Mechanism of Excitation of *Aplysia* Neurons by Carbon Dioxide

A. M. BROWN and P. R. BERMAN

From the Departments of Physiology and Medicine, University of Utah College of Medicine, Salt Lake City, Utah 84112

ABSTRACT The abdominal ganglion of *Aplysia californica* was perfused with artificial seawater equilibrated at different P_{CO_2}'s and pH's for 5 min or less. 5% CO_2 dropped perfusate pH from 8.0 to 6.5 and produced depolarization and increased discharge rate in visceromotor neurons. Half the giant cells studied had a similar response, whereas the other half were hyperpolarized. Pacemaker neurons showed little, if any, response to such changes in pH or CO_2. Membrane conductance of responsive cells was always increased. The effect of CO_2 occurred even when synaptic transmission was blocked by low calcium and high magnesium, and therefore must have been a direct result of CO_2 or the concomitant fall in pH. When extracellular pH was lowered to 6.5 using HCl or H_2SO_4 and no CO_2, the same effects were observed. Also, local application of HCl or H_2SO_4 to the external surface of the cell soma elicited depolarization and spike discharge. When extracellular pH was held constant by continual titration, 5–50% CO_2 had no effect. Intracellular pH was probably decreased at least one pH unit under these circumstances. Thus CO_2 per se, decreased intracellular pH, and increased bicarbonate ion were without effect. It is concluded that CO_2 acts solely through a decrease in extracellular pH.

INTRODUCTION

The reported effects of CO_2 on nervous tissue are highly variable but rarely inconsequential. It stimulates respiration by excitation of neurons in or near the respiratory center of the medulla (von Euler and Söderberg, 1952; Comroe, 1966), provokes depolarization and increases spike discharge of some neurons in the visceral ganglion of *Aplysia fasciata* (Chalazonitis, 1963; Brown, 1969), and reduces the transmembrane potential of frog skeletal muscle (Ling and Gerard, 1949). On the other hand, Shanes (1948) described an increase in demarcation potential in frog sciatic nerves exposed to CO_2 and Krnjević, Randić, and Siesjö (1965) reported hyperpolarization of cortical cells of the cat during hypercapnia.

The mechanism by which CO_2 produces these effects is unknown (Lambertsen, 1961). Changes in extracellular or intracellular pH, or direct effects of

molecular CO_2 or bicarbonate ion could be implicated either singly or in combination. The purpose of these experiments was to identify the precise mechanism by which CO_2 excites some neurons in the abdominal ganglion of *Aplysia californica*. The results show that CO_2 acts solely through the concomitant fall in extracellular pH that it produces.

METHODS

Aplysia californica were obtained from Pacific Biomarine Supply Company (Dr. R. Fay, Venice, Calif.) and kept at 14°C in a seawater aquarium (Instant Ocean, Inc., Wickliffe, Ohio). The abdominal ganglion was excised and pinned to the paraffin bottom of a Perspex chamber having a volume of 1.5–2.0 ml. Both connectives and the branchial and siphon nerves were placed on stimulation electrodes (Fig. 1). The ganglion was superfused with artificial seawater (ASW) having the same composition as the extracellular fluid of *Aplysia* (Hayes and Pelluet, 1947; Geduldig and Junge, 1968). The following solutions (mM) were used:

	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	Tris*	Sucrose
Control-ASW	494	10	10	20	30	10	—
Low $(Ca)_o$, high $(Mg)_o$	494	10	1	29	60–80	10	—
Hypertonic	494	10	10	20	30	10	105
Hypotonic	428	10	10	20	30	10	—

* Tris (hydroxymethyl) aminomethane-maleate (Tris-maleate) buffer made according to Gomori (1948).

The osmolality of all solutions was measured with an osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.); ASW and low (Ca) solutions were 950 milliosmols/kg. The pH of each solution was measured with a Beckman model pH meter having an expanded scale with an accuracy of ±0.05 pH. Control pH was adjusted to 7.6–8.0 and was subsequently set at any desired level using 0.5 MHCl, H_2SO_4, NaOH, or $NaHCO_3$. Volume changes of the solution were less than 1.0 ml/liter and there was no measurable effect on osmolality. The titration curve for the control ASW had a constant buffer value over the range of pH used.

The CO_2 mixtures were obtained from Matheson Company, Newark, Calif. The % CO_2 was kindly checked for us by Dr. F. Ukradyha by means of the microScholander technique. The relationship between pH and log P_{CO_2} was linear over the range of P_{CO_2}'s used (1–60 mm Hg). By measuring pH in the chamber, any loss of CO_2 between the reservoir and chamber could be detected and the flow rate adjusted to prevent such loss. The flow was usually 15 ml/min and the dead space from the reservoirs to the chamber was 10 ml.

Cells on the dorsal surface of the abdominal ganglion (Fig. 2) were impaled using glass micropipettes having tip diameters less than 1 μ and resistances of 2–10 mΩ. The electrodes were filled with 3 M KCl, 0.6 M Na_2SO_4, 0.6 M K_2SO_4, or 0.1–3.0 M HCl

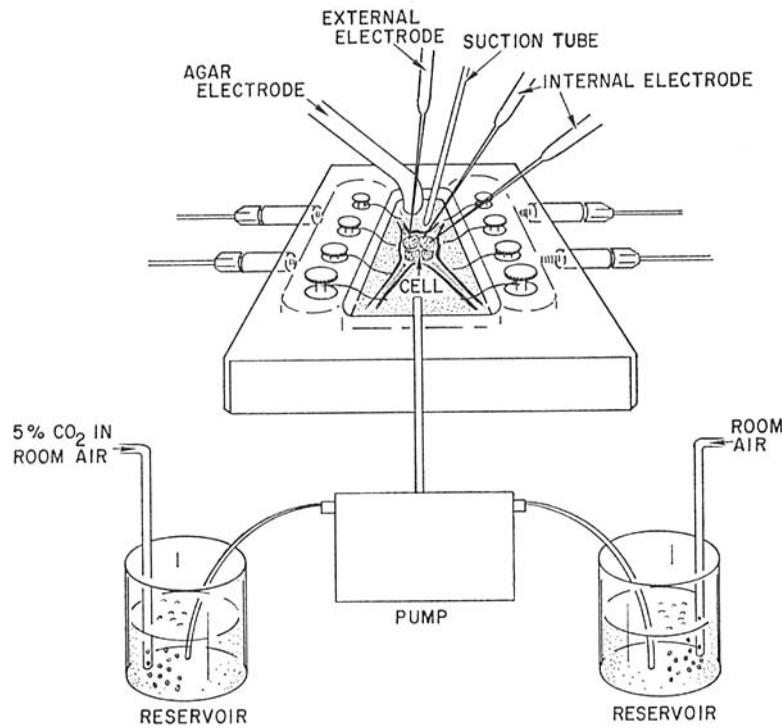

FIGURE 1. Experimental setup. The volume of the chamber was 1.5–2.0 ml. The nerves entering the ganglion were placed on Ag–AgCl electrodes for stimulation. Constant flow was maintained by the pump from either the right or left reservoir, as desired.

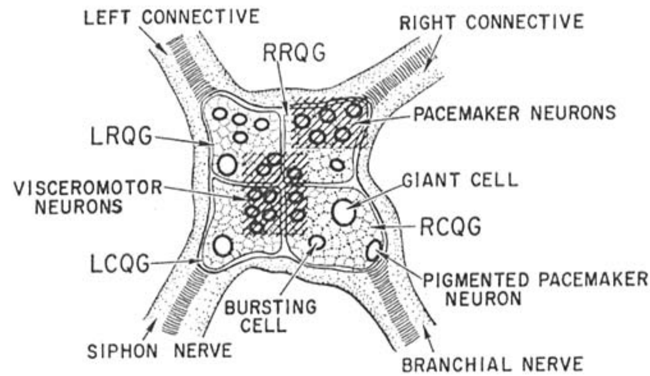

FIGURE 2. Schematic view of the dorsal surface of the abdominal ganglion of *Aplysia californica* indicating the most common position of some identified cells. *RRQG*, right rostral quarter ganglion; *RCQG*, right caudal quarter ganglion; *LRQG*, left rostral quarter ganglion; *LCQG*, left caudal quarter ganglion. Mapping scheme modified from that of Frazier et al. (1967). The areas in *RRQG* containing pacemaker neurons and in the center of the ganglion containing visceromotor neurons very responsive to CO_2 are cross-hatched.

or H_2SO_4. 3 M KCl-filled electrodes were generally used to record voltage and pass current. The indifferent electrode was an Ag-$AgCl_2$ electrode connected to the bath through an agar-ASW bridge. For accurate recording of DC potentials, the reference electrode was a micropipette of 2–10 MΩ resistance placed immediately outside the impaled cell (Hutter and Noble, 1960). Addition of CO_2 and/or hydrogen ion to the control ASW altered liquid junction potentials by less than 1 mv. Tip potentials were determined according to Adrian (1956) and were 0–1.0 mv. Experiments were begun 20–30 min following impalement; this allowed the cell to seal around the electrode and the membrane potential to attain its maximum value. All experiments were performed at room temperature (18–20°C).

Current-voltage curves were obtained using two intracellular micropipettes, one for passing current and one for recording voltage. Current pulses of varying intensity and 1.5–2.0 sec duration were passed through a 5×10^8 Ω series resistor. Current-voltage curves were also obtained using a steadily varying DC current according to the method of Furshpan and Potter (1959). The signal proportional to the current is fed to the external horizontal input and the signal proportional to the membrane voltage is fed to the vertical input of a Tektronix 564 storage oscilloscope. This method was faster and allowed inscription of the curves before, during, and after exposure of the cells to CO_2.

In some experiments, a glass pipette having a tip diameter of 200–500 μ and filled with 10^{-1} M HCl or H_2SO_4 was placed immediately outside the cell being studied and acid was applied to the external surface from a manually operated Hamilton microsyringe (Hamilton Co., Whittier, Calif.).

Data were recorded on tape (Ampex FR 1300) and subsequently displayed on an oscilloscope screen for photographic recording. Interspike intervals were measured by a Hewlett Packard 5325A universal counter and recorded graphically after passing through a digital analogue converter (Hewlett Packard 580A).

RESULTS

Response of Aplysia Neurons to CO_2 The abdominal ganglion of *Aplysia californica* (Fig. 2) includes pacemaker and visceromotor neurons. As discussed by Frazier, Kandel, Kupferman, Waziri, and Coggeshall (1967) and Alving (1968), the pacemaker cells are easily identified since they have an endogenous rhythm, little or no synaptic input, and send out few axons to the periphery. Cells with prominent synaptic inputs and many connections to the viscera have been called visceromotor neurons.

The ganglion was superfused first with ASW, then with ASW which had been equilibrated with 5% CO_2 in room air. Pacemaker neurons showed little, if any, response to 5 or 10% CO_2 (30 experiments) whereas visceromotor neurons showed a marked increase in rate of discharge (50 experiments) (Fig. 2 A and B, Walker and Brown, 1970). Thus, interspike intervals showed little changes for pacemaker cells (Fig. 3 B) but showed 20- to 40-fold peak reductions for visceromotor cells (Fig. 3 A). Depolarization ranged from 3 to 20 mv in cells that were stimulated and was easily measured in

quiescent cells. In cells which were spontaneously active, there was no well-defined transmembrane resting potential, and therefore, the mean value between the undershoot of one action potential and the point of inflection preceding the upstroke of the following action potential was taken as an approximation of the resting potential. At 18°C, this value ranged from −40 to −55 mv, depending upon the cell and its size. Smaller cells seemed to have lower resting potentials, perhaps as a result of injury following impalement.

The giant cell which was normally quiescent had a resting potential of −55 ± 5 mv (mean ± SEM). CO_2 produced depolarization (3–10 mv) and

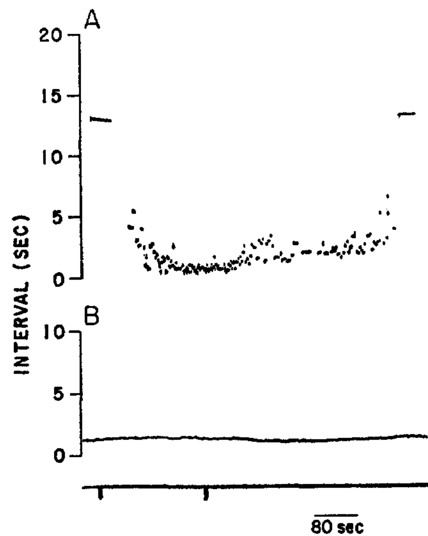

Figure 3. Effect of ASW–5% CO_2, pH 6.5, applied between the signal marks (bottom trace) on the interspike intervals of a visceromotor cell (A) and a pacemaker cell (B). The visceromotor cell was firing spontaneously with an interspike interval of 13 sec (4.6/min) which decreased to a minimum of 0.3 sec (200/min). The pacemaker cell had an interval of 1.2 sec which showed little change.

spiking in half the giant cells studied (Fig. 4 B) and hyperpolarization of 2–8 mv was observed in the other half (Brown, Walker, and Sutton, 1970) (48 experiments). The effect on the "bursting cell" (10 experiments) described by Strumwasser (1965) was similar to that shown by spontaneously active visceromotor cells.

In the case of the visceromotor cell shown in Fig. 5 A and the giant cell shown in Fig. 4 B, increases in synaptic activity may have been responsible for the depolarization observed during CO_2-ASW perfusion. In order to test this possibility, synaptic activity was abolished in low $(Ca)_o$, high $(Mg)_o$ ASW. As shown in Fig. 5 B, depolarization and increased rate of discharge persisted; in fact, the rate of discharge was now greater. Moreover, in the presence of tetrodotoxin-zero $(Ca)_o$ solutions, the depolarization produced by CO_2-ASW perfusion persisted, although action potentials were absent (Brown et al., 1970). Since CO_2 acts in the absence of greatly increased synaptic activity, CO_2 must act on the responsive cells directly.

The capsule of the ganglion which was left intact provided an unknown

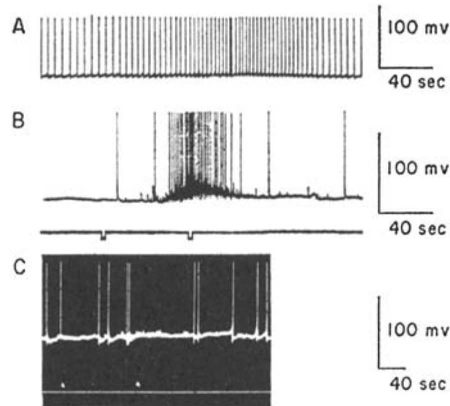

FIGURE 4. Effect of simultaneously superfusing a pacemaker cell (A) and a giant cell (B) with ASW–5% CO_2 in room air, pH 6.5. At the first signal mark (bottom trace, B), solution switched from ASW–room air, pH 8.0 (control) to test solution; at second mark solution switched back to control. The smaller voltage deflections recorded from the giant cell were EPSP's. Pacemaker cell showed a small increase in discharge rate. (C) effect of superfusing the same giant cell (between signal marks) with ASW equilibrated with 50% CO_2 in room air, pH held at 7.6 by continuous titration with 0.5 M $NaHCO_3$. Note the complete lack of effect on membrane potential and spike discharge. Time base is slower in C.

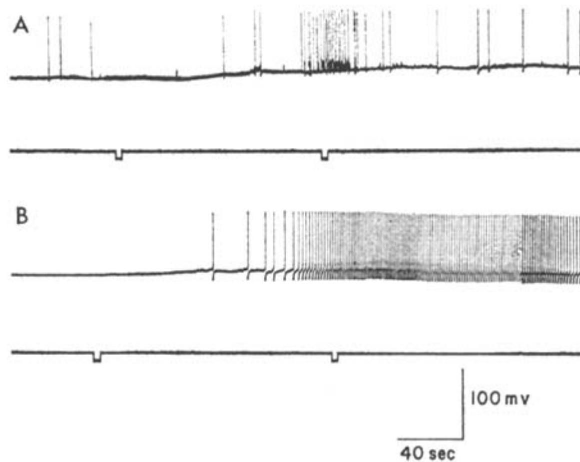

FIGURE 5 A. Effect on a visceromotor cell of ASW–5% CO_2 applied between signal marks. Smaller voltage deflections were EPSP's. B. Same cell now superfused in low $(Ca)_o$, high $(Mg)_o$ ASW (solution 2, Methods). At the signals, solution 2 equilibrated with 5% CO_2 was applied. Note the absence of synaptic activity. The depolarization is as great as that shown in A and the increase in rate of discharge is even greater.

diffusion barrier. Moreover, the earliest changes in depolarization and increased rate of discharge were not easily determined. Although latency measurements were only approximate, the response to CO_2 was definite within 30 sec after the test solution had arrived at the chamber.

When the effect of CO_2 was maximal (which required from 30–240 sec), the perfusion was switched back to the control fluid. The effects of 5 min exposure to 5% CO_2 in air were reversible although recovery periods of 30–60 min were often necessary. Equilibration of ASW for 3–5 min with 1% CO_2 which dropped the extracellular pH 0.8 unit, produced less than 1 mv depolarization in five visceromotor cells with no change in rate of discharge. 3% CO_2 which dropped extracellular pH 1.0 unit, evoked less than 4 mv depolarization in three cells with a 10% or less increase in discharge rate. The effect of 5% CO_2 was the same whether it was given in room air or 95% O_2.

Deliberate changes in osmolality of ±15% with the hyper- and hypotonic solutions listed did not alter the results. Changes in calcium ion concentration due to binding by the buffer system were considered unimportant since the content of $CaCl_2$ could be varied from 1 to 20 mM without affecting the results.

Effect of Equivalent Changes of Extracellular pH Equilibration of ASW with 5% CO_2 in room air caused a fall in extracellular pH from 8.0 to values ranging from 6.3 to 6.5. Hence the action of CO_2 could have been due to an effect of molecular CO_2 per se, a decrease in extracellular pH, a decrease in intracellular pH, or an increase in extracellular HCO_3. Fig. 6 B shows that

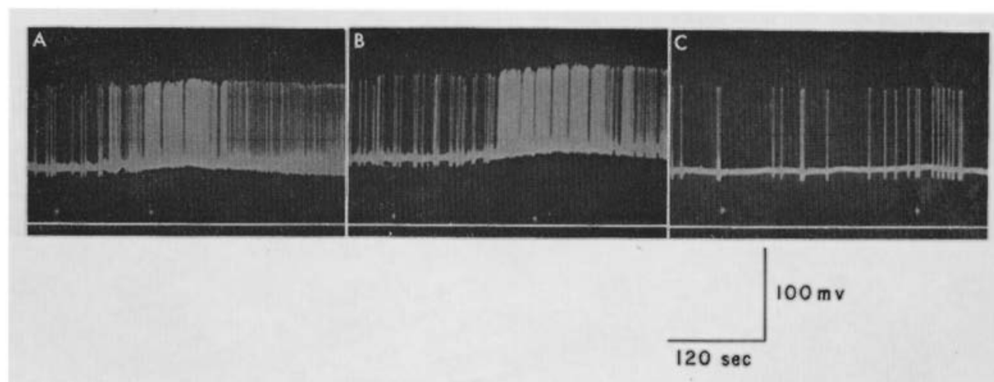

FIGURE 6 A. Effect of ASW–5% CO_2, pH 6.5, on a visceromotor cell. Depolarization and increased spike discharge were elicited. B. Effect of ASW–room air, pH 6.5 (adjusted with 0.5 M H_2SO_4), on the same cell. After a longer latency, depolarization and spike discharge similar to that in A were provoked. C. Effect of ASW–5% CO_2 pH held constant at 8.0 by continuous titration with 0.5 M $NaHCO_3$. No effect on transmembrane resting potential or spike activity was observed. *Figure reprinted by permission from Science. 1970. 167:1502. Copyright 1970 by the American Association for the Advancement of Science.*

an equivalent change in pH produced by 0.5 M H_2SO_4 had an effect of almost identical magnitude to that produced by 5% CO_2 (Fig. 6 A). Either pH change induced a depolarization of about 8 mv and a large increase in discharge frequency. In this experiment, the latency and the time to peak effect were always greater with lowered pH alone than with increased CO_2 and an equivalent fall in pH. This may have been due to slower changes of extracellular pH immediately outside the cell under study when the pH was lowered using H_2SO_4 or HCl because hydrogen ion diffuses more slowly than CO_2 through the capsule and other lipid-containing tissue barriers (Gray, 1968).

Since it takes many hours for intracellular pH to be reduced by decreasing extracellular pH alone (Caldwell, 1958; Waddell and Bates, 1969), the changes produced by lowering extracellular pH using H_2SO_4 or HCl occurred far too quickly to have been associated with any change of intracellular pH. However, in the case of 5% CO_2 (Fig. 6 A), intracellular changes might have occurred. For example, Chalazonitis and Romey (1964) injected an indicator dye into *Aplysia* cells and reported that intracellular pH fell almost as much as extracellular pH, when the cells were exposed to 80% CO_2. In the present experiments, when ASW was equilibrated with 5% CO_2 and the extracellular pH held constant at 8.0 by continual addition of 0.5 M NaOH (volume changes of perfusate were negligible), CO_2 had no effect (Fig. 6 C). ASW equilibrated with (*a*) 5% CO_2 at pH 6.5; (*b*) room air at pH 6.5; and (*c*) 5% CO_2 at pH 8.0 provoked responses similar to those shown in Fig. 6 in 15 other neurons.

The effect of decreased extracellular pH was the same whether HCl or H_2SO_4 was used to adjust pH. However, these adjustments would be accompanied by a loss of CO_2 from the perfusate and possibly the ganglion, as well as by a fall in pH. Therefore, experiments were done in which pH was lowered equally either by adding 5% CO_2 to the perfusate or by decreasing the amount of buffer base (HCO_3) at constant P_{CO_2}. In these experiments, a freshly made CO_2–HCO_3 buffer system was substituted for Tris-maleate. $(HCO_3)_o$ was 2.0 mM and pH 7.46. P_{CO_2} was 2.78 mm Hg as calculated from the equation:

$$\log P_{CO_2} = pK^1 + \log (HCO_3)_o - pH - \log \frac{\alpha}{640 \times 22.40}$$

where pK^1 at 20°C was 6.39 and α, the solubility of CO_2 in ml CO_2/ml water at 1 atmosphere, was 0.88 (Umbreit, Burris, and Stauffer, 1964), and the barometric pressure at Salt Lake City (elevation 4200 ft) was 640 mm Hg. When the solution was equilibrated with 5% CO_2 in room air giving a P_{CO_2} of 32.0 mm Hg, the pH fell to 6.49. This produced depolarization and increased spike discharge (Fig. 7 A) in the cell which gave the responses shown in Fig. 6. When $(HCO_3)_o$ was lowered to 0.2 mM, pH fell to 6.46 and

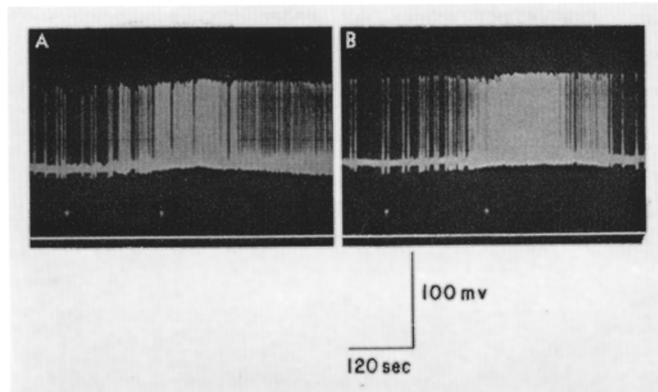

Figure 7 A. Effect on a visceromotor cell of 5% CO_2 using ASW containing CO_2–HCO_3 buffer in place of Tris-maleate. In control, $(HCO_3)_o$ 2.0 mM, pH 7.46, and P_{CO_2} 2.78 mm Hg (see text). Test solution applied at signal mark was equilibrated with 5% CO_2 ; P_{CO_2} was 32.0 mm Hg, pH 6.49. B. Same cell superfused (at signal mark) with ASW containing $(HCO_3)_o$ 0.2 mM, pH 6.46, and P_{CO_2} 2.78 mm Hg. There is no difference in the response to the two test solutions.

P_{CO_2} remained at 2.78 mm Hg. The response provoked by this acid solution shown in Fig. 7 B was very similar to the one elicited by the 5% CO_2 , 2.0 mM $(HCO_3)_o$ acid solution. The common stimulating factor in the experiments illustrated by Figs. 6 and 7 was the fall in pH.

Effect of CO_2 without a Concomitant Change of Extracellular pH Fig. 4 B showed the marked response of the giant cell to 5% CO_2 and Fig. 4 C showed the lack of any response to 50% CO_2 when the extracellular pH was held constant at 8.0. CO_2 had no effect when extracellular pH was not allowed to decrease (five giant cells). Seven visceromotor cells had responses similar to that shown by the giant cells. 10 other visceromotor cells exposed to 10% CO_2 and pH 8.0 also were not affected.

Without measuring intracellular pH, it is difficult to know whether it changed in the case of the cell shown in Fig. 6 C. If the graph relating intra- to extracellular pH of isolated rat diaphragm is applied (Fig. 4, Waddell and Bates, 1969), then intracellular pH should have fallen. In the case of Fig. 4 C, there can be little doubt that the addition of 50% CO_2 would produce a considerable fall in intracellular pH (Waddell and Butler, 1959; Chalazonitis and Romey, 1964; Waddell and Bates, 1969), yet there was no change in electrical activity. In 3 of 12 instances, a small depolarization of 3 mv or less and an increase in discharge rate of 15% or less occurred when 50% CO_2 at pH 8.0 was added. This may have been due to a local decrease of pH in the fluid immediately surrounding the impaled cell produced by the CO_2 which diffuses faster than the ionic constituents of the bulk fluid in the chamber (Gray, 1968).

Continuous titration of CO_2–ASW, which added bicarbonate ion to the perfusing solution, lacked any effect. This suggested that increased (HCO_3) had no part in the response evoked by 5% CO_2 at pH 6.5. This is analyzed further in the following section.

Effect of Changes in HCO_3 and Tris-Maleate The possibility that changes in $(HCO_3)_o$ might be involved was ruled out by the type of experiment shown in Fig. 8. In three such experiments, a CO_2–HCO_3 buffer system was used

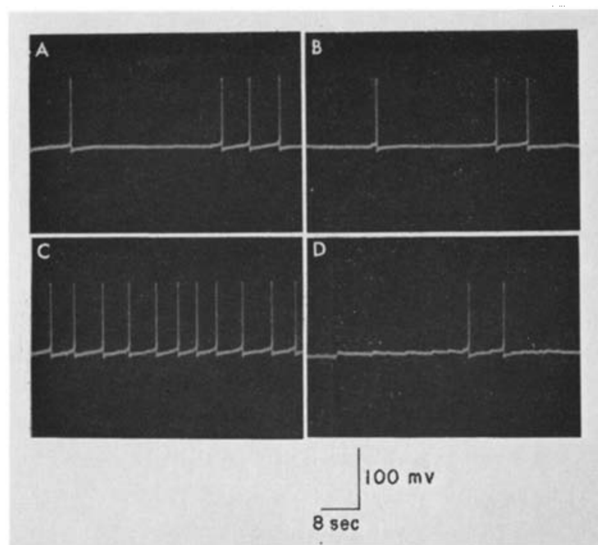

FIGURE 8 A. Effect of changes in (HCO_3). Visceromotor cell superfused with ASW in which Tris-maleate 10.0 mM was replaced by $NaHCO_3$ 2.0 mM, pH 8.0. B. Same cell, $NaHCO_3$ lowered to 0.2 mM and pH adjusted from 6.4 to 8.0 with Tris-maleate. Recorded 2 min after change of solution. C. Same cell, $NaHCO_3$ 0.2 mM and pH 6.4. Note increased discharge, recorded 2 min after change of solution. D. Same cell, 30 min after recovery from C. $NaHCO_3$ 2.0 mM, pH 8.0.

and similar results were obtained in each case. In Fig. 8 A $(HCO_3)_o$ was 2.0 mM, pH 8.0, and in Fig. 8 B $(HCO_3)_o$ was 0.2 mM and pH was adjusted to 8.0 with Tris-maleate. There was no difference in discharge. In unadjusted 0.2 mM $(HCO_3)_o$ ASW, pH fell to 6.5 and a marked increase in activity occurred (Fig. 8 C).

A final possibility that Tris-maleate might be involved was ruled out by two experiments in which its concentration was varied 10-fold without affecting the cell's electrical activity (Fig. 9 A–D). Thus, in Fig. 9 B, Tris-maleate was lowered from 10.0 to 1.0 mM, the pH adjusted to 8.0 with $NaHCO_3$, and no effect was observed.

Effect of CO_2 and Lowered pH on the Current-Voltage Curve of Responsive Cells

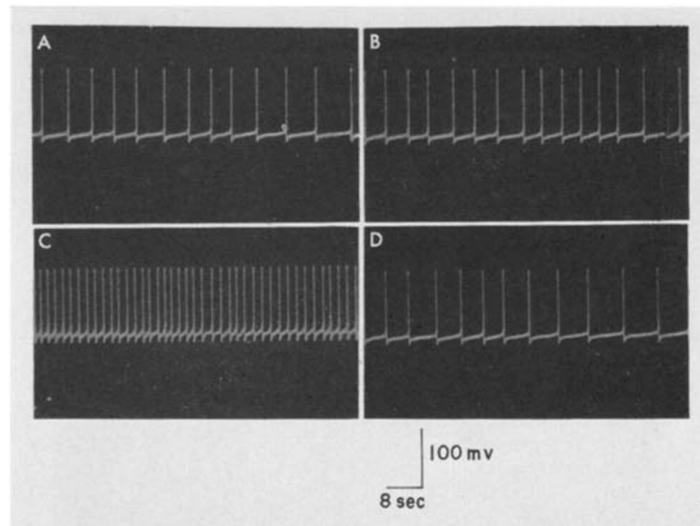

FIGURE 9 A. Effect of changes in Tris-maleate. Visceromotor cell superfused with ASW buffered with Tris-maleate 10.0 mM, pH 8.0. B. Same cell, Tris-maleate 1.0 mM, pH adjusted to 8.0 with 0.5 M $NaHCO_3$. Recorded 2 min after change of solution. C. Same cell, Tris-maleate 1.0 mM, pH 6.4. Note the increased discharge recorded 2 min after change of solution. D. Same cell after recovery in control solution.

Further evidence that the CO_2 effect was due to a change in extracellular pH came from studies of its effect on the current-voltage curves of visceromotor cells, and the giant cell. Curves identical to those obtained by the method of using current pulses of varying amplitude were obtained by continuously varying the current according to the method of Furshpan and Potter (1959), and this more convenient method was employed to study the effects of CO_2. The results were the same whether the current-passing or recording micropipettes contained 3 M KCl, 0.6 M Na_2SO_4, or 0.6 M K_2SO_4.

Current-voltage curves of the giant cell, bursting cell, pacemaker cells, and all visceromotor cells were nonlinear (Fig. 10 A; Kandel and Tauc, 1965). Thus, membrane slope conductance of the cell shown in Fig. 10 A was lowest around the resting potential of -55 mv (set at the origin) where it was 1.2 μmhos. It increased to 2.9 μmhos as the cell was depolarized to -40 mv indicating the onset of delayed rectification (Grundfest, 1961). Hyperpolarization to -60 mv also increased slope conductance to 3.3 μmhos indicating the onset of anomalous rectification (Kandel and Tauc, 1965).

The addition of 5% CO_2–ASW, pH 6.5, for 3 min always caused the current-voltage curve to become linear, and this linearity was due to an increase of the slope conductance from 1.2 to 2.4 μmhos, at or near the resting potential (Fig. 10 B). The resting potential of -55 mv had been set at the origin and when the membrane was depolarized to -45 mv, this value was also

set at the origin (Fig. 10 B). Thus, the curve was actually displaced 10 mv to the right along its entirety. The slope conductance at −45 mv increased from 0.5 to 2.4 μmhos. Note also that the slope at the hyperpolarized end of the curve has decreased from 3.3 (Fig. 10 A) to 2.4 μmhos (Fig. 10 B). The recovery curve (Fig. 10 C) was taken 20 min later. The slope conductance at the origin (−55 mv) has fallen to 1.3 μmhos, at the −45 mv point to 0.4 μmho, and at the hyperpolarized end, it has risen to 3.9 μmhos. When the current-voltage curves were extended over twice the ranges shown in this figure, equivalent changes were observed. The changes of slope conductance were probably due to changes of membrane conductance. In fact, axoplasmic

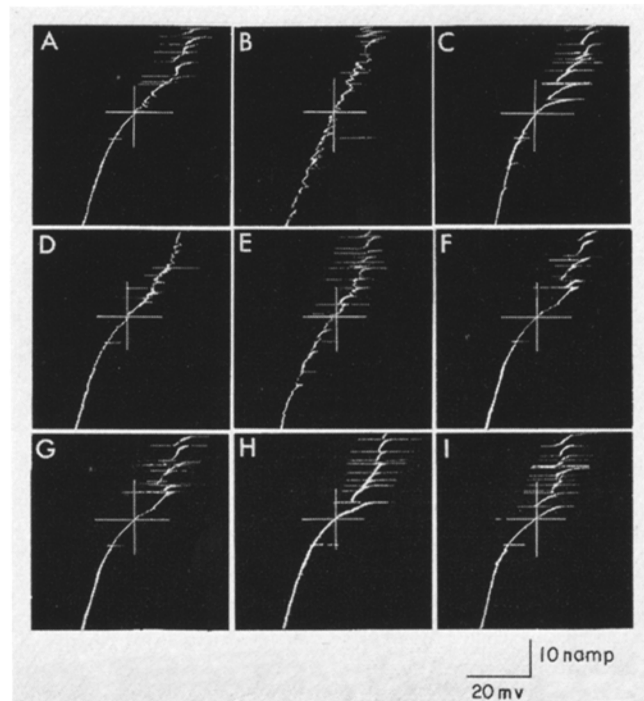

FIGURE 10. Effect of CO_2 and pH on the current-voltage curves of the giant cell. Current axis is vertical, voltage axis is horizontal, depolarization is to the right of the origin along the voltage axis and hyperpolarization to the left. Depolarizing current is upwards from the origin. Resting potential of −55 mv was set at the origin. Columns I and III, cell perfused with ASW, pH 8.0. Column II, B. Cell superfused for 3 min with ASW–5% CO_2, pH 6.5. Note the marked increase in membrane conductance at or near the origin. The conductance at the extreme end of the hyperpolarization range is decreased compared with A and C. The cell was depolarized from −55 to −45 mv but the membrane potential was arbitrarily set at the origin. The truncated horizontal lines that appear as the membrane is depolarized are action potentials. Column II, E. Cell superfused for 3 min with ASW–room air, pH 6.5. Effect similar to that in B. Column II, H. Cell superfused for 3 min with ASW–5% CO_2, pH held constant at 8.0 by continuous titration with 0.5 M NaOH. Current-voltage curve is unchanged compared with G and I.

resistance in frog skeletal muscle is not altered by changes in pH (Meves and Volkner, 1958).

Similar changes in the current-voltage curve were produced by an exposure of 3 min to zero CO_2–ASW, pH 6.5 (Fig. 10 D–F). Thus, the slope conductance near the origin (−55 mv), and at −45 mv, rose from 1.1 (Fig. 10 D) to 1.8 μmhos (Fig. 10 E), and fell during recovery to 0.9 μmho (Fig. 10 F). At the hyperpolarized end, the corresponding values went from 3.4 to 2.7 to 3.3 μmhos in control, test, and control recovery solutions, respectively. The cell was then depolarized from −55 to −46 mv. When 5% CO_2 was added and extracellular pH held constant at pH 8.0, there was virtually no change in the current-voltage curve or membrane potential (Fig. 10 G–I). Visceromotor cells responded in a like manner to the stimuli used in Fig. 10. By contrast, the current-voltage curve of pacemaker cells was unaffected even by more prolonged exposure to 5% CO_2, pH 6.5 (Fig. 11). When 10% CO_2 was used, or the pH lowered below 6.0, membrane conductance increased, and the current-voltage curve showed qualitative changes such as those shown in Fig. 10; the changes were, however, much less marked.

In two experiments, hydrogen ion as HCl or H_2SO_4 was successfully applied directly to the cell soma and produced 8 and 13 mv depolarizations accompanied by spike discharge in previously quiescent cells. Chalazonitis and Takeuchi (1966) reported similar results for giant cells of *Helix pomatia*. However, in our experiments, it was impossible to know what changes in extracellular pH occurred immediately outside the cell soma. In three experiments, attempts were made to decrease intracellular pH by ejecting hydrogen ion from a micropipette containing 3 M H_2SO_4 (100 namp for 30 min). No effect

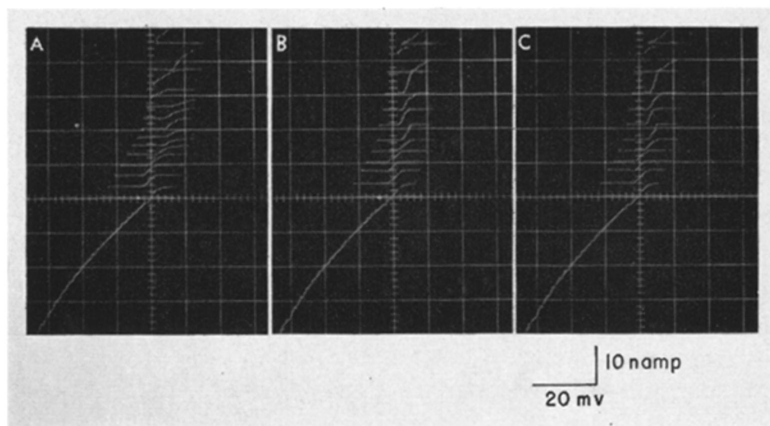

Figure 11. Effects of ASW–5% CO_2, pH 6.5, on the current-voltage curve of a pacemaker cell. Notation as in Fig. 10. A. Control. Resting potential of −50 mv was set at the origin. B. After 10 min exposure to test solution. Current-voltage curve unchanged. C. 15 min after return to control solution.

was observed, but it was not known whether intracellular pH was actually lowered.

DISCUSSION

Response to CO_2 Brief exposure of visceromotor cells of *Aplysia* to 5% CO_2 results in increased membrane conductance, membrane depolarization, and increased spike activity. Half the giant cells studied had a similar response, whereas hyperpolarization occurred in the other half. The nature of the underlying conductance changes in the giant cell is the same in either case (Brown et al., 1970; Walker and Brown, 1970). These results agree with those of Chalazonitis (1968) who reported that the membrane resistance of *Aplysia* neurons was reduced by CO_2, although in a later note (1969), he reported an increase in membrane resistance.

Mechanism by Which CO_2 Acts CO_2 exerts its effect solely through a decrease of external pH. Thus, cells studied at P_{CO_2} of 2.78 mm Hg (room air) showed an effect identical to that observed with cells studied at P_{CO_2} of 32.0 mm Hg (5% CO_2 in room air) when each solution was buffered to pH 6.5. Furthermore, when P_{CO_2} ranged from 30 to 60 mm Hg and pH was held constant at 8.0, no effect was observed. In fact, P_{CO_2}'s of 310–320 mm Hg were ineffective as stimuli provided extracellular pH was held at 8.0. Thus, CO_2 itself has no effect. Another possible stimulus, namely increased $(HCO_3)_o$ was ruled out since 10-fold changes in concentration had no effect provided external pH was not allowed to change.

The increased $(H)_o$ appears to act on the external surface of responsive cells, since its effect is rapid (less than 3 min) compared with the many hours required for increased $(H)_o$ to increase $(H)_i$ (Conway, 1957; Caldwell, 1958; Spyropoulos, 1960). Moreover, it seems that increases of $(H)_i$ do not have much effect on membrane conductance or membrane potential (Figs. 4 C and 10 H), since exposure to 50% CO_2 at constant external pH which must have increased $(H)_i$ (see below), had little or no effect on membrane potential or impulse activity in either visceromotor cells or the giant cell. What little effect there was could be attributed to faster diffusion of CO_2 compared with that of hydrogen ion through lipid-containing tissue barriers resulting in a temporary fall in pH, immediately outside the cells. Other supporting evidence for different diffusion rates is the fact that the response to CO_2 always occurred with a shorter latency than the response to an equivalent increase of $(H)_o$. Similar results were reported by Gray (1968) for carotid body chemoreceptors. He also suggested that the excitatory effect of CO_2 was mediated by the increased $(H)_o$.

An increase in CO_2 from 5 to 50% should increase $(H)_i$ by 0.5–1.5 units assuming that the intracellular buffering capacity of responsive *Aplysia* neu-

rons does not differ appreciably from that of other tissues in different species (Waddell and Butler, 1959; Adler, Roy, and Relman, 1965; Waddell and Bates, 1969). More direct evidence for this was provided by Chalazonitis and Romey (1964) although the colorimetric method they used is open to criticism (Caldwell, 1958). If such an increase of $(H)_i$ occurred, it did not appear to alter membrane conductance or potential. Nevertheless, in the absence of direct measurements of intracellular pH in our experiments comparison of the effects of increased $(H)_o$ with $(H)_i$ are speculative.

Differences in Response to CO_2 and pH amongst Neurons Far greater increases in CO_2 and $(H)_o$ are required to evoke a response in pacemaker neurons and the changes in membrane conductance, although qualitatively similar to those shown by visceromotor cells and the giant cell, are considerably smaller. Membrane depolarization and increased spike discharge are also considerably less. Nonetheless, the fact that similar responses can be obtained with larger increases in $(H)_o$ would account for the results of Chalazonitis (1963), who showed that all cells in the abdominal ganglion of *Aplysia depilans* could be depolarized by 50% CO_2. As he pointed out, however, some cells are much more responsive than others. It is possible that the difference between visceromotor neurons and the giant cell, on the one hand, and pacemaker cells on the other, can best be related to the number of sites in the membrane at which $(H)_o$ can act; the greater the number of these sites the greater the response.

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