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Carbon dioxide sensitivity and its role in multifunctional neurons in the mollusk *Onchidium*

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ABSTRACT

Intrinsically photoresponsive neurons in the abdominal ganglion of the amphibious mollusk *Onchidium* named Ip-1 and Ip-2 (Ip-1/2) react to several different stimuli. These neurons respond to light with slow hyperpolarization and to CO_2 stimulation with slow depolarization. In this study, increasing the concentration of CO_2 in the air caused hyperventilation and enlargement of the pneumostome in the intact animal. In a semi-intact preparation, pouring artificial seawater (ASW) with dissolved CO_2 onto the central ganglia caused the previously closed pneumostome to open. In an ASW environment, Ip-1/2 neurons depolarized even under conditions of constant pH (alkaline ASW) and after dissolution of CO_2. This depolarization prolonged the firing of action potentials in Ip-1/2 neurons. Adding protons (H^+) to ASW caused Ip-1/2 depolarization only when the neurons' membranes were depolarized to a potential above the resting potential. Furthermore, in the presence of the carbonic anhydrase inhibitor acetazolamide (AZ), CO_2-induced excitation in Ip-1/2 neurons was increased in both normal and alkaline ASW. These results suggest that when dissolved in ASW, CO_2 directly induced the depolarizing response in Ip-1/2 neurons. Since Ip-1/2 neurons participate in pneumostome opening, these results suggest that increased CO_2 levels in ASW directly stimulate CO_2-sensitive central neurons, promoting ventilation.

1. Introduction

Ip-1 and Ip-2 (Ip-1/2) neurons in the abdominal ganglion, a constituent of the central ganglia of the amphibious mollusk *Onchidium,* are multifunctional. These neurons intrinsically respond to light with hyperpolarization and act as secondary neurons, receiving inhibitory presynaptic inputs from stimuli such as water pressure and/or touch arising from the body surface. Furthermore, Ip-1/2 neurons are also involved in air-breathing behavior (pneumostome activity) when the animal is on land (Gotow and Nishi, 2009; Shimotsu et al., 2010). Because this amphibious species stays underwater during high tide, many inhibitory inputs to Ip-1/2 neurons are necessary to prevent the pneumostome from opening in the water.

Respiration (inspiration of O_2 and expiration of CO_2) is a common animal behavior. High concentrations of CO_2 in the air will induce hyperventilation up to a level approaching suffocation. A simple model of respiration is found in the related pulmonate mollusk *Helix*. There are many CO_2-sensitive neurons in the central ganglia of *Helix,* for which focal stimulation with CO_2 causes increased ventilation. Both mechanisms of increasing ventilation are reminiscent of the central control of respiration in vertebrates (Erlichman and Leiter, 1997; Putnam et al., 2004).

It has been believed that CO_2-induced adjustments in ventilation are related to protons (H^+) in solution. Since CO_2 in water produces H^+ (Loeschcke, 1982), a cell's sensitivity to CO_2 is commonly represented by its H^+ detection ability (Huckstepp and Dale, 2011). However, neurons directly sensitive to gaseous CO_2 have been discovered in the rat olfactory system (Hu et al., 2007), as well as in *Drosophila* (Suh et al., 2004) and the nematode *Caenorhabditis elegans* (Hallem and Sternberg, 2008). Taken together, these data render plausible the hypothesis that CO_2-sensitive neurons are also part of the respiratory system (Huckstepp and Dale, 2011).

This study was performed to investigate the effects of CO_2 on the multifunctional, photoresponsive Ip-1/2 neurons of the amphibious mollusk *Onchidium*. Furthermore, the potential for direct CO_2 stimulation of these neurons and its putative contribution to ventilation in *Onchidium* was also studied and discussed.

2. Materials and methods

2.1. Biological material

Specimens of the marine gastropod mollusk *Onchidium verruculatum* weighing 10–20 g were collected from the intertidal zone of Sakurajima, Kagoshima, Japan. The animals were kept in a natural seawater aquarium (20 °C) under a 12:12 LD cycle. Under these conditions, the animals tend to reside underneath a rock in the tank for extended periods without feeding, and can survive this way for more than 3 months. The

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abdominal ganglion was exposed by dissecting the mid-dorsal surface of the animal, and was isolated after the overlying connective tissue had been removed. The ganglion was pinned on the silicone rubber base of a 1.5 ml chamber and perfused continuously with saline using a gravity-driven perfusion system at a rate of 1 ml/min. The procedure used to prepare and condition neurons was similar to that described previously (Nishi and Gotow, 1992, 1998; Shimotsu et al., 2010). In some experiments, both semi-intact (reduced) and intact animal preparations were used, to examine the correlation between the observed electrophysiological and behavioral phenomena. For the reduced preparation, an animal was initially anesthetized by cooling in crushed ice. The cerebral ganglia were isolated along with abdominal nerves 1 and 2, and then dissected to prepare a dorsal side up, semi-intact preparation. The experimental procedures were begun about 1 h after the dissection.

2.2. Solutions

For the normal saline bath, artificial seawater (ASW) containing 450 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, and 10 mM $CaCl_2$ was used. For the control ASW, 10 mM Tris buffer adjusted to pH 7.80 using HCl was added. Ca-free (Nishi and Gotow, 1992) and high (50 mM) Mg (Gotow, 1985) ASW were used to examine synaptic transmission. CO_2 gas (100%) was dissolved in ASW, and the final concentration of CO_2 was measured with a CO_2 meter (DKK-TOA Co., CGP-1, diaphragm method) sensitive to a minimum concentration of 0.01% CO_2. The pH of the solution in the chamber was continuously measured by a micro pH electrode (OD 1.5 mm, Microelectrodes Inc. MI-508) located next to the preparation. The output of the pH sensor was converted to DC voltage changes and recorded with other electrical signals via a DAT tape recorder. The relationship between pH and concentration of CO_2 in ASW was determined after estimating the CO_2 concentration in the solution, as shown in Fig. 1. As long as any exchange of solutions was done at the same time point, the pH and CO_2 concentrations would change in an identical manner. For simplicity, all figures use the same representation for such changes. All saline baths were maintained at 22–24 °C.

2.3. Drugs and adjustment of pH of the solution

The carbonic anhydrase inhibitor acetazolamide (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and added to saline. The final concentration of DMSO was 0.01%. To investigate the effect of pH in ASW (e.g., Figs. 5 and 7D), the final pH was adjusted to 5.8 by 1 N HCl. To examine the composite effect of CO_2 and H^+ (Figs. 6 and 7C), 1 N NaOH was added after dissolving CO_2 in the solution. In the final solution, the change in CO_2 concentration upon adding NaOH was less than 0.5%, and the change in osmolarity was negligible (about 2 mM Na^+ was added).

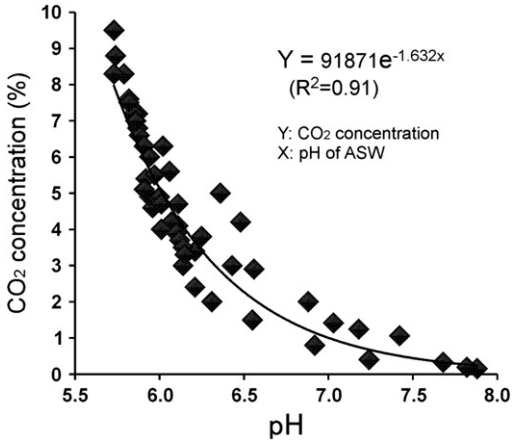

Fig. 1. The relationship between CO_2 concentration and pH in the control artificial seawater (ASW). The concentration of CO_2 was plotted against the pH of the ASW. The result is presented as an approximation curve and an accompanying equation calculated from the data shown in the figure.

2.4. Recording and stimulation

The methods used for recording voltage and for passing current through one electrode have been described previously (Nishi and Gotow, 1992). Briefly, one glass microelectrode filled with 2.5 M KCl, and with a resistance of less than 5 MΩ was inserted into the neuronal soma under visual control. A differential electrode (Ag–AgCl) was placed in the bath. Signals were recorded and displayed using a differential DC amplifier (CEZ-3100, Nihon Kohden) and an oscilloscope coupled via a bridge circuit, and stored using a digital audio tape (DAT) recorder for later analysis.

2.5. Quantification of membrane activity

In order to quantify membrane activity, I measured burst durations as an indicator of depolarization using the schema described in Fig. 2. Briefly, the raw sums of the burst durations were normalized to a per-minute basis. Normalized values were averaged for each condition, and then compared statistically via two-tailed Student's *t*-test. Comparisons were made between the control ASW and the 5% CO_2 ASW conditions, in either normal (series 1) or pH-constant (series 2) solutions (mean ± SEM, *n*; the number used represents multiple preparations). In addition, the above burst duration values for the 5% CO_2, pH-constant condition were compared between the presence or absence of AZ (series 3; mean ± SEM, *n*; the number used represents multiple preparations).

3. Results

3.1. Acceleration of ventilation induced by high CO_2 conditions in both intact and semi-intact preparations

The *Onchidium* is fully amphibious and lives in intertidal zones. For respiration, it uses a gill (gill-tree) in the water at high tide, and a pneumostome (an orifice of the lung) on the land at low tide. Normally, the pneumostome opens for several minutes, and only occasionally closes for a short time (several seconds), unlike the repetitive opening/closing alternations seen in the terrestrial snail *Helix* (Sommerville, 1973).

Fig. 3A shows the *Onchidium* in the experimental environment. The pneumostomal opening enlarged in response to an increase in the concentration of CO_2. The *Onchidium* would raise its labial palps and move around actively in this condition (5% CO_2 in air). *Onchidium* can survive at least 15 min in this condition.

The photograph in Fig. 3B demonstrates the semi-intact (reduced) preparation from the lateral side. The central (circumesophageal) ganglia with abdominal nerves 1 and 2 were exposed in a small pocket on the experimental plate. Under this condition, the pneumostome closed [Fig. 3C(1)]. Then ASW with 5% CO_2 dissolved in it was poured over the ganglia, inducing the pneumostome to open after about 15 s of exposure to the ASW [Fig. 3C(2)]. The pneumostome remained open even after the solution was replaced with control ASW. Similar results were obtained from five reduced preparations using the same procedure as described above. These results support the idea that the CO_2-sensitive Ip-1/2 neurons promote ventilation in hypercapnic conditions.

3.2. The relationship of pH and concentration of CO_2 in the solution

To determine the concentration of CO_2 in the solution, I utilized the relationship between pH and CO_2 concentration. As CO_2 is dissolved in ASW, the pH of the ASW decreases due to the production of H^+. These

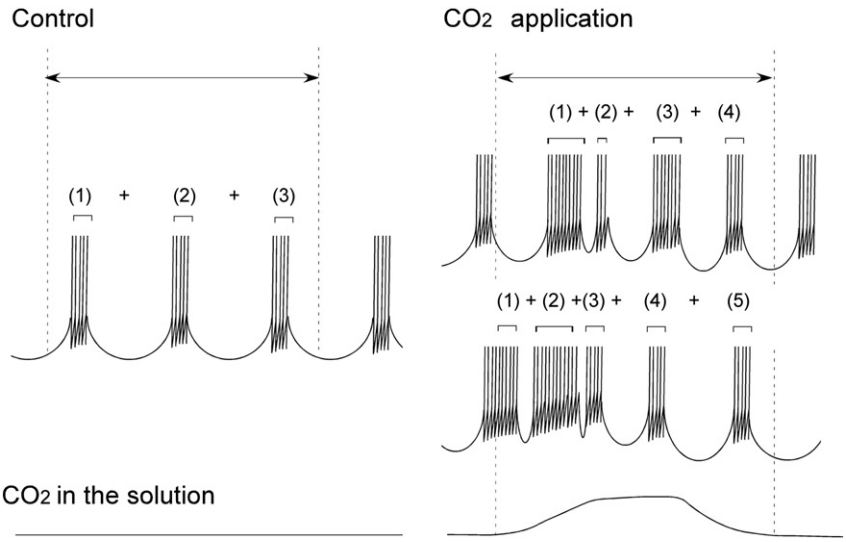

Fig. 2. Schematic illustration showing the analysis of burst firing. The sums of durations of repetitive firings were determined for control ASW (1) + (2) + (3) and experimental solutions (1) + (2) + (3) + (4), and when CO_2 was administered in the solution during the time course of the firings in (5). Durations of firings in the control were adjusted to the period in which CO_2 gas was dissolved in the solution. The sums of firing durations were calculated and normalized to a 1-min time period, and then compared between the control and CO_2-containing ASW conditions.

protons are generated in proportion to the duration of CO_2 gas application. A decrease in pH caused by CO_2 indicated that the buffering capacity of the Tris–HCl had been surpassed. The concentration of CO_2 reached saturation at about 10% in ASW. This saturated value is a far lower concentration than that used in a previous study of *Aplysia* (Brown and Berman, 1970), in which 50% CO_2-ASW was utilized. The difference in the saturating concentration of CO_2 may reflect the experimental conditions: I began the experiments about 1 h following the preparation of the CO_2-ASW, whereas the *Aplysia* study added the CO_2 gas to ASW just before the reservoir was placed in position. The CO_2 concentration in a solution is thought to decrease exponentially; thus, 5% CO_2-ASW could be stably maintained in this study. The concentration of CO_2 in the control ASW was plotted against the pH in Fig. 1, and the exponential fitting curve was produced empirically from these data.

3.3. Membrane responses to CO_2 application in the solution

The photoresponsive neurons Ip-1 and Ip-2 are located side by side in the peripheral region of the abdominal ganglion. Their appearances and photoresponsive characteristics, including the threshold to light stimulation and time course of responses, are almost identical.

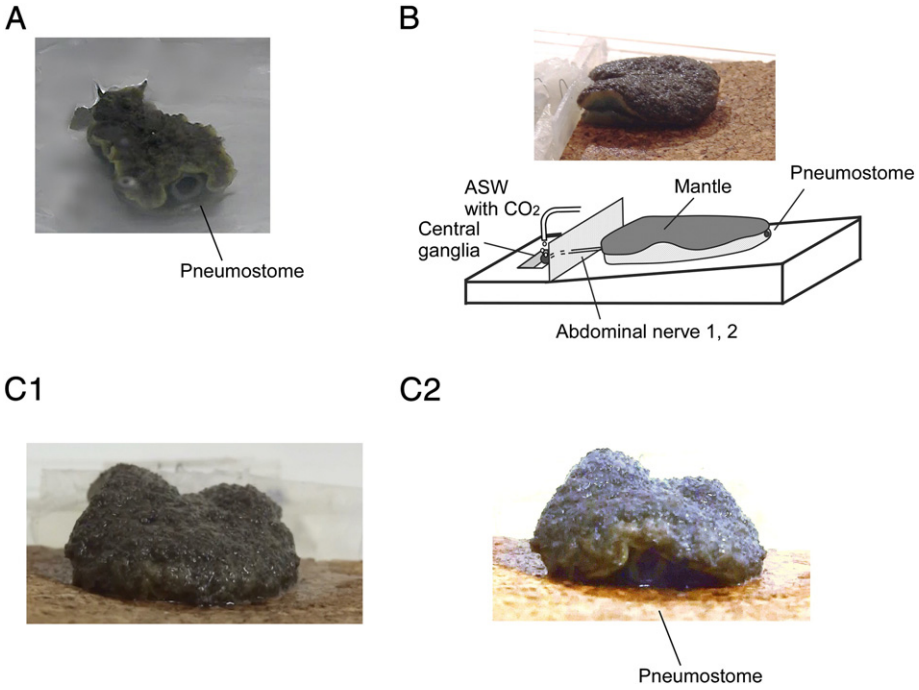

Fig. 3. Pneumostome opening evoked by CO_2 in intact and semi-intact preparations. A: An increase of CO_2 in air (5%) caused a larger pneumostome opening than that in normal conditions, as observed in the intact animal preparation. B (upper panel): A photograph of the semi-intact preparation taken from the lateral side. The left side of the mantle kept rising. The central ganglia with abdominal nerves 1 and 2 were exposed on the chamber and covered with wet gauze to prevent drying. The truncated posterior of the animal was on a tilted plane. B (lower panel): A schematic diagram of the experimental setup. C1: Photograph taken from the posterior side. C2: When CO_2-containing artificial seawater (ASW) was poured onto the ganglion, it evoked opening of the pneumostome with a short time delay (about 15 s) from the start of pouring.

Furthermore, Ip-1 and Ip-2 neurons are usually connected by electrical synapses (Shimotsu et al., 2010). Thus, in this study, I did not discriminate between the two neurons, and instead referred to them as Ip-1/2 neurons because they can be considered as a single functional unit.

Their resting membrane potential was around −48 mV (Nishi and Gotow, 1998). In control ASW, the membrane activities of Ip-1/2 neurons without any stimuli showed three different firing patterns, either spontaneous regular or irregular bursting, or beating. Fig. 4A shows an example of a relatively regular bursting pattern. The repetitive firing continued for a few minutes, and a silent period followed immediately. The duration of the repetitive firing and subsequent silent period varied among preparations, often with both firing and silent durations lasting more than 1 min. The threshold of firing was about 3 to 5 mV above the resting potential, though this too varied among preparations. Fig. 4B shows an example of an irregular bursting pattern, in which the duration of repetitive firing and silent periods was not constant. Fig. 4C shows a beating pattern of membrane activity. The interspike intervals were longer in the beating pattern than in the bursting pattern. The reasons for the difference in spontaneous membrane activities among preparations are still unclear, but they were dependent on neither the weight of the animal nor the duration of its stay in the laboratory. The bursting pattern of membrane activity was more commonly observed in summer than in winter, and may indicate a seasonal difference in endocrine activity, perhaps relevant to reproduction (Nishi, 2013).

When CO_2 was dissolved in the solution, the membrane potential of Ip-1/2 neurons was depolarized in parallel with the change in pH of the solution. CO_2-induced responses occurred regardless of background membrane activity (i.e., bursting/beating). The pH of the solution reached the minimum value about 2 min after introducing CO_2. I examined the effect of 5% CO_2 on Ip-1/2 neurons, similar to the value used for studies in other molluscan CO_2-sensitive neurons, including those of *Helix* (Erlichman and Leiter, 1993) or *Lymnaea* (Inoue et al., 2001).

As shown in Fig. 4, the ASW contained about 5% CO_2 when the pH of the solution was about 6.0. At a membrane potential of −40 mV, depolarizing responses of 3–5 mV occurred [Fig. 4(1)]. CO_2-induced depolarization became smaller as the membrane potential was hyperpolarized [Fig. 4(2)], such that the reversal potential was estimated to be around −55 mV [Fig. 4(3)]. CO_2-induced depolarizing responses were also produced in Ca-free (Nishi and Gotow, 1992) or high Mg (Gotow, 1985) conditions where the chemical synaptic inputs were blocked (data not shown), indicating that Ip-1/2 neurons respond directly CO_2 and not via synaptic input from other neurons. The membrane potential returned to the control level at the same time that the preparation was returned to the control ASW solution. Sometimes, however, the bursting pattern of background activity accompanied by a slow hyperpolarization was evoked in the preparations, as shown in Figs. in 4B(1), 5A(1) or Fig. 8(A, C) with asterisks.

Depolarizing responses induced by CO_2 were additive with the spontaneous slow fluctuation of the membrane potential. Due to the presence of a wide variation in background membrane activity, especially in the irregular burst pattern, it was impossible to quantify the exact magnitude of depolarizing responses to CO_2 using the degree of depolarization. Furthermore, gradual membrane depolarization often and unpredictably evoked abrupt bursting. However, the results obtained within a given preparation (individual organism) were consistent. Therefore, a typical example obtained from one individual preparation is provided where quantification was impossible. Results shown in the figures represent typical examples of the qualitatively measured responses in membrane activity patterns in Ip-1/2 neurons.

3.4. Membrane responses to H^+ application in solution

The depolarizing effects of CO_2 dissolved in ASW could have been due to CO_2 per se, to H^+ produced in the solution, or both. To determine the independent effects of CO_2 and H^+, I compared their effects in control ASW to those in ASW buffered at a pH of 7.80.

Fig. 5 shows the effects of dissolved CO_2 and a continuous infusion of H^+ (from HCl) on Ip-1/2 neurons in ASW. The membrane potentials were −40 mV (1) and −50 mV (2), and the recorded cells demonstrated the irregular burst-type background activity. The pH of the solution to which H^+ was added was slightly acidic (pH = 5.8) compared to

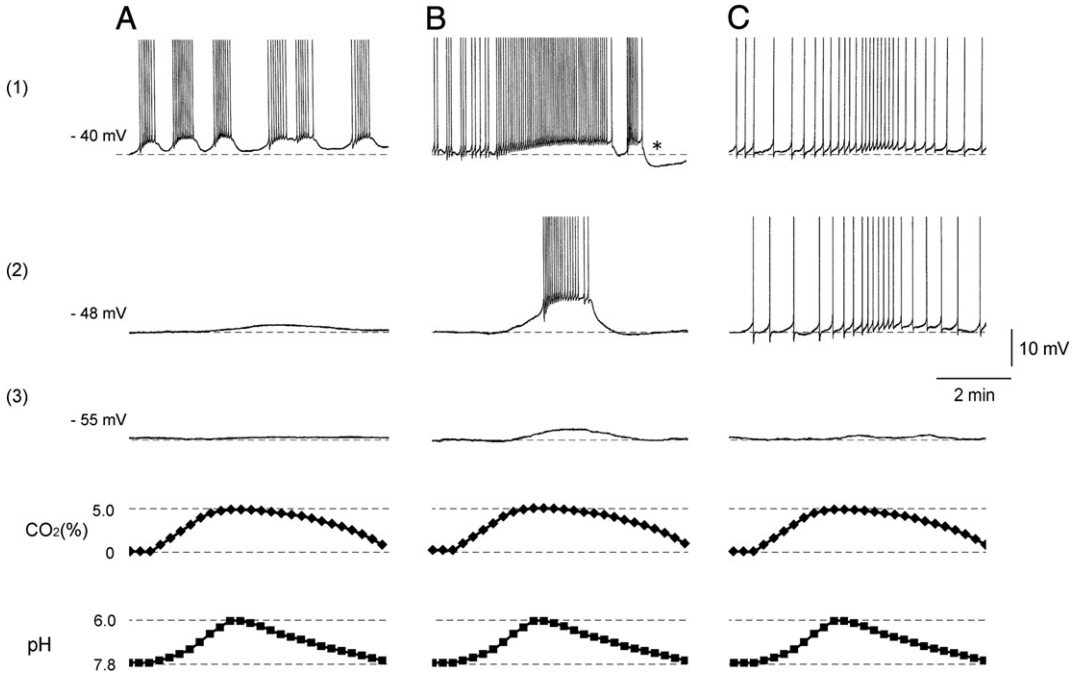

Fig. 4. The effects of CO_2 in artificial seawater (ASW) solution on the membrane potential of Ip-1/2 neurons. A, B: Bursting pattern of membrane activity, where B represents a pattern with a variable burst duration and subsequent silent period. (1) The Ip-1/2 neurons depolarize in response to CO_2 in ASW at a membrane potential of −40 mV. C: the beating pattern of spontaneous membrane activity, in which the frequency of spikes increased in response to CO_2. The depolarizing responses induced by CO_2 became smaller as the membrane potential was hyperpolarized to either −48 mV (2) or −55 mV (3). The concentration of CO_2 was estimated from the equation derived in Fig. 1. Similar results were obtained from ten recordings of burst type neurons and three beating pattern neurons. The asterisk represents hyperpolarization occurring upon return to control ASW. The tops of spikes are cut.

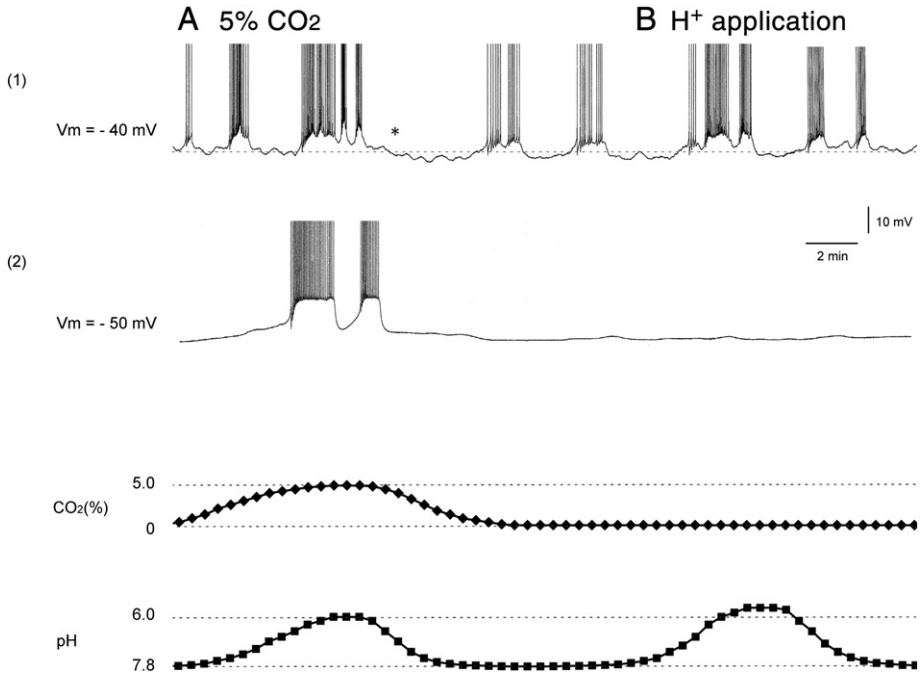

Fig. 5. Comparison of the effects of CO_2 and H^+ on the membrane potential. The background membrane activity pattern is of bursting type. (1) When the membrane potential was −40 mV, the depolarizing responses of Ip/1-2 occurred continuously with both CO_2 and H^+ administration. (2) When the membrane potential was −50 mV, CO_2-induced depolarization was still evoked, but the response to H^+ addition disappeared. Similar results were obtained from five recordings of burst-type of neurons. The concentration of CO_2 was estimated from the equation derived in Fig. 1. The asterisk represents hyperpolarization occurring upon return to control ASW. The tops of spikes are cut.

that in which the CO_2 was dissolved. The depolarizing responses induced by CO_2 were similar to those of H^+ addition when the membrane potential was −40 mV. However, CO_2 application produced a larger response at −50 mV than H^+ alone. The degree of the responses was independent of the order of administration. On repeated H^+ addition (i.e., acidification), the responsiveness of Ip-1/2 neurons often diminished. However, the CO_2-induced responses showed little reduction with repetitive stimulation. These results indicate that the effects of CO_2 and H^+ are not identical. Rather, the effect of CO_2 is a composite effect mediated in part by production of H^+ and in part by direct

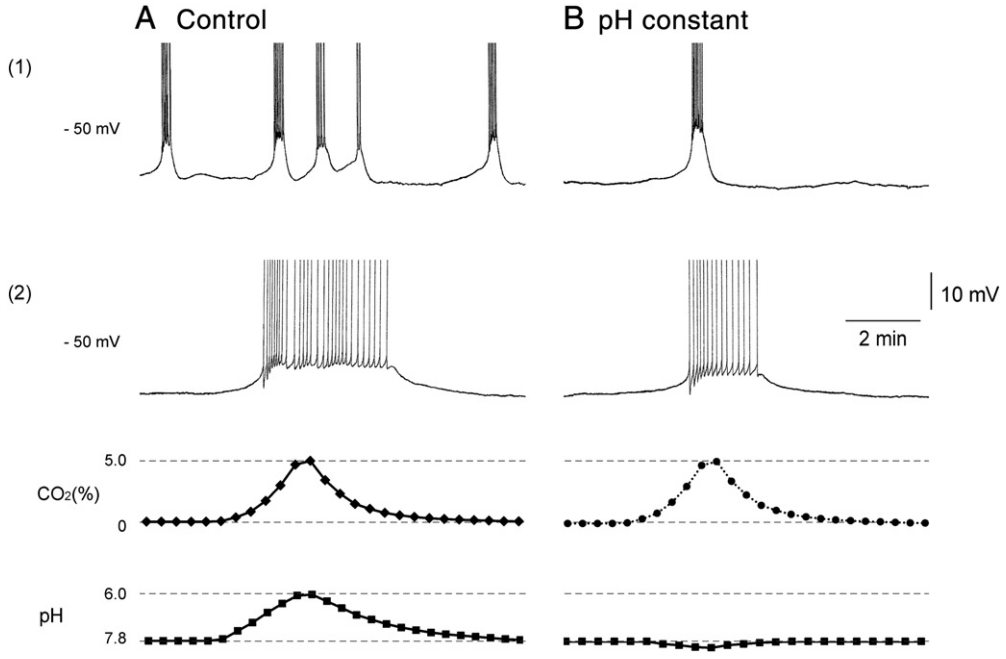

Fig. 6. The effects of CO_2 administration under slightly alkaline conditions. Depolarizing responses induced by CO_2 are shown in (1) burst-type or (2) non-burst type neurons. The membrane potential was held at −50 mV. A: Depolarizing responses to CO_2 administration in pH-controlled artificial seawater (ASW). B: Compared to the pH 6.0 ASW, smaller depolarizations occurred upon CO_2 administration to the slightly alkaline ASW (pH 8.0; for simplification, pH is indicated as "pH constant" in the figure title). The data for A(1) and B(1) were obtained from the same preparation, as were the data for A(2) and B(2). Similar results were obtained from five recordings of burst-type and five non-burst type neurons. The concentration of CO_2 in A was estimated from the equation derived in Fig. 1. CO_2 concentration in B (indicated as dots) was assumed to be the same as that in A. The tops of spikes are cut.

stimulation by the CO_2 gas itself. Furthermore, the direct effect of CO_2 is prominent in hyperpolarized conditions, whereas both effects are similar in neurons at or near their resting potential.

3.5. The effects of CO_2 in an alkaline solution

In order to further differentiate the effects of H^+ and CO_2, I investigated the effects of CO_2 in "pH constant" ASW. It should be noted that the pH of the "pH constant" ASW was slightly alkaline (pH 8.0) relative to the control ASW, thanks to the addition of hydroxyl (OH^-) ions (from NaOH) after the CO_2 gas was dissolved. Membrane potentials were held at −50 mV, at which the CO_2 response component predominates (see Fig. 5). Compared with the controls, smaller depolarizing responses were evoked by CO_2 in the alkaline solution. This was true for both burst-type [Fig. 6(1)] and non-burst type neurons [Fig. 6(2)]. These results indicate that CO_2 gas directly evoked depolarizing responses, though under experimental and not physiological conditions. These results also support the hypothesis that the depolarizing responses induced by CO_2 are mediated, at least in part, directly by CO_2 gas.

3.6. The effects of acetazolamide on CO_2 sensitivity

The above results indicate that the depolarizing effect induced by CO_2 is likely to be composed of both indirect (H^+ production) and direct (stimulation by CO_2) effects. It is commonly recognized that a cell membrane is freely permeable to CO_2. In rat olfactory CO_2-sensing neurons, CO_2 diffuses into the cell, where it activates an enzymatic cascade (Hu et al., 2007). It is eventually metabolized by carbonic anhydrase through

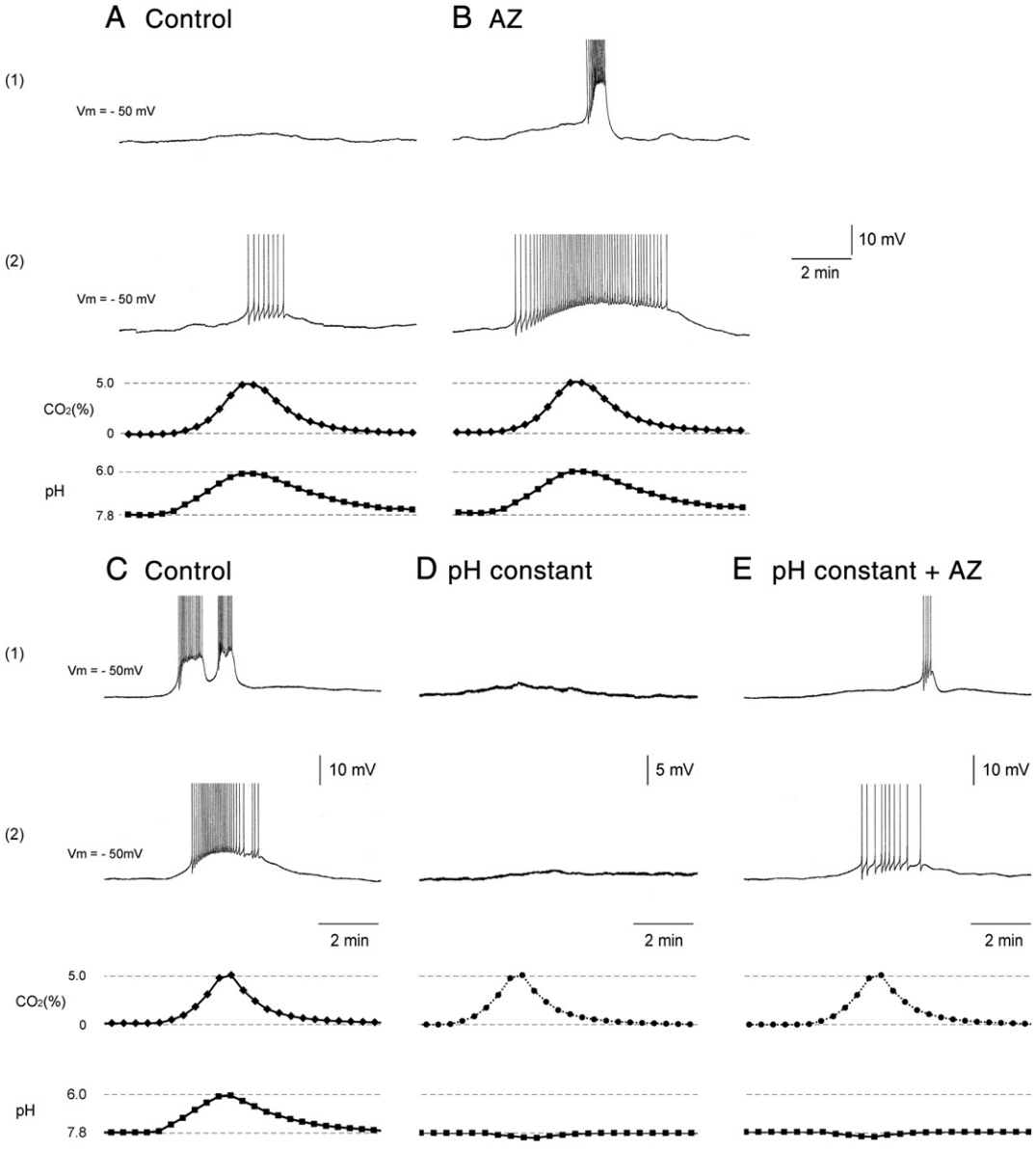

Fig. 7. The effect of acetazolamide (AZ) on CO_2-induced depolarization of neurons in normal and alkaline artificial seawater (ASW). Depolarizing responses induced by CO_2 are shown for (1) burst type and (2) non-burst type neurons at a membrane potential of −50 mV. A(1, 2) and C(1, 2) represent the conditions following application of CO_2 to control artificial seawater (ASW). B: The depolarizing responses became larger when both AZ (2 mM) and CO_2 were administered. B(1): Repetitive spikes coincided with a gradual membrane potential change. B(2): Repetitive spikes continued for more than 5 min in the presence of AZ. The results in A(1) and B(1) were obtained from the same preparation, as were those shown in A(2) and B(2). Similar results were obtained from six recordings of burst-type of neurons and five recordings from non-burst type neurons. D(1, 2): Smaller depolarizing responses were induced by CO_2 when the pH of the ASW was slightly alkaline (pH 8.0). E(1, 2): These responses became stronger in the presence of AZ. The results in C(1), D(1), and E(1) were obtained from the same preparation, as were those shown in C(2), D(2), and E(2). Similar results were obtained from five recordings of burst type of neurons and three recordings of non-burst type neurons. The concentration of CO_2 in all cases was estimated from the equation derived in Fig. 1. CO_2 concentration in D and E (indicated as dots) was assumed to be the same as that in C. The tops of the spikes are cut.

the reversible reaction: $CO_2 + H_2O \leftrightharpoons H^+ + HCO_3$. Acetazolamide (AZ), a carbonic anhydrase inhibitor, is thought to increase the local CO_2 concentration (pCO_2) (Voipio et al., 1991). It has been used clinically as an anti-epilepsy agent, which leads to suppression of neuronal hyperexcitation (Woodbury et al., 1984; Shorvon et al., 2009). Thus, to increase intracellular CO_2 levels for this preparation, I administered AZ to the ASW with dissolved CO_2.

Fig. 7 shows the effects of AZ on CO_2 infusion in control ASW at a −50 mV membrane potential. In the presence of 2 mM AZ, the depolarizing responses induced by CO_2 became larger in both burst-type [Fig. 7B(1)] and non-burst type [Fig. 7B(2)] Ip-1/2 neurons in ASW. The AZ was dissolved in a small amount of DMSO, which had no effect on the CO_2-induced response (data not shown).

As described in Fig. 6, the CO_2-induced response in the "pH constant" condition is thought to be a direct effect of CO_2. I examined the effects of increasing CO_2 concentration under the "pH constant" condition by the addition of AZ as described above. The data in Fig. 7D [(1), (2)] show that CO_2-induced depolarizing responses in the "pH constant" ASW were smaller than those in the control ASW, in terms of both magnitude and duration. These responses also grew stronger following AZ administration [Fig. 7E(1), (2)]. AZ-induced augmentation of the depolarizing responses to CO_2 in both normal and "pH constant" ASW strongly supports the idea that CO_2 directly stimulates Ip-1/2 neurons.

3.7. Membrane activity related to CO_2 in the depolarized condition

The results presented in Figs. 6 and 7 were obtained at a membrane potential of −50 mV, at which bursting was not observed. To investigate the effect of CO_2 in neurons with background bursting patterns, a preparation similar to those used in Sections 3.5 and 3.6 was used, although in this case the membrane potential was held at −40 mV. Fig. 8 shows examples of relatively regular bursting patterns. Fig. 8A shows the effect of adding 2.5% CO_2, in which the period of bursts increased slightly. As seen in Fig. 8B, when 5% CO_2 was added to the ASW, the burst duration lengthened and the between-burst silent period shortened. Ultimately, the separate bursts appeared to fuse into one long burst (Fig. 8B). The duration and number of between-burst silent periods paralleled membrane polarization, and returned to their original (pre-CO_2) values after the switch back to control ASW. Fig. 8C shows the effect of 5% CO_2 under "pH constant" ASW conditions (i.e., similarly to those shown in Fig. 6), in which prolongation of firing due to CO_2-induced depolarization was clearly visible even in an alkaline solution. Fig. 8D shows the effect of H^+ at −40 mV. H^+ produced prolongation of firings, but without the hyperpolarization (cf. Fig. 8A, C, asterisks) following the switch back to ASW.

In addition to the analyses above, I examined the effects of AZ when the membrane was depolarized to −40 mV. The results are demonstrated in Fig. 9, in neurons with a regular bursting pattern. When 5% CO_2 was added to the ASW, the burst durations were unambiguously longer (Fig. 9A). As depicted in Fig. 9B, no bursting occurred when the membrane potential was held at −50 mV. Rather, there was a gradual depolarization, which was additive with the cell's periodic fluctuation.

Fig. 9C shows the effect of 5% CO_2 at a −40 mV membrane potential, and under "pH constant" (alkaline) ASW conditions. In alkaline conditions, CO_2 was weakly depolarizing, similarly to the results presented in Fig. 7D, but unlike the former data, bursting was induced at −40 mV potential. In addition, AZ with CO_2 (Fig. 9D) augmented excitation similarly to the data presented in Fig. 7E. As Fig. 9D demonstrates, this increased excitation was expressed as increased burst durations.

In order to quantify the effects of CO_2, I measured the burst durations at −40 mV under different conditions, according to the schema described in Fig. 2. Group averages of normalized burst duration sums

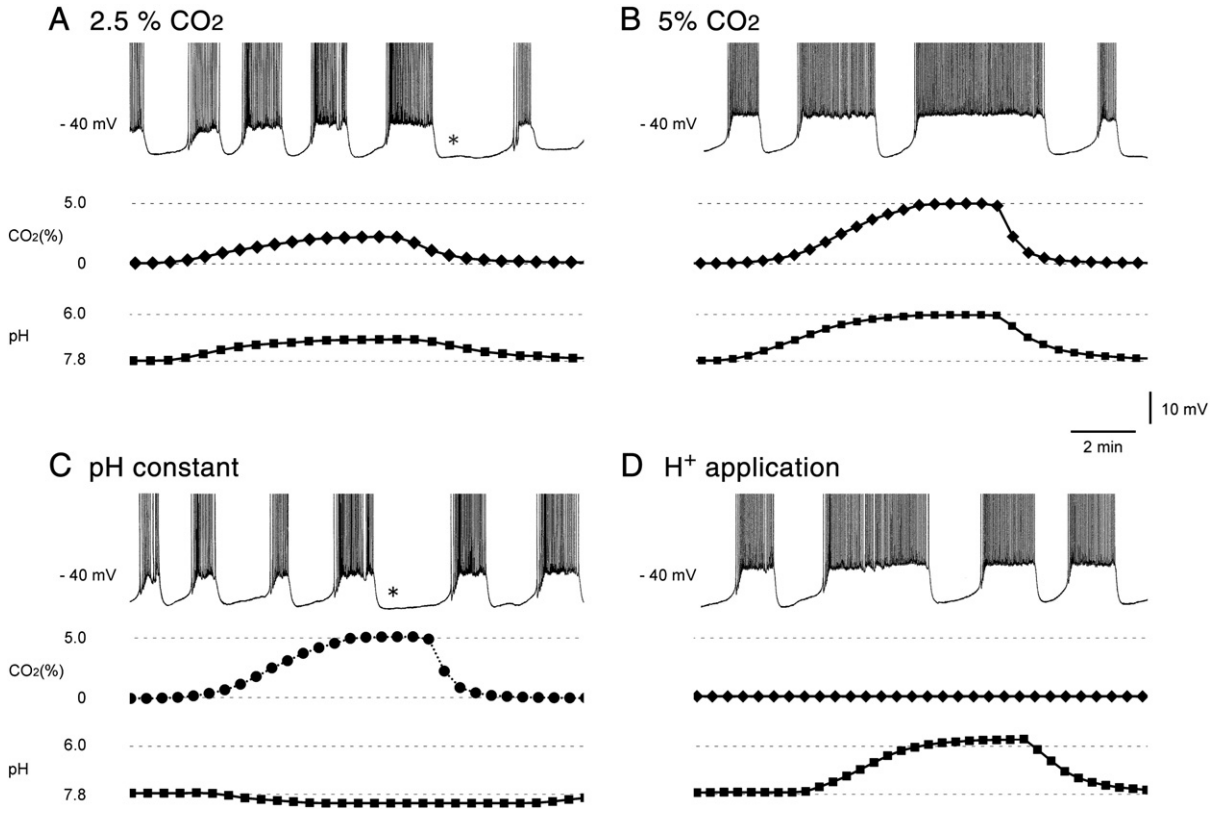

Fig. 8. Membrane activity related to CO_2 or H^+ under slightly depolarized conditions. All recordings were obtained at a membrane potential of −40 mV and from the same preparation. A, B: Effects of 2.5% CO_2 (A) and 5% CO_2 (B) infused into control artificial sea water (ASW). The asterisks in A and C represent hyperpolarization occurring upon return to control ASW. C: Effect of 5% CO_2 administration under slightly alkaline (pH constant) conditions. D: Effect of H^+ administration into control ASW. The concentration of CO_2 in A, B was estimated from the equation derived in Fig. 1. The CO_2 concentration in C (the dotted portion of the line) was assumed to be the same as that in A. Similar results were obtained from three separate burst-type of neurons. The durations of bursts in each condition were prolonged under depolarizing conditions. The tops of the spikes are cut.

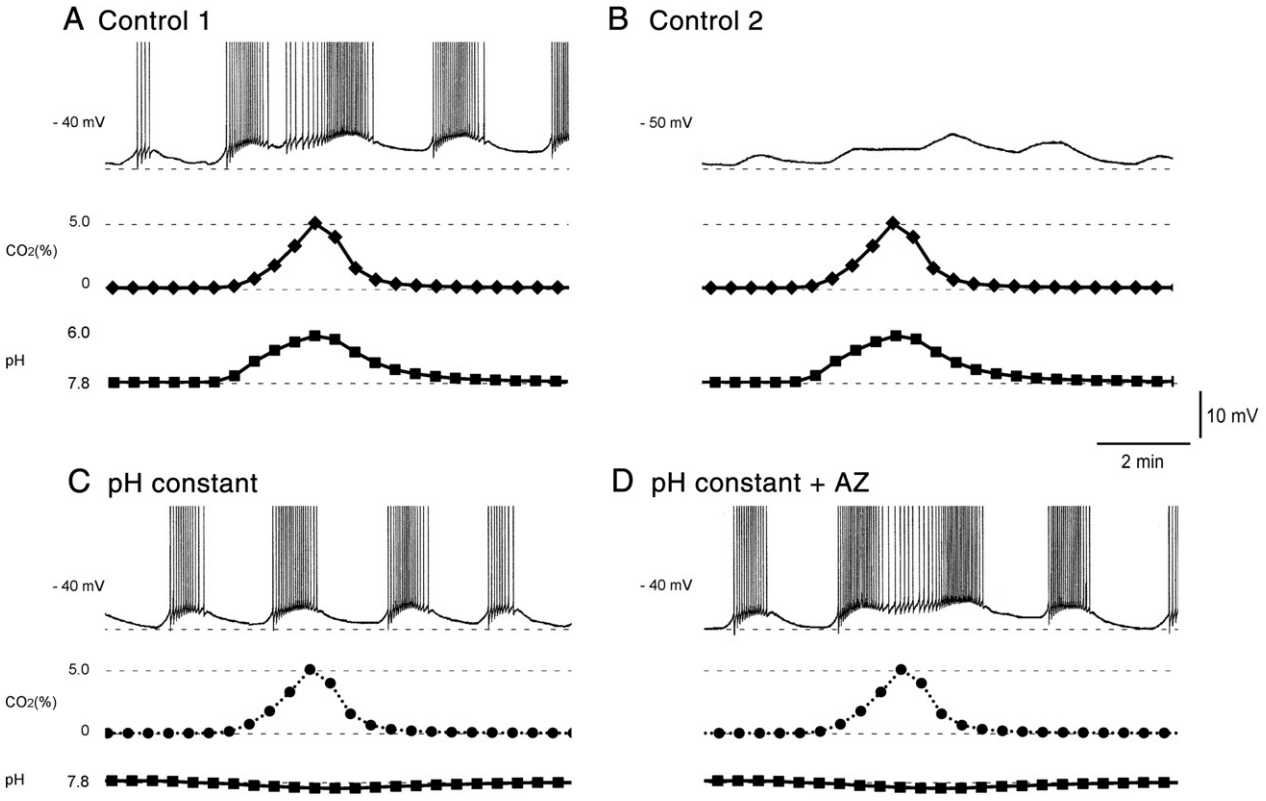

Fig. 9. Membrane activity related to acetazolamide (AZ) administration under slightly depolarized conditions. Recordings in A, C, and D were obtained at a membrane potential of −40 mV. The recording in B was obtained at −50 mV to demonstrate the effect of CO_2 without depolarization. All recordings were obtained from the same preparation. A, B: The effect of 5% CO_2 infused into control artificial seawater (ASW). C: The effect of 5% CO_2 infusion under slightly alkaline (pH constant) conditions. D: The effect of AZ administration on the responses to 5% CO_2 under pH constant conditions. The concentration of CO_2 in A, B was estimated from the equation derived in Fig. 1. The CO_2 concentrations in C, D (the dotted portions of the respective lines) were assumed to be the same as that in A. Similar results were obtained from four recordings of burst-type of neurons. The tops of the spikes are cut.

were analyzed (see Section 2.5) and are presented in Table 1. Representative physiological traces of the analyzed neurons are found in Figs. 8 and 9. When tested via Student's *t*-test, no significant differences were found between the control groups, or between the 5% CO_2 groups, across series. Series 1 compares control and 5% CO_2-infused ASW. In control ASW, the sum of the burst durations was significantly longer with CO_2 than without it (control, 21.9 ± 2.1 s/min; 5% CO_2 added, 31.1 ± 2.1 s/min; $n = 7$; $P < 0.01$ via paired *t*-test). Series 2 compares alkaline (pH constant) ASW with and without 5% CO_2 added. Addition of CO_2 evoked a statistically significant prolongation of the burst duration (control, 15.0 ± 2.4 s/min; CO_2 added, 24.2 ± 1.5 s/min, $n = 5$; $P < 0.04$). Series 3 compares the effect of AZ in pH-constant ASW infused with 5% CO_2. The addition of AZ also evoked a significant prolongation of burst duration (control, 21.3 ± 5.9 s/min; AZ added, 29.8 ± 6.9 s/min; $n = 4$; $P < 0.01$).

Table 1
Effects of CO_2 on the membrane activities.

	Group compared	Burst duration[a]	*N*	*P* value[b]
Series 1	Control (normal ASW)	21.9 ± 2.1	7	0.01
	5% CO_2	31.1 ± 2.1	7	
Series 2	Control (normal ASW)	15.0 ± 2.4	5	0.04
	5% CO_2 (pH constant)	24.2 ± 1.5	5	
Series 3	5% CO_2 (pH constant)	21.3 ± 5.9	4	0.01
	5% CO_2 (pH constant) + AZ	29.8 ± 6.9	4	

[a] Values are normalized on a per-minute basis (i.e., seconds/minute) and presented as mean ± standard error of the mean.
[b] Groups within each series were compared by 2-tailed paired *t*-test.

4. Discussion

4.1. Excitation induced by CO_2 and H^+

The intrinsically photoresponsive neurons Ip-1/2 in *Onchidium* responded to CO_2 gas dissolved in the bathing solution with depolarization when at a resting membrane potential of around −48 mV. Regardless of the type of background membrane activity, depolarization induced by CO_2 stimulation prolonged serial firing, due to intrinsically weak inactivation of Ip-1/2 neurons after spiking. Interestingly, the only known synaptic inputs to Ip-1/2 neurons are inhibitory synaptic inputs that convey information (e.g., tactile stimulation) from the body surface (Shimotsu et al., 2010). Given their role in promoting pneumostomal opening, the triggering of repetitive spiking in Ip-1/2 neurons by CO_2 stimulation seems to be of great importance.

The depolarizing responses induced by CO_2 were similar to those of H^+ addition when the membrane potential was −40 mV (slightly depolarized). However, there were also several key differences in the responses to CO_2 and H^+ application. First, the CO_2-induced responses were larger than those of H^+ application with the membrane hyperpolarized (Fig. 5). Second, the time-course of the responses was different, in that the depolarizing response induced by H^+ was monotonous and fully dependent on the concentration of H^+. Indeed, it paralleled the time course of the pH change in the solution. On the other hand, the responses induced by CO_2 were rather complex. Hyperpolarization was sometimes (28 of 50 preparations) observed in the burst-type neurons after they were returned to the control ASW [see Figs. 4B(1), 5A(1) and 8(A, C)]. Furthermore, repetitive application of H^+ to Ip-1/2 neurons often produced diminished responses over time. These results demonstrate that the effects of CO_2 are not identical to those of H^+, and imply that multiple conductance changes are involved

in the CO_2 responses. This is different from the mechanism utilized by CO_2-sensitive neurons in *Aplysia*. Studies in *Aplysia* found that many neurons responded to CO_2 with depolarization, and only a few with hyperpolarization. Furthermore, they showed that the effects of CO_2 were brought about solely by the production of H^+ when CO_2 was dissolved in saline. A drop in pH elicited a large increase in Cl^- and smaller increase in K^+ conductance. Differences in the intracellular Cl^- concentration in individual neurons generate both depolarizing and hyperpolarizing responses (Brown and Berman, 1970; Brown et al., 1970). Using the terrestrial mollusk *Helix*, Denton et al. (2007) reported that excitatory responses to CO_2 were caused by a decrease in several types of K^+ conductance, and that this effect derived from a production of H^+. These former studies, however, did not consider the participation of a direct CO_2 effect on the neurons. In *Onchidium*, the reversal potential of the CO_2-induced response is around −55 mV (Fig. 4), which is higher than E_K (Nishi and Gotow, 1998). This can be explained by a mechanism in which CO_2 first increases Cl^- conductance, as it does in *Aplysia* (Brown and Berman, 1970; Brown et al., 1970). After a delay, this increase is accompanied by a decrease in K^+ conductance, as seen in *Helix* (Denton et al., 2007). The differences in responses to CO_2 across preparations may be directly related to the different distributions of Cl^- and K^+ channels.

4.2. CO_2 can cause excitation directly

Recently, direct detection of CO_2 by olfactory neurons in the rat (Hu et al., 2007), *Drosophila* (Suh et al., 2004), and *C. elegans* (Hallem and Sternberg, 2008) has been reported. These CO_2-sensitive cells are too small to record intracellular electrical activity, though, making it impossible to compare their data to those of the former studies directly. Additionally, the gating mechanisms of excitation brought about by CO_2 remain unclear (Luo et al., 2009). In contrast to these molecular biological experiments, CO_2 gas detection has been demonstrated behaviorally in the honeybee (Sugahara et al., 2012). These data indicate that direct detection of CO_2 is a common property of the nervous systems of both vertebrates and invertebrates. They also support the likelihood that direct CO_2 gas detection has a role in neuronal respiratory control.

For many years, increased ventilation induced by CO_2 has been explained by acidosis of blood or other bodily fluids. The central respiratory center and/or the peripheral CO_2 sensors (e.g., the carotid body) have been believed to increase ventilation under hypercapnic conditions (Huckstepp and Dale, 2011). In this study, I discriminated between the effects of CO_2 and H^+ by using a solution of ASW in which the pH is kept constant during CO_2 application, using the *Onchidium* Ip-1/2 neuron model. Specific, CO_2-induced excitation clearly occurred in the alkaline ASW solution, though it was of smaller amplitude than that of the control solution (Fig. 6). This observation differs from data collected on CO_2-sensitive neurons in *Aplysia,* although both studies used similar procedures. In fact, in one of the *Aplysia* studies, a concentration of 50% CO_2 dissolved in the solution had no effect on the external pH, which was maintained at 8.0 (Brown and Berman, 1970).

Furthermore, when the carbonic anhydrase inhibitor AZ, which increases intracellular levels of CO_2, was applied along with CO_2 in ASW, the CO_2-induced excitation became larger both in the normal solution (Fig. 7) and in the solution in which pH was held constant (Figs. 8, 9). These results support the idea that CO_2 directly evokes excitation. In conclusion, CO_2-induced excitation of the multifunctional Ip-1/2 neurons is brought about by both the production of H^+ and direct CO_2 stimulation, and the effects of CO_2 and H^+ are composite or cumulative.

4.3. CO_2 increases ventilation in Onchidium

Onchidium live in the intertidal zone, and are fully amphibious. They can live under the water for at least two weeks in aquaria (Arey and Crozier, 1921). As *Onchidium* is related to the pulmonate mollusks, many of its activities (including feeding and reproduction) are performed on land. The pneumostome, an orifice of the lung used for air-based respiration, typically opens for long periods and only closes for brief periods, to facilitate diffusion of air across the lung. This diffusion lung system indicates that *Onchidium* is a lower, more primitive species of pulmonata, like *Melampus*. It is speculated that these primitive pulmonates never left the seashore during the evolutionary process (McMahon and Russell-Hunter, 1981). On the other hand, the primarily aquatic pond snail *Lymnaea* feeds on weeds in the water, and periodically surfaces to refresh the air supply to its lung (Syed et al., 1991). Thus, its mode of respiration is entirely different from that of *Onchidium*. Initially, CO_2-sensitive neurons were found in *Aplysia* (Chalazonitis, 1961), including many of them in the species' abdominal ganglion (Brown and Berman, 1970). However, the properties of CO_2-sensitive neurons are still obscure. *Aplysia* is completely aquatic animal which uses a gill for respiration, rather than the lung noted in *Helix*, *Onchidium,* and *Lymnaea*. Therefore, the CO_2 sensitivity of the neurons may also be related to expression of other behaviors, such as the avoidance of inadequate environments seen in *C. elegans* (Hallem and Sternberg, 2008).

The pneumostomal opening of *Onchidium* became larger following an increase in CO_2 concentration in both the intact and semi-intact preparations (Fig. 3A, C). In *Lymnaea*, it has been reported that central pattern-generating (CPG) neurons generate the basic respiratory rhythmic activity (Syed and Winlow, 1991). Hypercapnic water prolonged the duration of pneumostome opening in intact *Lymnaea*, but in an isolated ganglionic preparation, it suppressed this activity (Inoue et al., 2001). These findings suggest that CO_2-sensitive chemoreceptors are located outside the central CPG neurons, possibly in the foot or wall musculature (Inoue et al., 2001). In contrast, focal stimulation of the central chemoreception area of the brain (the subesophageal ganglia) with a hypercapnic solution in a semi-intact preparation of *Helix* increased the pneumostome opening (Erlichman and Leiter, 1993). The similarities in ventilation control between *Helix* and *Onchidium* may reflect their phylogenetic relationship, in that both belong to the group Eupulmonata, whereas *Lymnaea* belongs to the group Basommatophora (Klussmann-Kolb et al., 2008).

When the pneumostomal opening becomes larger due to high CO_2 concentrations in air in the intact animal, the rim of the dorsum rises and it forms a shape reminiscent of the Greek letter omega. In order to show this behavior, coordination between the neurons that control the mantle would be necessary. Besides the Ip-1/2 neurons, it is hypothesized that a large number of CO_2-sensitive neurons exist in the central ganglia, as noted in *Helix* (Erlichman and Leiter, 1997) or *Aplysia* (Brown and Berman, 1970), and that they work cooperatively to perform ventilatory behavior in *Onchidium*.

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