Opposing effects of calcium and barium in vertebrate rod photoreceptors

(visual transduction/ionic control of vertebrate rods/adaptation in vertebrate rods)

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ABSTRACT Intracellular recording in outer segments of red rods of the toad retina showed that increasing Ca²⁺ concentration in the perfusate mimicked certain aspects of light adaptation. Light sensitivity was reduced, the amplitude of light responses was reduced, the time course of light responses was altered by shortening the delay to the peak and increasing the decay rate, and the resting membrane potential was generally increased. These results provide further support for the hypothesis that Ca²⁺ acts as an internal transmitter that is subject to light-induced release from the rod saccules. The addition of Ba²⁺ to the perfusate mimicked dark adaptation by having effects opposite to all those described for Ca²⁺. The Ba²⁺ effects were specific, Mg²⁺ and Sr²⁺ being ineffective in similar or greater concentration. Also, Ba²⁺ gave clear effects at concentrations as low as 0.1 mM. It is proposed that Ba²⁺ enters the cell and reduces the uptake of Ca²⁺ into the rod saccules, because the various effects of Ba²⁺ upon light responses all seem explainable from this hypothesis. Ba²⁺ also reduced the resting membrane potential, probably by reducing the membrane conductance for K⁺. The demonstrated effects of Ba²⁺ indicate an important role for Ba²⁺ in analyzing the ionic aspects of transduction in vertebrate photoreceptors. They also suggest a critical physiological role for Ba²⁺ in controlling both the resting properties and light-induced responses of vertebrate rods, because barium has been reported to be concentrated in outer and inner segments of cat photoreceptors.

Recently, much interest has focused upon the role of ions in generating light responses in the outer segments of vertebrate photoreceptors. Yoshikami and Hagins (1) have proposed that Ca²⁺ plays a critical role in rods by being concentrated in the saccules of the outer segment and then being released to the cytoplasm after a light-induced conformational change of rhodopsin in the saccule membrane. The released Ca²⁺ then diffuses to the outer membrane and decreases the membrane conductance for Na⁺, thus giving rise to the hyperpolarizing light responses recorded intracellularly in vertebrate photoreceptors. Although supported by several lines of evidence (1–4), there are outstanding questions (4), especially concerning the light-induced release of sufficient Ca²⁺ at low light intensities (4).

Bellhorn and Lewis (5) have recently shown by secondary ion mass spectroscopy that barium is much more concentrated in outer and inner segments of photoreceptors than at other sites in the cat retina. This suggests that Ba²⁺ may also play a crucial role in the normal physiology of rod outer segments. Hence, we examined the effects of both Ca²⁺ and Ba²⁺ in the perfusate while recording intracellularly from rod outer segments.

METHODS

Preparation. Studies were conducted in the isolated and inverted retina of the toad Bufo marinus. A glass contact lens with the convex surface upward was sealed into a perfusion chamber mounted on a microscope stage, and the retina was placed on the glass contact lens. Well-focused stimuli were delivered from below through the condensing lens of the microscope. The receptor surface was viewed continuously by using infrared illumination from below and a Zeiss ×40 water-immersion lens. The magnified image was focused upon a silicon diode image tube, which acted as an infrared image converter, in a high-resolution TV camera whose output was displayed upon a monitor. Microelectrodes were introduced at an angle of about 25° from the retinal surface, and contact of an electrode with the receptor surface could be visualized by a slight movement of the tip of the outer segment initially contacted. Scanning electron microscopy has shown that rod outer segments are the only structures available for penetration at shallow depths in this preparation (6). Red and green rods were distinguished by their different wavelength sensitivities, and all recordings in this work were from red rods. At the angle of penetration used, only the outer segments of red rods would be penetrated at depths up to about 155 μm, as measured along the electrode track. We accepted as outer segments only cells recorded at electrode depths of less than 100 μm, and many recordings were obtained very close to the tips of the outer segments.

Microelectrode Technique. Fresh outer segments of red rods, when detached from the inner segments and examined in control perfusate under a light microscope, had diameters that measured 5.0–7.5 μm and averaged about 5.9 μm (6). We used microelectrode techniques developed recently for intracellular work in such small cells (6). A new micropipette puller provided tips that were reliably only 0.02–0.07 μm in diameter. These tips were also quite short and hence relatively stiff. For this work they were sometimes beveled as we have described (7), and all electrodes were filled with 4.0% Procion yellow in 0.15 M KCl. Electrodes were advanced by a new high-speed stepping hydraulic advance that provided 1-μm steps featuring high acceleration to a velocity of about 1 μm/msec (which pertained through most of the step) followed by negligible vibrations (6). With these techniques we readily obtained intracellular recordings that exhibited membrane potentials as large as 40 mV and hyperpolarizing light responses with amplitudes up to 90 mV, both of which sometimes remained stable for 2-3 hr.

Perfusion. The control perfusate contained NaCl (94 mM), KCl (2 mM), MgCl (1 mM), NaHCO₃ (15 mM), glucose (10 mM), and CaCl₂ (1.8 mM); after it was bubbled with 98.5% O₂/1.5% CO₂, it had a pH of 7.7–7.8. Effects of Ca²⁺ concentration outside the cell [(Ca²⁺)ₒ] were studied with experimental solutions that either raised or lowered [Ca²⁺]ₒ from control

Abbreviations: [Ca²⁺]ₒ, calcium concentration outside cell; Eₘ, resting membrane potential; [Ca²⁺]ᵢ, calcium concentration inside cell (in cytoplasm); gₘ, membrane conductance for potassium.
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Our main results were usually in significant variation of pH among the solutions used. After a change in the perfusing solution, a period of about 3 min was usually required to flush the perfusion chamber and establish the steady-state effects of the new solution upon the cell, as determined by monitoring both the resting membrane potential \(E_m\) and the light response. Reported results were obtained under these steady-state conditions, unless specifically indicated otherwise. Successful experiments were conducted on 16 retinas and 33 cells, and the effects of \([Ca^{2+}]_o\) and \([Ba^{2+}]_o\) that will now be described were found consistently.

**RESULTS AND DISCUSSION**

Our main findings are illustrated in Fig. 1. The results in Fig. 1 were all obtained from a single cell that exhibited no significant change of either \(E_m\) or the light response, upon each return to control conditions, for more than 2 h. Starting from a control solution containing \(Ca^{2+}\) at 1.8 mM, the \([Ca^{2+}]_o\) was increased to 7.2 mM and then decreased successively to 1.0 and 0.6 mM before it was returned to the control value. At each steady-state \([Ca^{2+}]_o\), light responses were elicited by a fixed flash intensity that yielded a response in the control solution of about 7.0-mV peak amplitude, which was well below the maximum response amplitude of the cell. Also, \(E_m\) was measured just before each light response by signal averaging with a computer. The same series of solution changes was then repeated, and at each steady-state \([Ca^{2+}]_o\), the attenuation of light intensity was determined for eliciting a response with a peak amplitude of 1.0 mV. This yielded data for the column showing relative sensitivity as a function of \([Ca^{2+}]_o\). The same types of information were obtained with \(Ba^{2+}\) by using a control solution containing 1.8 mM \(Ca^{2+}\) and no \(Ba^{2+}\), then increasing \([Ba^{2+}]_o\) by three steps to a maximum of 1.2 mM before returning to the control solution.

**Effects of \([Ca^{2+}]_o\).** An increased \([Ca^{2+}]_o\) over the illustrated range, markedly decreased the amplitude of light responses. Also, when \([Ca^{2+}]_o\) was raised from normal to 7.2 mM, the cell’s sensitivity was decreased by 0.7 log unit, whereas decreasing \([Ca^{2+}]_o\) from normal to 0.6 mM increased the cell’s sensitivity by 1.0 log unit. Decreasing \([Ca^{2+}]_o\) from 1.8 to 0.6 mM consistently depolarized \(E_m\), but this depolarization was usually less than the accompanying increase of response amplitude. After a change in \([Ca^{2+}]_o\), from 1.8 to 7.2 mM, \(E_m\) consistently showed a large initial hyperpolarization that declined to a small value in the steady state and sometimes even culminated in a small depolarization. The light responses in Fig. 1A appear to decay more rapidly to the baseline at higher \([Ca^{2+}]_o\). This is a true effect because it was still evident when appropriate gains were used to match response amplitudes. When \([Ca^{2+}]_o\) was changed from 0.6 to 7.2 mM, these records showed that the time required for decay to one-half the peak amplitude decreased from 785 to 523 msec. The initial time course of the light response was likewise altered, as illustrated by the superimposed records at faster time base in Fig. 2A. When the values of \([Ca^{2+}]_o\), were 0.6 and 1.8 mM, respectively, the time from stimulus onset to the response peak was shortened from 400 to 330 msec.

**Fig. 2.** Intracellular recordings at expanded time scale to illustrate the effects of varying \([Ca^{2+}]_o\) (A) and \([Ba^{2+}]_o\) (B) on the initial time course of the light response. Recording and stimulating conditions were as described for light responses in Fig. 1. Effects of \([Ca^{2+}]_o\) were from the same cell illustrated in Fig. 1. The effects of \([Ca^{2+}]_o\) were better illustrated by another cell that was impaled 60 μm beyond the tips of the outer segments, as measured along the electrode track. \([Ca^{2+}]_o\) was decreased from 1.8 to 0.6 mM, and two intermediate records are also shown that were obtained as the \([Ca^{2+}]_o\) in the recording chamber fell to the new steady-state level.
Effects of \([\text{Ba}^{2+}]_0\). In most respects the effects of \(\text{Ba}^{2+}\) were just the opposite of \(\text{Ca}^{2+}\) effects. With increased \([\text{Ba}^{2+}]_0\), \(E_m\) became more depolarized (Fig. 1B). When \([\text{Ba}^{2+}]_0\) was increased to 0.6 mM, the cell's sensitivity increased by 0.4 log unit; the sensitivity then declined at the higher concentration of 1.2 mM. Also, when \([\text{Ba}^{2+}]_0\) was increased to 0.6 mM, the response amplitude increased markedly; at 1.2 mM the response amplitude became difficult to assess because of the large oscillations that preceded the light response. With increased \([\text{Ba}^{2+}]_0\), decay of the response was delayed progressively and markedly, and the time course of the decay assumed a different form. When \([\text{Ba}^{2+}]_0\) was increased from 0.0 to 0.6 mM, the time required for decay to one-half the peak amplitude increased from 524 to 1367 msec. Also, the expanded time scale of Fig. 2B shows that, when \([\text{Ba}^{2+}]_0\) was increased from 0.0 and 0.6 mM, the time from stimulus onset to the response peak increased from 340 to 440 msec. Finally, light-evoked oscillations of the membrane potential occurred consistently at the higher concentrations of \(\text{Ba}^{2+}\); these oscillations began just after decay of the light response to the baseline and they sometimes persisted for several minutes. If they were occurring prior to the stimulus, as shown especially in the top record of Fig. 1B, they always ceased during the stimulus. The \([\text{Ba}^{2+}]_0\), that gave the most prolonged light-evoked oscillations was often close to 0.55 mM but was sometimes higher, as in the case of Fig. 1B.

**Specificity and Sensitivity of \(\text{Ba}^{2+}\) Effects.** The described effects of \(\text{Ba}^{2+}\) have thus far proved specific to that ion. In agreement with a previous study (8), we found no significant effect when \(\text{Mg}^{2+}\) was either removed entirely or increased to 4.0 mM (4 times normal). Also, \(\text{Sr}^{2+}\) (1.0 mM) has had no significant effect. The negative results with \(\text{Mg}^{2+}\) and \(\text{Sr}^{2+}\) seem particularly interesting because \(\text{Mg}^{2+}\) has competitive effects with \(\text{Ca}^{2+}\) in presynaptic transmitter release (9), whereas \(\text{Sr}^{2+}\) lies between \(\text{Ca}^{2+}\) and \(\text{Ba}^{2+}\) in the periodic table and has given significant effects in other preparations influenced by \(\text{Ca}^{2+}\) and \(\text{Ba}^{2+}\) (10, 11). The photoreceptor is highly sensitive to \(\text{Ba}^{2+}\), which has given clear effects at concentrations as low as 0.1 mM. Also, Fig. 1 shows that the cell is similarly sensitive to \([\text{Ca}^{2+}]_0\) and \([\text{Ba}^{2+}]_0\), when judged by the effect upon response amplitude when the concentration of either ion is varied.

**Ionic Mimicking of Light and Dark Adaptation.** Whereas \(\text{Ca}^{2+}\) can mimic light adaptation by reducing the photoreceptor's sensitivity, \(\text{Ba}^{2+}\) can mimic dark adaptation by increasing sensitivity. These ions also mimic well-known effects of adaptation on the time course of photoreceptor responses. Light adaptation speeds the response by decreasing the delay to its peak and causing more rapid decay to the baseline (12). Fig. 2 shows that increased \([\text{Ca}^{2+}]_0\) decreased the delay to the response peak, and increased \([\text{Ba}^{2+}]_0\) had the opposite effect. Similarly, Fig. 1 shows that increased \([\text{Ca}^{2+}]_0\) speeded the decay of the response to the baseline, and increased \([\text{Ba}^{2+}]_0\) greatly slowed the decay. Hence, these findings suggest that \(\text{Ca}^{2+}\) or \(\text{Ba}^{2+}\) or both may control some of the nonphotochemical aspects of light and dark adaptation that occur in vertebrate photoreceptors.

**Interpretation of \(\text{Ca}^{2+}\) Effects.** We shall assume, following previous authors (2, 8), that increasing \([\text{Ca}^{2+}]_0\) will increase \(\text{Ca}^{2+}\) concentration in the cytoplasm ([\(\text{Ca}^{2+}\)]). According to the \(\text{Ca}^{2+}\) transmitter hypothesis, this increased \([\text{Ca}^{2+}]_0\) should hyperpolarize \(E_m\); it should also reduce the amplitude of light responses because fewer \(\text{Na}^{+}\) channels would be available for closure by the light-induced release of additional \(\text{Ca}^{2+}\) from rod saccules. Both these effects have been observed previously (8), and they have been confirmed in this work, aside from the failure of supernormal \([\text{Ca}^{2+}]_0\) to consistently hyperpolarize \(E_m\) in the steady state. The decreased light sensitivity that we observed with increasing \([\text{Ca}^{2+}]_0\), may be assumed to have a basis similar to that of the reduced amplitude of light responses. When Brown and Pinto (8) decreased \([\text{Ca}^{2+}]_0\) from 1.8 to 0.6 mM, while recording intracellularly from toad rods, they found the depolarization of \(E_m\) to be matched by the increased response amplitude. In this same range of \([\text{Ca}^{2+}]_0\), however, we had found that, after a given increase or decrease of \([\text{Ca}^{2+}]_0\), to new steady-state conditions, the change of response amplitude usually exceeded the change of \(E_m\) by about 50%. Also, after a reduction of \([\text{Ca}^{2+}]_0\) in the perfusate, \(E_m\) always showed a marked initial depolarization that subsided to a much smaller depolarization in the steady state, whereas the amplitude of light responses increased monotonically to the new steady state value. Hence, in this subnormal range of \([\text{Ca}^{2+}]_0\), the effects of \([\text{Ca}^{2+}]_0\) on the amplitude of light responses cannot be understood entirely from the effects on \(E_m\). For the case of supernormal \([\text{Ca}^{2+}]_0\), a similar conclusion has already been reached (8) and is strongly indicated by our results because we sometimes even observed a depolarization of \(E_m\) in conjunction with the smaller light response, as shown in Fig. 1A.

**Oscillations of \(E_m\).** A recent study (13) of spontaneous and light-evoked oscillations, recorded from rods of the isolated retina of *B. martinius* in control perfusate, showed that such oscillations depend on receptor interactions with second-order cells having large receptive fields. The oscillations were thus abolished by markedly reducing the size of a focused and centered stimulus spot or by treating the retina with 2.0 mM \(\text{Na}^{+}\) aspartate, which consistently blocks synaptic transmission to second-order cells in this preparation (8, 13). Although light-evoked oscillations were commonly seen in control perfusate during early stages of our work, they became rare as techniques improved for isolating the retina and maintaining it in good physiological condition. By contrast, light-evoked oscillations were consistently induced by the higher concentrations of \(\text{Ba}^{2+}\). These oscillations could be markedly reduced or abolished by limiting the size of a stimulus spot that was carefully centered and focused upon the receptor. Hence, stimulus spots only 50 \(\mu\)m in diameter were used in Fig. 1 when determining relative sensitivities with both \(\text{Ba}^{2+}\) and \(\text{Ca}^{2+}\); this was necessary to eliminate \(\text{Ba}^{2+}\)-induced oscillations and thus permit a response amplitude of 1.0 mV to be measured accurately at the higher concentrations of \(\text{Ba}^{2+}\). The \(\text{Ba}^{2+}\)-induced oscillations were also reduced by the application of 2.0 mM \(\text{Na}^{+}\)-L-aspartate, but they were not abolished by this procedure when stimulating with large stimulus spots. Hence, the \(\text{Ba}^{2+}\)-induced oscillations may be enhanced by receptor-to-receptor interactions, and they may be modulated by influences from second-order cells, but they appear not to be critically dependent upon interactions with second-order cells. Clarification of the mechanisms underlying the oscillations seems to require much further study. Although \(\text{Na}^{+}\)-aspartate reduced the oscillations, it had no clear influence on any of the other \(\text{Ba}^{2+}\) effects. Hence, all of the other \(\text{Ba}^{2+}\) effects appear to be exerted directly upon the photoreceptors.

**Interpretation of \(\text{Ba}^{2+}\) Effects.** It seems evident that certain effects of \(\text{Ba}^{2+}\) upon light responses, such as the altered decay rate, cannot derive from the effect of \(\text{Ba}^{2+}\) upon \(E_m\). We shall assume that alterations of \([\text{Ba}^{2+}]_0\), will alter \([\text{Ba}^{2+}]_0\). This assumption seems reasonable because \(\text{Ba}^{2+}\) has been shown to pass through \(\text{Ca}^{2+}\) channels even more readily than \(\text{Ca}^{2+}\) in giant muscle fibers of a barnacle (10). All of the main effects of \(\text{Ba}^{2+}\) on the light response then seem explicable on the hypothesis that \(\text{Ba}^{2+}\) interferes with the uptake of \(\text{Ca}^{2+}\) from the cytoplasm into the rod saccule, a process that has been proposed to occur
continuously through the action of a Ca\(^{2+}\) pump (2). Our hypothesis predicts that, after light-induced release of Ca\(^{2+}\) from the saccule, Ba\(^{2+}\) will reduce the re-uptake of this released Ca\(^{2+}\) during the period from release until the Ca\(^{2+}\) reaches Na\(^{+}\) channels of the outer membrane. The amount of the released Ca\(^{2+}\) that reaches the outer membrane would thus be increased, and response amplitude should be increased. This would also sensitize the cell by reducing the flash intensity required to deliver a given amount of Ca\(^{2+}\) to the membrane bounding the outer segment. Because Ba\(^{2+}\) would reduce Ca\(^{2+}\) re-uptake during the entire period while Ca\(^{2+}\) is diffusing to the outer membrane, the net rate at which Ca\(^{2+}\) arrives at the outer membrane should be increased throughout the rising phase of the light response. The slope of the response rise should thus be increased, from the very onset of the response, as shown to occur with increased \([\text{Ba}^{2+}]_0\) in Fig. 2B. With reduced re-uptake, the released Ca\(^{2+}\) may also continue to reach the outer membrane over a longer period and thus account for the observed increase in delay to the response peak. Finally, Ba\(^{2+}\) should interfere with the re-uptake of Ca\(^{2+}\) that has reached the outer membrane and contributed to the light response. Hence Ba\(^{2+}\) should slow decay of the light response, as has been observed. In summary, this working hypothesis seems promising for explaining the various effects of Ba\(^{2+}\) on light responses, but detailed testing remains to be conducted. Perhaps the greatest strength of this hypothesis is that it can explain both the increased amplitude and the prolonged decay of the light response. Other hypotheses considered to date, such as a competitive action of Ca\(^{2+}\) and Ba\(^{2+}\) at Na\(^{+}\) channels of the outer membrane, fail to explain one or both of these Ba\(^{2+}\) effects.

If Ba\(^{2+}\) decreases Ca\(^{2+}\) uptake from the cytoplasm, it should also increase the steady state \([\text{Ca}^{2+}]_s\). Whereas the effect of Ba\(^{2+}\) on light-released Ca\(^{2+}\) should increase light sensitivity, the increased steady-state \([\text{Ca}^{2+}]_s\) should decrease sensitivity. These opposing effects may account for the observation that with increased \([\text{Ba}^{2+}]_0\), the photoreceptor's sensitivity first increased and then decreased again at the highest \([\text{Ba}^{2+}]_0\), 1.2 mM. This could well occur if the lower values of \([\text{Ba}^{2+}]_0\) exert their predominant effect on sensitivity by reducing the re-uptake of light-released Ca\(^{2+}\), but at sufficiently high \([\text{Ba}^{2+}]_0\), the predominant effect on sensitivity results from an increased steady-state \([\text{Ca}^{2+}]_s\).

Because increased \([\text{Ba}^{2+}]_0\) should increase the steady-state \([\text{Ca}^{2+}]_s\), this effect would hyperpolarize the outer segment membrane. The observed depolarization of \(E_m\) with increased \([\text{Ba}^{2+}]_0\) thus requires a different explanation. Ba\(^{2+}\) has been reported to reduce membrane conductance for K\(^+\) (\(g_K\)) in several preparations (14-16). The \(g_K\) of the rod outer segment has been reported to be too low to detect (17), and this would seem to preclude any significant further lowering of \(g_K\) in the outer segment. However, proximal portions of the cell appear to be permeable to K\(^+\) (18) and thus could be depolarized by a Ba\(^{2+}\)-induced reduction of their \(g_K\). Because the recorded \(E_m\) is probably an integrated result from the entire cell, it is thus suggested that increased \([\text{Ba}^{2+}]_0\) causes a net depolarization because of a Ba\(^{2+}\)-induced decrease of \(g_K\) that depolarizes proximal portions of the cell and that predominates over the hyperpolarizing effect of a Ba\(^{2+}\)-induced increase in \([\text{Ca}^{2+}]_i\) in the outer segment.

Significance of Ba\(^{2+}\) Effects. Because Ba\(^{2+}\) profoundly affects the signals generated by toad rods, it offers considerable promise for analyzing the ionic basis of signal generation in vertebrate photoreceptors. Thus far, no physiological function appears to have been demonstrated for barium, which has been treated as a trace element found in certain tissues but having little or no significance in normal functions (19). Hence, a critical question is whether Ba\(^{2+}\) is consistently present at relatively high concentration in rod outer segments. The results of Bellhorn and Lewis (5) in the cat retina are highly suggestive, especially since barium was found localized mainly in the outer and inner segments, a result that seems difficult to interpret on other grounds. If their finding is confirmed in other species, and if the barium is in free ionic form, then our findings will also indicate that Ba\(^{2+}\) has a functional role in the normal control of signal generation in vertebrate photoreceptors.

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