward low blood physiological values shown in Figure 1*B* (for example Po_2 = 2 kPa, O_2 concentration 25 μM) modified the pyloric output (Fig. 3*B*). By comparison with the reference situation (Fig. 3*A*), two striking and reliable changes were observed. These included an increased pyloric cycle period and dramatic increases in LP and PY burst durations. Surprisingly, all pyloric neuron membrane potentials remained unchanged. Note that after such exposures (up to 6 hr in some experiments), these modifications were reversible (Fig. 3*C*). The action of Po_2 = 1 kPa (O_2 concentration 12.5 μM) on the pyloric cycle period is shown in Figure 4*A*, in comparison with the reference situation at Po_2 = 20 kPa. At 1 kPa, pyloric activity was characterized by a mean cycle period >1.2 sec (n = 294/296 in 11 different experiments). In contrast, most of the cycle periods occurring at Po_2 = 20 kPa were briefer than 1.2 sec (n = 238/330 in 11 experiments). This shows that in our experimental conditions, Po_2 = 1 kPa prevented the expression of pyloric cycle periods shorter than 1.2 sec. In addition to this global analysis, it must be noted that the percentage by which the pyloric cycle period increased was variable among experiments (32 ± 23%, mean ± SD). This nonhomogeneous result recalls previously described actions of neuromodulators on the pyloric network. Indeed, it has been shown that the actions of neuromodulators depend on the previous physiological state in the STG (Hooper and Marder, 1987; Nusbaum and Marder, 1989; Turrigiano and Selverston, 1989). Figure 4*B* shows that the low Po_2 effect was also state-dependent. Thus, the effect was minor or null in preparations exhibiting slow pyloric rhythms at 20 kPa (T > 1.4 sec) but dramatic in the most active preparations. Figure 4*C* shows that in addition to this state-dependency, the O_2 effect was also dose-dependent. This was studied while a series of seven different Po_2 plateaus was performed, which were presented in the following order: Po_2 = 20, 6, 4, 3, 2, 1, and 20 kPa. The duration of each exposure was 60 min, with the new rhythm occurring at each Po_2 attaining equilibrium within 20–30 min. It is clear that the effect on the pyloric cycle period was linked in a dose-dependent manner to the Po_2 value. The Po_2 threshold was below 6 kPa (75 μM), and the effect was maximum at the lowest tested Po_2 = 1 kPa (12.5 μM; p = 0.01, paired t tests). Note again that the O_2-induced effect was reversible, because a 5 hr exposure period at Po_2 ranging from 1–6 kPa did not lead to any statistical difference between reference (*closed symbol*) and recovery (*open symbol*) periods at 20 kPa (paired t tests).

Finally, in addition to the state- and dose-dependent effects of O_2 on the pyloric cycle frequency, O_2 also influenced the pattern of the pyloric rhythm. The change that occurred in the relative phasing and duty cycles (i.e., the fraction of the cycle during which a neuron is active) after 1 hr exposure periods at 20, 1, and 20 kPa are presented in Figure 5 (n = 8 different preparations). At Po_2 = 1 kPa, the main effect is an increase of the LP and PY duty cycles (by 50% and 150%, respectively), with an increased amount of overlap between their discharges. Additionally, PD duty cycle decreased, although it must be noted that the absolute PD neuron burst duration was statistically unchanged, whatever the Po_2 (Fig. 6*B*). Note also that (1) the absolute mean value of the silent gap between the PD and LP discharges remained constant regardless of Po_2 (0.25 ± 0.03 sec at 20 kPa and 0.24 ± 0.09 sec at 1 kPa, paired t tests), as did (2) the mean latency between the onset of the LP and PY bursts during the reference and test periods (0.25 ± 0.09 sec and 0.22 ± 0.10 sec, respectively), and of course (3) the sum of both delays, which corresponds to the latency between the end of the inhibitory PD burst and the onset of the PY firing (also see Fig. 3).

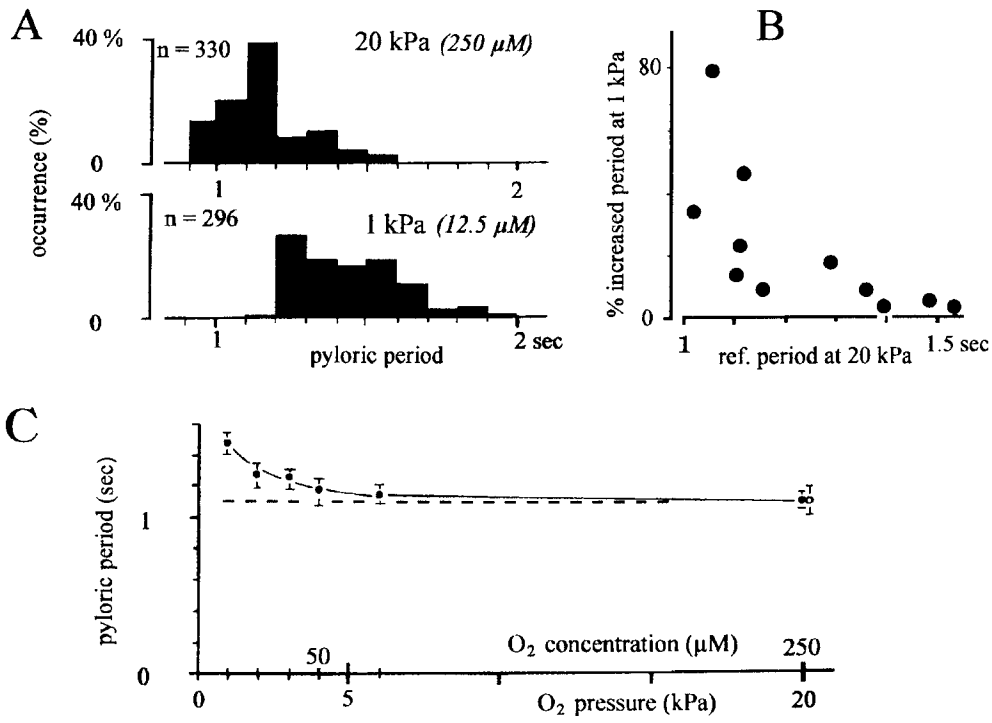

Figure 4. Characterization of the O_2 effect on the pyloric network. *A*, Distribution of pyloric cycle period at Po_2 = 20 kPa and after 60 min at Po_2 = 1 kPa (n = 11 preparations). At Po_2 = 20 kPa, 75% of the cycle periods are <1.2 sec, whereas at Po_2 = 1 kPa, all pyloric cycle periods are >1.2 sec. O_2 concentration that corresponds to the applied Po_2 is shown in parentheses. *B*, The O_2 effect is state-dependent. Percentage increase in pyloric cycle period at Po_2 = 1 kPa as a function of the reference cycle period at 20 kPa. Each data point is the mean value of >30 cycles from 11 preparations. *C*, The O_2 effect is dose-dependent and reversible in O_2-sensitive preparations. Data represent mean ± SE. *Closed circles* indicate reference and test values, and *open circle* indicates recovery value (n = 8 preparations).

Specificity of the network O_2 effect via a unique neuron, the LP neuron

The above data set showed that the pyloric cycle period increased by ~30% at low physiological Po_2 when compared with the standard, albeit artificial reference at 20 kPa. We next addressed the issue regarding the origin of this altered pyloric rhythm. Specifically, we examined whether the change in cycle frequency resulted from a direct O_2 effect on the pacemaker group or as an indirect result of its influence on other network components. With regard to the latter possibility, it seemed more likely that a key element of the O_2 actions would be the LP neuron and not the PY neurons, because only the LP neuron directly inhibits the pacemaker group and could thereby delay their firing (Fig. 1*D*). Consequently we focused our attention first on the LP neuron and the pacemaker group.

Figure 6 presents a burst analysis for these two cellular groups. It is evident from these data that the decreased Po_2 influenced the LP burst duration considerably more than the PD burst duration (Fig. 6*A–B*). Specifically, between Po_2 = 20 and 1 kPa, the LP burst duration increased significantly (+87%: from 0.31 ± 0.02 sec to 0.58 ± 0.06 sec; n = 7 preparations). In contrast, the PD burst duration did not change statistically (0.45 ± 0.05 sec at 20 kPa and 0.49 ± 0.03 sec at 1 kPa). Interestingly, the intraburst firing frequency remained independent of Po_2 in both neurons. The LP firing frequency was 33.5 ± 3.4 Hz at 20 kPa (n = 9) and 32.0 ± 4.5 Hz at 1 kPa (n = 7; Fig. 6*C*), whereas for PD at the same O_2 partial pressures, it was 45.1 ± 3.3 Hz (n = 7) and 43.7 ± 1.7 Hz (n = 5; Fig. 6*D*), respectively. This absence of any increase in firing frequency contrasts strongly with what is known in so-called hypoxic or asphyxic preparations. It suggests, together with the recovery pattern shown in Figures 3, 4*C*, and 5, that cellular integrity was maintained in our experimental preparations regardless of the Po_2 value. Because the LP neuron seemed to be the only network neuron whose activity was both modified by O_2 in the studied range and able to delay the firing of the pacemaker group, we tested its role at low Po_2 by (1) manipulating its burst duration and (2) deleting it from the network.

Figure 7 presents a typical experiment in which we studied the effect on the pyloric rhythm of injecting hyperpolarizing pulses of current (2–3 nA) in LP to shorten its burst duration at 1 kPa (n = 3 experiments). It illustrates again that at Po_2 = 1 kPa, the pyloric cycle period increased (compare Fig. 7, *A* and *B1*), but more importantly, that under these conditions the shortening of the LP burst reversed the O_2-induced slowing of the pyloric rhythm (compare Fig. 7, *B1* and *B2*). This suggested that it was not the cyclic activity of the pacemaker group by itself that was directly responsible for the decreased cycle frequency at low Po_2. Instead, the increased LP burst duration apparently inhibited the pacemaker group sufficiently well to slow its bursting activity. To confirm this LP-mediated mechanism, we experimentally deleted LP from the network by photoinactivation (see Materials and Methods). For this experimental series (n = 4 experiments), we used preparations with fast pyloric rhythms under control conditions, because it is in this situation that the O_2 effect is strongest (Fig. 4*B*). It must be noted at first that after LP deletion the remaining pyloric cells still generated rhythmic activity, with an alternation between bursts in the PD and PY neurons and a constant cycle period (Fig. 8*B1*). In these LP-deleted preparations, when Po_2 was reduced to 1 kPa, there was no increase in the pyloric cycle period (compare Fig. 8, *B1* and *B2*). Even after long-term exposures to low Po_2 (>1 hr), the LP-deleted pyloric rhythm remained unchanged. Figure 9*A* presents a plot of the pyloric cycle periods pooled from the four experiments. Contrary to what occurred in the intact network (Fig. 4*A*), the LP-deleted network displayed a constant pyloric cycle period regardless of Po_2 (1–20 kPa). This implies that the low Po_2 had no direct effect on AB, PD, and PY activity. Moreover, although in the intact network the pattern of activity was reliably modified at 1 kPa (Fig. 5), this effect disappeared when LP was deleted (Fig. 9*B*). Thus,

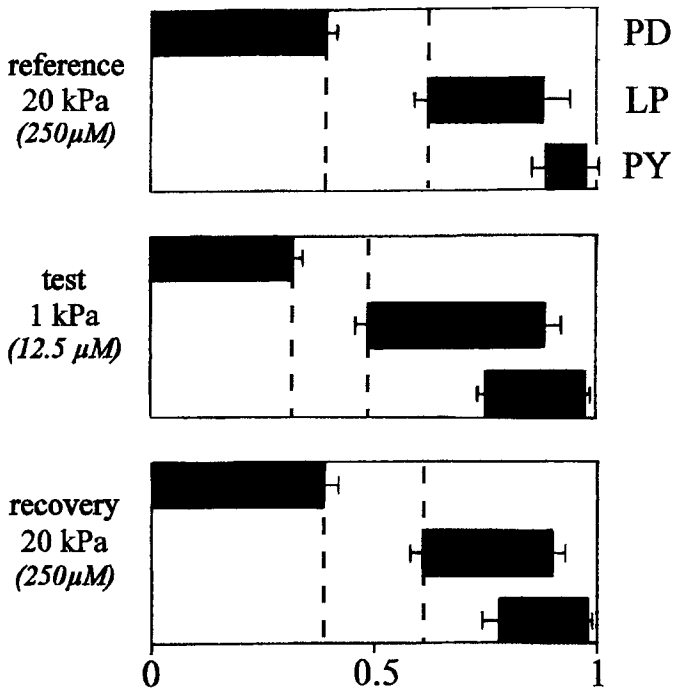

Figure 5. Normalized phase relationship of the pyloric neurons under different Po_2 levels. The global pyloric activity pattern is altered in a reversible manner between Po_2 = 20 kPa (*reference*) and 1 kPa (*test*). *Bar diagram* represents mean phases of the pyloric neuron bursts during one normalized cycle of the pyloric rhythm. Phase onset and phase offset are calculated as the fraction of the cycle that has elapsed before the onset and offset, respectively, of the burst in a pyloric neuron divided by the cycle period. The onset of consecutive bursts in the PD neuron is arbitrarily designated as the beginning and end, respectively, of a pyloric cycle. Mean ± SE (n = 8 preparations).

without the LP neuron, the pyloric network became insensitive to O_2 changes in the studied range.

DISCUSSION

The present results show that changes in O_2 levels over the physiological range (Po_2 = 1–6 kPa; corresponding to $[O_2]$ = 12.5–75 μM) reversibly alter the output of the lobster pyloric network in a manner similar to neuromodulatory transmitters. Our data demonstrate that (1) the pyloric rhythm remained unchanged when Po_2 varied from 6–20 kPa, (2) the pyloric cycle period increased in a reversible manner by ~30–40% when Po_2 decreased from 6 kPa to 1 kPa, and (3) the relative phasing and duty cycles of the three pyloric neuron subsets were altered when Po_2 was decreased. These effects were dose- and state-dependent. Among the 12 pyloric neurons, O_2 acted specifically via the single LP neuron. Thus, rather than globally changing the activity of all pyloric network neurons, the O_2 effect was quite similar to that reported previously for exogenously applied modulatory transmitters in the STNS (Hooper and Marder, 1987; Harris-Warrick et al., 1992).

Specificity of the LP neuron in the O_2 network effect

When the STG was superfused with Po_2 in the physiological range, we observed dramatic effects on both the cycle period and the pattern of the pyloric rhythm relative to the rhythm in the presence of standard, aerated saline. As discussed below, we found that the LP neuron played a key role in these effects. This conclusion comes from experiments performed in intact (Figs. 3–6) and LP-deleted networks (Figs. 7–9). It will be interesting to determine whether Po_2 changes directly influence the LP neuron. Synaptic interactions within the pyloric network are fairly complex, and the way in which a neuron responds to a neuroactive substance when that neuron is embedded in a network can differ from its response when isolated from that network. For example, in the STNS, dopamine and proctolin indirectly activate the PD neurons when they belong to the intact network, but dopamine inhibits and proctolin is without effect when these neurons are isolated experimentally and therefore influenced only directly (Flamm and Harris-Warrick, 1986a,b; Hooper and Marder, 1987).

Although the cellular mechanisms of O_2 action on LP were outside the scope of this paper, we have presented the effects induced by decreasing Po_2 on the pyloric rhythm cycle frequency and on the phasing and duty cycle of the pyloric component neurons. We suggest that the increased pyloric cycle period is entirely an LP-mediated effect. Specifically, in the intact network at low Po_2, the increased LP burst duration enhanced its synaptic inhibition of the pacemaker neurons (PD and AB), thereby delaying the onset of each subsequent burst in the pacemaker neurons and increasing the pyloric cycle period. On the contrary, at Po_2 = 20 kPa, the LP inhibition of the pacemaker neurons

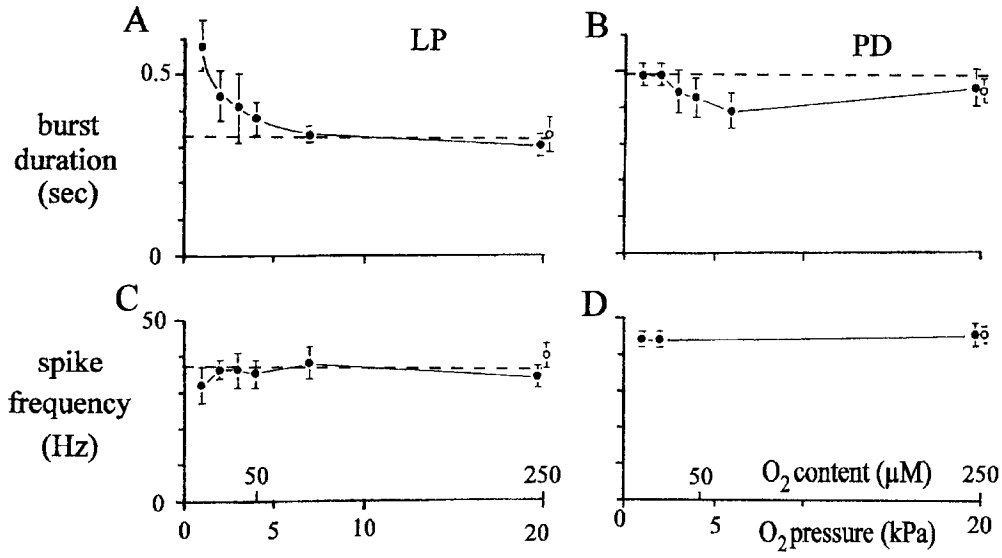

Figure 6. Low physiological Po_2 levels increase, reversibly, the LP neuron burst duration (*A*) but not the PD burst duration (*B*). The change in Po_2 does not modify the intraburst spike frequency in either *LP* or *PD* (*C*, *D*). Each mean value was measured at the end of a 60 min exposure period. Mean ± SE (n = 7–8 preparations). *Closed circles* represent reference and test values, and *open circle* indicates recovery value.

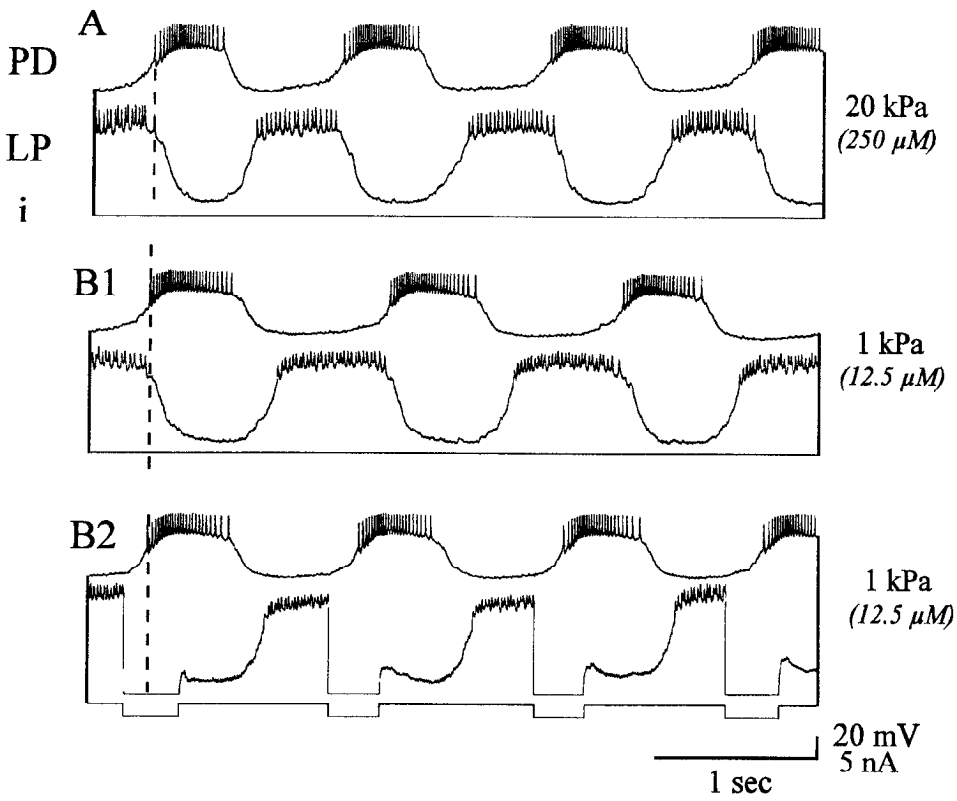

Figure 7. Reducing the duration of the LP burst via hyperpolarizing current injection at low Po_2 levels restores the pyloric period toward its value at high Po_2. *A*, Intracellular recordings of the PD and LP neurons at Po_2 = 20 kPa. *B1*, The LP burst duration and the delay between subsequent PD bursts are increased at Po_2 = 1 kPa. *B2*, The rhythmic reduction of the LP burst at Po_2 = 1 kPa by rhythmic hyperpolarizing current injection (*i*) reduced the pyloric period.

seems to have no regulatory effect on the pyloric rhythm. Indeed, when LP was deleted from the network at this high unphysiological Po_2, the PD cycle period remained unchanged (Figs. 8*A*,*B*1, 9*A*). Obviously, this O_2-related change in LP synaptic efficacy requires further study. Nevertheless, the present work reports the first documented modulation of the pyloric rhythm that alters the pyloric cycle period via an indirect influence on the pacemaker neurons. Previously, several sources of modulatory input have been shown to influence the pyloric cycle period via a direct effect on the pacemaker neurons (Nagy and Dickinson, 1983; Hooper and Marder, 1987; Nusbaum and Marder, 1989; Cazalets et al., 1990a,b).

It is also noteworthy that different mechanisms were responsible for the modifications of phasing and duty cycle in PD and PY, and yet their intrinsic properties were O_2-insensitive, as demonstrated in the reduced network (Fig. 9*A*,*B*). Thus, the PD duty cycle decreased in the intact network at low Po_2 (because the pyloric period increased and the PD burst duration was unchanged), whereas both the PY burst duration and the duty cycle increased. Any explanation for the latter effect remains speculative, but because the latency between the onset of the LP and PY neuron bursts remained constant when Po_2 decreased (Fig. 5), it seems likely that the LP inhibition of the PY neurons is time-dependent. Time-dependent synaptic effects are known to occur within the STG. Indeed, depolarization of a presynaptic neuron via a long-lasting plateau evokes a biphasic response in the postsynaptic neurons that includes an early transient sharp peak followed by a smaller sustained response (Graubard et al., 1983). In the present situation, we propose that when the LP burst was shortened at Po_2 = 20 kPa, only the initial transient component was expressed, and it evoked a strong and constant inhibition of the PY neurons. Conversely, at low Po_2, the LP burst duration increased and allowed the activation of the second, weaker inhibitory component. This latter component may be unable to sustain reliably the inhibition of the PY neurons, and a longer PY burst duration occurs. It must now be explained, however, how the LP neuron can fire despite the classical view that the PY neurons inhibit LP. For this, it must be kept in mind that the PY population can be divided into two subpopulations. The first one inhibits the LP neuron and has been reported to systematically fire after the LP burst. The second group does not inhibit LP (Maynard, 1972; Hartline and Gassie, 1979; Eisen and Marder, 1984). An increase in pyloric period at low Po_2 could simply allow the firing of this second type of PY neuron during the second weaker part of the LP burst. In this respect, note that the time latency between the end of the inhibitory PD burst and the onset of the PY firing remains constant, whatever the Po_2. This means that the inhibition by PDs of the PYs remains constant. In the present work, because the different PY neurons are difficult to identify, we treated them as a single population.

In summary, the selective alteration of LP neuron activity accounts for all of the O_2 effects on the pyloric network, and an LP-deleted network becomes O_2 insensitive. At physiological Po_2, the LP neuron has two functional roles. It changes the phase relationships in the pyloric network, and it reduces the network activity by increasing the pyloric rhythm cycle period.

Why reduce neuronal activity at low Po_2?

During the pyloric rhythm, the membrane potential of each neuron oscillates, generates spikes, and receives rhythmic synaptic inputs. Each of these events changes the membrane potential via a change of ionic fluxes for which there must be active compensation to maintain electrochemical gradients. Reuptake, resynthesis, and repacking of neurotransmitters also require a constant flux of energy. The physiological processes involved in this perpetual rebuilding represent an energy expenditure that is difficult

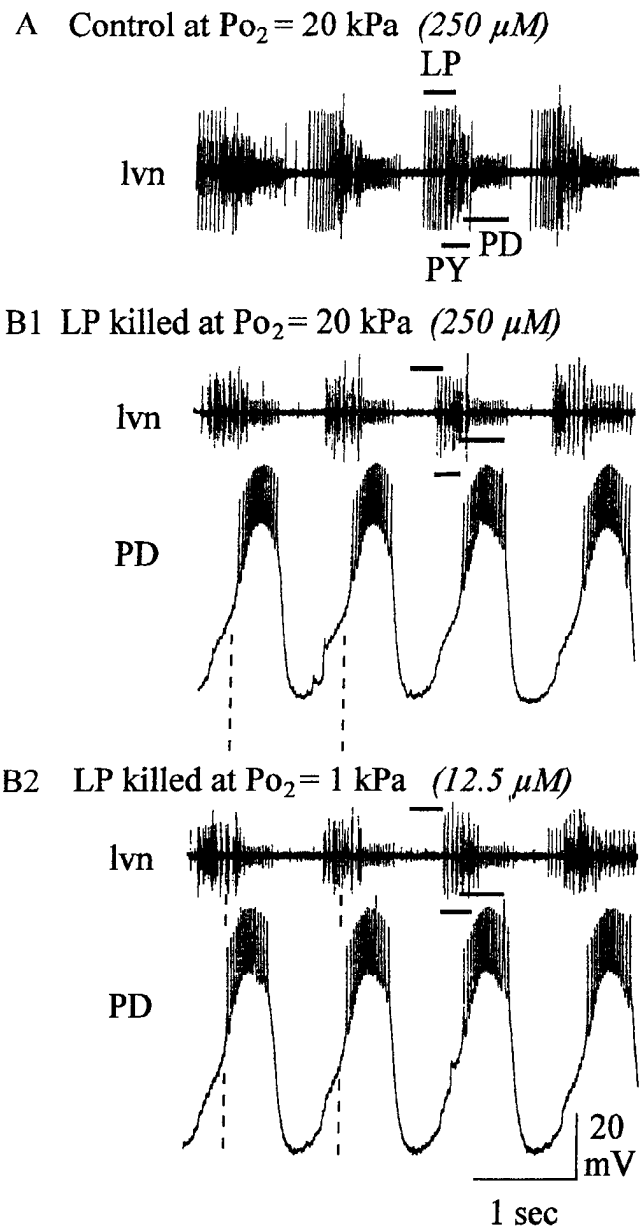

Figure 8. The pyloric network loses its O_2 sensitivity after experimental deletion of the LP neuron. *A*, The pyloric rhythm is monitored extracellularly (*lvn*) in an intact pyloric network at Po_2 = 20 kPa. Under these conditions, the pyloric network generates ongoing rhythmic activity involving LP (*largest spikes*), PY (*middle spikes*), and PD (*smallest spikes*) neurons. *B1*, After photoinactivation of LP and with Po_2 = 20 kPa, the pyloric network continues to generate spontaneous rhythmic output in which the PY neurons (*lvn*) burst in alternation with the PD neurons (recorded intracellularly and *lvn*). *B2*, A 60 min exposure period with saline at Po_2 = 1 kPa has no effect on the pyloric cycle period of the LP-deleted network. All records were from the same preparation.

to estimate but is several times higher in nervous tissue than elsewhere (for review, see Siesjo, 1978). For example, in resting nerve of crab, the O_2 consumption that reflects this energy expenditure has been estimated to be $\approx 70\ \mu mol \cdot min^{-1} \cdot kg^{-1}$ (Treherne, 1966). In contrast, the mean value for all tissues, as measured in intact resting crustaceans, is only 15–20 $\mu mol \cdot min^{-1} \cdot kg^{-1}$ (Forgue et al., 1992b). In the mammalian brain, it is estimated that >50% of the energy released is used just for active Na-K transport (Clausen et al., 1991). Similar figures were proposed in a crustacean preparation: Giacobini (1965) showed that ouabain induced a 40% decrease in O_2 consumption in an isolated crayfish stretch receptor. Surprisingly, despite this important specific O_2 requirement, it is remarkable that in all the crustaceans that we studied (as well as in some fishes and molluscs, i.e., in the three main groups of water breathers; Forgue et al., 1992b), the arterial Po_2 in unfed animals at rest is regulated in the 1–3 kPa range, as illustrated in Figure 1*B* for *H. gammarus*. This corresponds to an apparent set-point, which is maintained regardless of Po_2 in the inspired water in the 3–40 kPa range (Massabuau and Burtin, 1984; Forgue et al., 1989; Massabuau et al., 1991). In many physiologically different water breathers, this constancy of the O_2 status in the *milieu intérieur* in the low range appears as a characteristic property of gas-exchange regulation.

In vivo, under unfed conditions, i.e., at low arterial Po_2, the pylorus is not as rapidly cycling as it is in postprandial conditions (Rezer and Moulins, 1983). *In vitro*, we found that superfusion at this low arterial Po_2 level slows down the rapidly cycling pyloric rhythm observed at 20 kPa (Figs. 3, 4*A*,*C*). Therefore, we propose that the mechanism of O_2 action that we report here is a way to reduce energy expenditure when a high level of neuronal activity is not required. *In vivo*, we are currently studying the blood gas changes that occur after a meal of lobster and their role in the postprandial, increased pyloric frequency.

Comparison with previous data

Our results do not deal with pathological events and therefore must be distinguished from studies of cerebral hypoxia (Siesjo, 1978; Choi, 1990). Indeed, we observed in *H. gammarus* that bathing the STG with exceptionally low Po_2, between 0 and 1 kPa, had significantly different effects from those shown here. Briefly, in the presence of these exceptionally low Po_2 levels, large changes in the global pattern of pyloric activity started within the first hour. The activity pattern then became disrupted, and the membrane potentials of the pyloric network neurons became lightly depolarized (Massabuau and Meyrand, unpublished observations). Finally, it is important to recall that in the present experiments we never observed rapid changes in membrane potential and/or increases in firing frequency, as is usually seen in preparations exposed briefly to anoxic conditions (Leblond and Krnjevic, 1989).

Although the presence of a physiological, nonlethal, and reversible O_2 effect on a neuronal network seems original, the idea that tissues *in situ* could be O_2-limited, i.e., in a state of permanent "slight hypoxia," has been proposed previously for various mammalian cell types (Rosenthal et al., 1976; Siesjo, 1978; Rennie, 1983; Chinet and Mejsnar, 1989). Moreover, regarding the brain tissue, some authors suggested that it could work *in situ* on the verge of O_2 insufficiency (Davies and Bronk, 1957; Rosenthal et al., 1976). Indeed, as stressed earlier, in every organ that was studied, most tissue Po_2s are in the low range, whereas this is (1) very close to, or even below, K_mO_2 for many O_2-dependent reactions (Vanderkooi et al., 1991) and (2) just above the critical Po_2 below which O_2 consumption falls in isolated mitochondria (Chance, 1957; Jobsis,1972). To our knowledge, however, we have presented here the first study demonstrating that physiological levels of O_2 can modulate neural network activity.

Conclusion

Present data demonstrate that the physiological Po_2 occurring at the cellular level can induce fundamental functional changes in neural network activity. We propose that in resting *H. gammarus*,

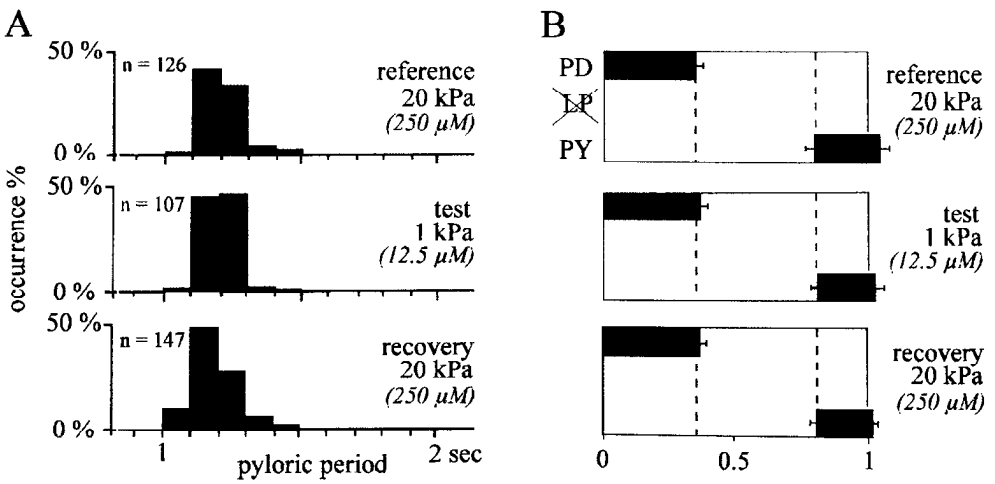

Figure 9. Characterization of the O_2 effect on the pyloric network after experimental deletion of the LP neuron. *A*, Distribution of pyloric cycle periods at Po_2 = 20 kPa (*top*), after 60 min at Po_2 = 1 kPa (*middle*), and after 60 min recovery at Po_2 = 20 kPa (*bottom*) (n = 30 cycles/preparation; n = 4 preparations). *B*, Normalized phase relationship of the remaining pyloric neurons at the same Po_2 levels as in *A*. Mean ± SE (n = 4 preparations). Compare Figure 9*A* with Figure 4*A*, and Figure 9*B* with Figure 5.

by increasing the pyloric rhythm cycle period, the LP neuron forces the network to slow down, to function at a lower energy cost, and to be less sensitive to a limitation in the O_2 supply. Such a mechanism could be a key adaptation for tolerating hypoxia in sensitive neural networks that are operating in poorly oxygenated environments.

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