Vol. 96, No. 2, 1980
September 30, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 785-792

REMOVING BICARBONATE/CO_2 REDUCES THE cGMP CONCENTRATION OF THE VERTEBRATE PHOTORECEPTOR TO THE LEVELS NORMALLY OBSERVED ON ILLUMINATION

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Received August 11,1980

SUMMARY: Illuminating the toad retina or removing bicarbonate/CO_2 from the incubation solution reduces the cGMP concentration of the rod outer segment by approximately 65%. In the absence of bicarbonate/CO_2 the maximum light-induced changes in cGMP concentration were reduced by 75%. Electrophysiological experiments done under these conditions showed approximately a 45% linear reduction in receptor potential amplitude at all measured light intensities with no major changes of cell sensitivity or certain other properties. In many respects decreasing the cellular cGMP concentration by eliminating bicarbonate/CO_2 did not mimic the effect of light on the phototransduction or light adaptation properties of the vertebrate photoreceptor.

INTRODUCTION: The biochemistry of the vertebrate photoreceptor cell has been intensely studied in attempts to elucidate the processes associated with visual transduction and adaptation. The possible role of the light-induced decreases in cGMP (cyclic GMP) concentration (1) has been a major concern and several models have been proposed linking this decrease with phototransduction (2, 3). The methods of testing the possible role of cGMP in the phototransduction or light adaptation processes of the photoreceptor have involved measuring the rate of the light-induced change in cGMP concentration (4, 5), observing the electrophysiological effects of perfusion with cGMP, dibutyryl cGMP, or IMX (isobutylmethylxanthine, a phosphodiesterase inhibitor) (6), or injection of cGMP into the photoreceptor (7, 8). We report here that elimination of bicarbonate/CO_2 from the perfusate can be used to reduce the cGMP concentration of the vertebrate photoreceptor to the level normally attained by illumination. Thus in the dark, we are able to alter the concentration of cGMP within its physiological levels without requiring injection

0006-291X/80/180785-08$01.00/0

or the addition of extrinsic artificial inhibitors. We have also observed the effect of these changes on the electrophysiological properties of the photoreceptor.

METHODS AND MATERIALS: Marine toads (Bufo marinus) were dark adapted for 24 hours before all experiments. The composition of the bicarbonate solution was: Na^+ 108 mM, Cl^- 90.5 mM, HCO_3^- 24 mM, K^+ 2.5 mM, SO_4^- 0.6 mM, Ca^{++} 1.0 mM, Mg^{++} 1.6 mM, glucose 5.6 mM, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid 3 mM. The solution was equilibrated with a gas mixture of 95% O_2/5% CO_2 and the pH was adjusted to 7.8. The solution without bicarbonate/CO_2 was identical to the control solution except that 24 mM NaCl was substituted for 24 mM $NaHCO_3$, and the solution was equilibrated with 100% O_2. The small difference in free Ca^{++} concentration between the two solutions due to bicarbonate binding (1.0 mM vs 0.9 mM) was found to have minimal effects on the measurements. For extracellular recording, 10 mM Na aspartate was added to both solutions. The methods for extracellular and intracellular recording have been described previously (9, 10). For the cGMP measurements the retina was excised free of pigment epithelium under infrared illumination. Incubations were carried out for 2 to 30 min in approximately 20 ml of solution. Full bleaching was attained by exposing the retina to room illumination for 5 minutes. To break off the rod outer segments the retina was transferred to a beaker containing 0.5 ml of the appropriate solution, shaken gently for 10 sec and the retina was then removed. To stop all reactions, 0.5 ml of 30% TCA (trichloroacetic acid) was then added. To insure disruption of the rod outer segments, the mixture was frozen and thawed twice. The mixture was then centrifuged at 27,000x g for 15 min. The supernatant was washed four times with 5 ml of water-saturated ether to remove the TCA, and the acid free solution was air dried. The residue was reconstituted with 0.3 ml of 50 mM acetate buffer (pH 6.2) and the cGMP content was measured by the radioimmunoassay technique (Collaborative Research, Waltham, MA (11)). The TCA precipitates were dissolved in 0.3 ml of 1.0 N NaOH and protein amounts were determined using the method of Lowry et al. (12).

RESULTS: As presented in Fig. 1 the cGMP concentration of toad photoreceptor outer segments is reduced by the elimination of bicarbonate/CO_2 from the incubating solution and by illumination. In the dark, in the presence of bicarbonate/CO_2 there were 77 ($\pm$ 5, standard error) pmoles cGMP/mg protein. Illuminating the retinas or eliminating the bicarbonate/CO_2 caused similar decreases in cGMP concentration, respectively to 26 ($\pm$3) and 28 ($\pm$2) pmoles cGMP/mg protein. Thus removing bicarbonate/CO_2 from the incubating solution mimicked the effect of light on the cGMP concentration of the toad photoreceptor outer segment. There were further decreases in cGMP concentration on illumination of the retinas incubated in the absence of bicarbonate/CO_2, (to 15 ($\pm$1) pmoles cGMP/mg protein). This indicates that the effect of light on the cGMP concentration is not completely eliminated by the removal of bicarbonate/CO_2. Approximately 25%

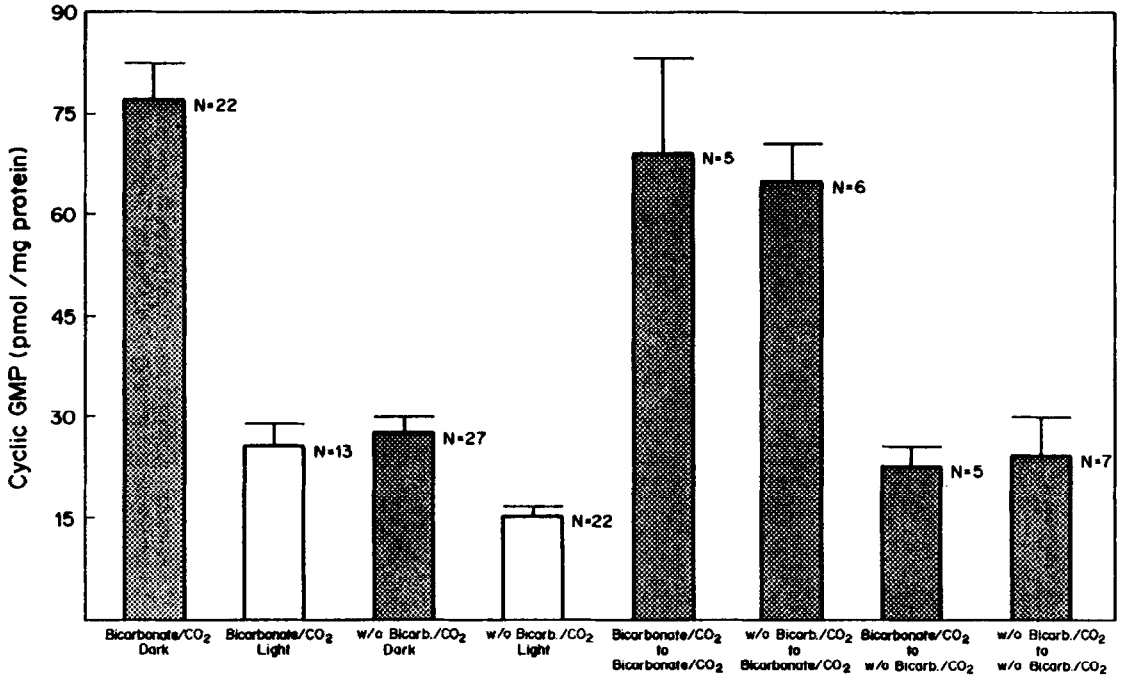

Figure 1. Cyclic GMP Concentrations of Rod Photoreceptor Outer Segments. Error bars indicate standard error of the mean. First four bar graphs present effects of various solutions with differing light-dark conditions. Second four bar graphs present effects of incubating only dark-adapted retinas in one solution for five minutes and transferring the retinas to a second solution for five minutes.

$(\frac{28-15}{77-26})$ of the effect still appears to be present. To be certain that we were not permanently injuring the photoreceptor, or permanently altering the ability of the photoreceptor to regulate cGMP, we performed a series of experiments in which, in the dark, the retinas were transferred between the different incubating solutions. This is also illustrated in Fig. 1. Although the additional transfer seemed to slightly reduce the cGMP concentrations, the final concentrations were determined by the final incubating solutions. These experiments indicate that the effect of the incubating solution on cGMP is reversible. These experiments also show that the changes in cGMP concentration occur within 5 minutes of transfer between solutions. No time-dependent effects on the cGMP concentration were observed from 2 to 30 minutes of incubation.

It was of some interest to observe the effect of bicarbonate/CO_2 on the electrophysiological responses of the photoreceptor. Representative experi-

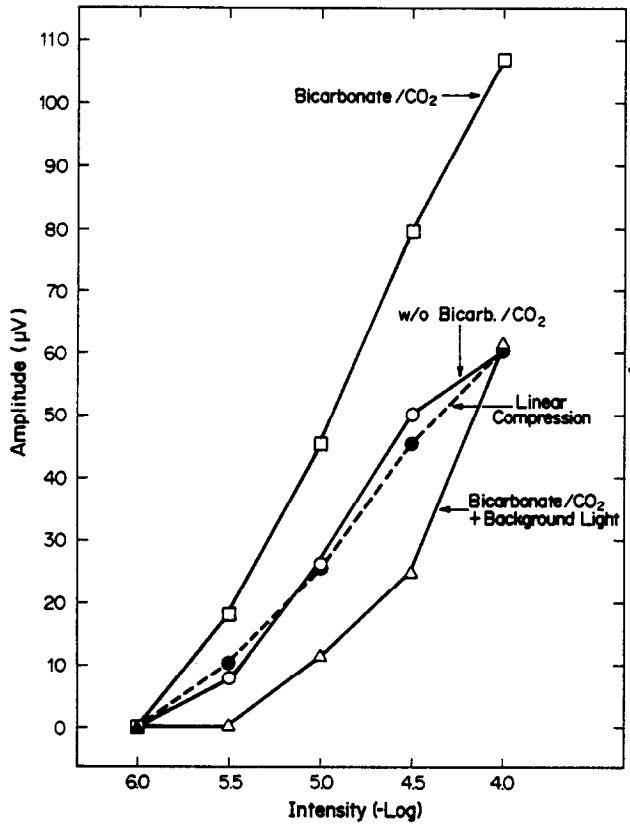

Figure 2. Representative Extracellular Experiment Comparing the Effect of Bicarbonate/CO_2 and Light on the Distal PIII Responses of the Toad Retina (Bufo marinus). Light stimuli were 300 msec in duration with 3 min between stimuli. The unattenuated stimulus light intensity (500 nm) was 1.4×10^{-5} W/cm^2. Background light intensity (500 nm) was 1.4×10^{-11} W/cm^2. Intensities were selected so that no stimuli were presented above cone threshold. The amplitude-light intensity relationship (V-log I) was determined after 84 min in the initial perfusion medium without bicarbonate/CO_2 (perfusion rate 8 ml/min). The perfusate was then changed to include bicarbonate/CO_2. The bicarbonate/CO_2 V-log I curve was determined after 84 minutes in that perfusate and the curve + background light after 107 min. The linear compression curve illustrates a 57% decrease of the bicarbonate/CO_2 curve. This experiment is representative of 10 such solution changes with 8 separate retinas. (Observed decreases without bicarbonate/CO_2, range 40-65%, average 53% $\pm$ 6 standard error.)

ments illustrating the data obtained with both extracellular and intracellular recording techniques are presented in Figures 2 and 3. Both techniques gave similar results. On removal of bicarbonate/CO_2, a decrease in response amplitude was observed (57% of maximum, Fig. 2; 55% of maximum, Fig. 3). This effect of the removal of bicarbonate/CO_2 was then compared with the effect of

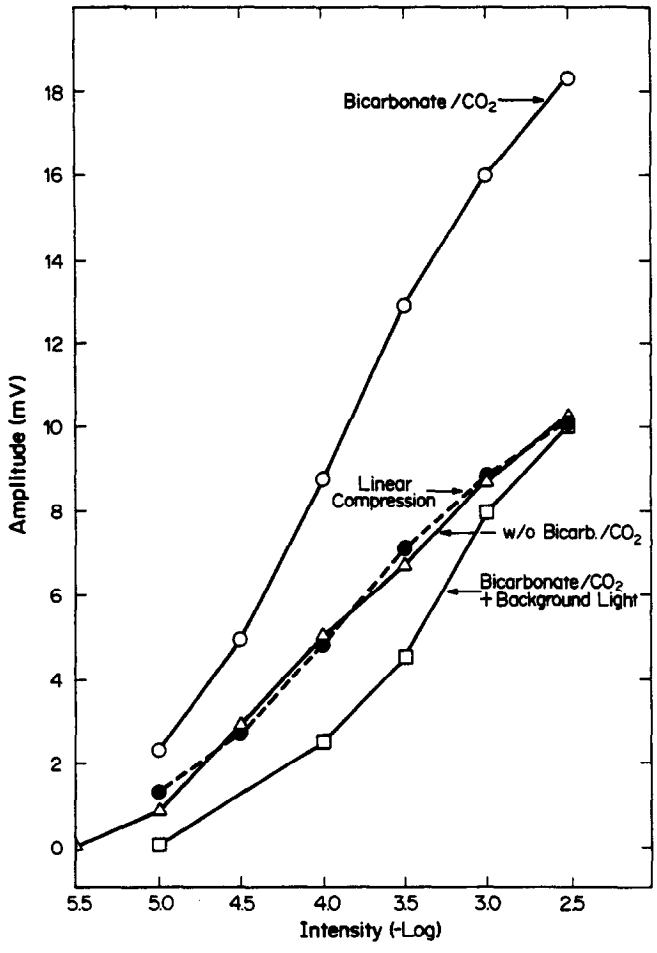

Figure 3. Representative Intracellular Experiment Comparing the Effect of Bicarbonate/CO_2 and Light on the Photoreceptor Responses of the Toad Retina (Bufo marinus). Light stimuli were 200 msec in duration with 15 sec between stimuli. The unattenuated stimulus light intensity (500 nm) was 2.3×10^{-6} W/cm^2. The background light intensity (500 nm) was 1.5×10^{-10} W/cm^2. Perfusion rate was approximately 4 ml/min. After a minimum of 5 minutes in the initial perfusion solution, bicarbonate/CO_2, a V-log I curve was determined. The V-log I curve without bicarbonate/CO_2 was started 9.5 min after changing solution. The linear compression curve illustrates a 55% decrease of the bicarbonate/CO_2 curve. This experiment is representative of 3 individual cells which were studied completely and 7 cells which were studied under various conditions and whose data were averaged. The only observed difference between the averaged cells and the data presented here was that some of the cells in solution without bicarbonate/CO_2 showed a somewhat greater sensitivity.

a steady background light selected so that it induced the same reduction in maximum signal amplitude. The effects are clearly different. While elimination of bicarbonate/CO_2 produced decreases in signal amplitude which were a linear compression of the original Signal Amplitude-Light Intensity relation-

ship (see Linear Compression curve in Figs. 2 and 3), the steady background light caused significant signal amplitude changes at the lower light intensities (i.e., sensitivity changes). The observed changes in maximum amplitude are consistent with previous reports of the effects of bicarbonate on the vertebrate receptor potential (13, 14, 15) and contrast with the results reported for an invertebrate (16). Bicarbonate/CO_2 also affects the membrane potential of the photoreceptor. Using intracellular recording, it was observed that changing to perfusate without bicarbonate/CO_2 caused a depolarization of the cell (in 8 of 11 changes, with 8 cells); changing to perfusate with bicarbonate/CO_2 caused a cellular hyperpolarization (in 14 of 18 changes, with 15 cells). The changes in membrane potential are consistent with the report of Pinto and Ostroy (14). In preliminary experiments we have also compared the effect of light and bicarbonate/CO_2 on the time-course of the photoreceptor responses. Whereas steady background light caused decreases in the latency, (2 of 3 comparisons of receptor potentials with similar amplitudes), decreases in the time to peak (3 of 3 comparisons), and decreases in the total time-course of the photoresponses (3 of 3 comparisons); the only effect of the elimination of bicarbonate/CO_2 were decreases in the latency (3 of 3 comparisons; maximum latency change 0.11 sec, a factor of 1.8). Elimination of bicarbonate/CO_2 caused no clear effect on the time to peak (2 of 3 comparisons) or the total time-course of the responses (2 of 3 comparisons).

DISCUSSION: The amplitude effects without changes of sensitivity that we observed under conditions that reduce the cGMP concentration of the photoreceptor, are comparable to the results obtained by Lipton et al. (6). Since Lipton et al. (6) used a 0.13 mM bicarbonate solution and then added IMX, cGMP, or dibutyryl cGMP, it is possible that their experiments altered cGMP concentration within the same range. We have not observed the 5-50 fold changes in latency reported by Nicol and Miller (7). However, they used 24 mM bicarbonate in their perfusion media and then injected cGMP. Therefore they presumably started at our higher levels of cGMP and went to even higher

levels. We have not performed comparable experiments. We do not have any definitive explanations for the cell depolarizations that we [and Pinto and Ostroy (14)] observed on eliminating bicarbonate/CO_2 from the perfusate as compared to the hyperpolarizations observed on altering cGMP by Lipton et al. (6) and Nicol and Miller (7, 8). Because we do not yet understand the mechanisms of the phototransduction or light adaptation processes, it is not possible to interpret the present data in a singular way with regards to the role of cGMP. We have been able to reduce the cellular concentration of cGMP to the levels normally attained by complete bleaching. However, some light-induced concentration changes of cGMP can still be produced. In many respects decreasing the cGMP concentration by eliminating bicarbonate/CO_2 did not mimic the effect of light on the phototransduction or light adaptation properties of the vertebrate photoreceptor. The data may indicate that concentration changes of cGMP in the photoreceptor are not critical to these electrophysiological processes. However, the data could also be interpreted as indicating that while the cellular concentrations of cGMP are not a critical factor, the remaining small light-induced changes of cGMP do reflect some critical step.

ACKNOWLEDGEMENTS: Supported in part by grants from the United States Public Health Service EY00413 and GM07211 (E.M.). We thank Peter J. Stein for some preliminary experiments and Roberta A. Svoboda for her comments.

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