Electrically Evoked Calcium Responses in Rods of the Frog Retina

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Abstract In the rod of the isolated frog retina perfused with normal Ringer solution, a transretinal current pulse flowing from the receptor side to the vitreous side evoked a response consisting of a transient depolarization and a subsequent damped oscillation. The transient depolarization was not an all-or-none response, but was a graded one which depended on the intensity and duration of the electrical stimulation. The amplitude of the initial depolarization varied with Ca\(^{2+}\) concentrations of the perfusate, being enhanced in the high Ca\(^{2+}\) and reduced in the low Ca\(^{2+}\) medium. Furthermore, it was enhanced by perfusion with a Ba\(^{2+}\)-containing solution, and suppressed by exposure to Co\(^{2+}\). These observations suggest that the initial depolarization can be attributed to an increase of Ca\(^{2+}\) conductance. The late hyperpolarization which followed the initial depolarization was suppressed by membrane depolarization and was enhanced by membrane hyperpolarization. In addition, the evoked responses were not affected by application of either L-aspartate or L-glutamate, both of which blocked the synaptic transmission from the photoreceptors to the second order neurons. Thus it is unlikely that the late hyperpolarization is an IPSP-like response evoked by the negative feedback input from horizontal cells. Rather the rod membrane itself seems to have an oscillatory property.

Key Words: frog retina, rod, Ca\(^{2+}\) response, Ba\(^{2+}\), Co\(^{2+}\).

In the cone system, a majority of the reports have indicated the presence of the negative feedback from horizontal cells to receptor cells (BaylOR et al., 1971; Fuortes et al., 1973; O'Bryan, 1973; BurKHARDT, 1977; Gerschenfeld and Piccolino, 1980; Piccolino and Gerschenfeld, 1980; Lasansky, 1981). In particular, Murakami et al. (1978, 1982) reported that a GABA-mediated IPSP resulting from the negative feedback was detected in carp cones when horizontal cells were depolarized by application of a transretinal current pulse. In the rod system, on the other hand, there have been controversies as to whether a nega-
tive feedback operates from horizontal cells to receptor cells; some experiments are affirmative (PINTO and PAK, 1974; NORMANN and POCHOBRADSKÝ, 1976), whereas others are negative (BROWN and PINTO, 1974; COPENHAGEN and OWEN, 1976; FAIN et al., 1977, 1980).

In the present study, using a transretinal current pulse, we examined whether there is negative feedback between the horizontal cells and rods in the frog retina. No evidence was found in support of the presence of negative feedback in the rod system of the frog. We further found that the rods are capable of producing Ca²⁺ responses in a graded manner depending on the strength of the electrical stimulation. A preliminary report was presented at the 60th Annual Meeting of the Physiological Society of Japan (MIYACHI et al., 1983).

METHODS

Preparation. Experiments were performed on retinas of the bullfrog, Rana catesbeiana. After the frogs were dark-adapted for more than 3 hr, the animals were decapitated and the eyes were enucleated. The eye was hemisected and the corneal half was discarded together with the lens. The retina was detached from the pigment epithelium and was placed, receptor side up, on a piece of filter paper. All surgical operations were carried out under dim light.

Perfusion system and solutions. The preparation was mounted in a perfusion chamber with a capacity of about 0.7 ml, which has been described elsewhere in detail (MURAKAMI et al., 1982). The Ringer solution was continuously supplied at a flow rate of about 1 ml/min. The normal Ringer solution, modified from BROWN and PINTO (1974), contained 106 mM NaCl, 2.5 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 0.13 mM NaHCO₃, 1.8 mM Na₂SO₄, 5.6 mM glucose, and 3.0 mM HEPES. The solutions containing high Ca²⁺ (3.0 mM) and low Ca²⁺ (1.0 mM) were prepared by either raising or lowering CaCl₂. The Ba²⁺-containing solution was prepared by replacing CaCl₂ with 1.8 mM BaCl₂. In order to avoid precipitation in the Ba²⁺ solution, neither NaHCO₃ nor Na₂SO₄ was added. Lack of NaHCO₃ or Na₂SO₄ did not affect the light-induced or electrically evoked responses. The Co²⁺-containing solution was made by adding 1 mM CoCl₂ to normal Ringer solution. The solution containing aspartate or glutamate was made by dissolving 2 mM sodium L-aspartate or L-glutamate to normal Ringer solution. The pH of the solutions was adjusted to 7.8 with NaOH. All chemicals used were from Kanto Chemical Co. (Tokyo). The temperature of the solutions was regulated at about 20°C by means of a water jacket attached beneath the perfusion chamber.

Transretinal current application and intracellular recording. Details of the methods have been fully described elsewhere (MURAKAMI et al., 1982). Transretinal current pulses were applied between two current electrodes; one was an Ag-AgCl wire ring 2.6 mm in diameter placed in a perfusate above the retina and
the other was a grounded Ag-AgCl plate placed on the bottom of the perfusion chamber. Intracellular microelectrodes were positioned at the center of the wire ring electrode.

Intracellular glass micropipettes were made with a Livingston-type puller and their resistance ranged from 80 to 200 MΩ when filled with 4 M potassium acetate. In order to minimize artifacts caused by transretinal current pulses, a differential recording was made between the intracellular microelectrode and the reference electrode (Ag-AgCl wire) appropriately placed in the perfusion medium. Responses were fed to a preamplifier (WPI, KS-700) and displayed on an oscilloscope (Nihon Kohden, VC9). The responses were simultaneously recorded with a pen recorder (Nihon Kohden, RJG-4100), of which the frequency characteristic was fast enough to follow the evoked responses.

Light stimulation and cell identification. The photostimulator used has been described elsewhere (TOMITA et al., 1967). The retina was illuminated with 500 msec diffuse light of 500 nm (8.2 × 10³ photons cm⁻² sec⁻¹).

Cell identification was achieved by intracellular staining with Lucifer Yellow CH (Aldrich Chemical Co.) in several initial experiments, thereafter, by the recorded depths, and by the shapes and time courses of the light-induced responses. Cells were identified as “red” rods by their spectral responses peaking at about 500 nm.

RESULTS

Responses in rods evoked by transretinal current pulses in normal Ringer solution

In the rod of the isolated frog retina perfused with normal Ringer solution, the membrane potential usually ranged between —35 and —40 mV in the dark. A transretinal current pulse evoked a transient depolarization frequently followed by an oscillation. Figure 1 shows such evoked responses to 10 mA brief current pulses, which were given at different timings during the course of light-induced responses. The response showed a damped oscillation which varied with timing of electrical stimulation. The frequency of the oscillation was about 2 Hz, being comparable with that of the spontaneously occurring oscillation observed by other investigators (NORMANN and POCHOBRADSKÝ, 1976; YAMADA and TAUCHI, 1982). The light stimulus was not a necessary condition for producing the electrically evoked response, since it was also observed in the dark. Electrically evoked responses were detectable in almost all recorded rods.

Transretinal current pulses flowing in the opposite direction, i.e., from the vitreous side to the receptor side, rarely evoked potential deflections in rods.

Graded property of evoked responses

In the toad rods, FAIN et al. (1977, 1980) reported that a regenerative depolarizing potential occurred following the light-induced response when the retina

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was perfused with solution containing tetraethylammonium chloride (TEA) or Ba²⁺. We examined whether the electrically evoked response is all-or-none or graded in normal Ringer solution.

Figure 2 shows the effect on the evoked response of the intensity of transretinal current with a fixed duration of 5 msec applied in normal Ringer solution. In each of six sample records shown in Fig. 2A, three pulses were successively applied at the timings indicated by triangles. The amplitude of transient depolarization increased in a graded manner as the intensity of pulse current was increased to about 10 mA, at which the response amplitude was saturated. Further increase of intensity resulted in an increase of amplitude of the following oscillation. Figure 2B illustrates an intensity-response curve plotting the amplitudes of the depolarizations elicited by the first stimuli recorded from the same cell in Fig. 2A. The relative amplitude of the transient depolarization increased in proportion to current intensity up to about 10 mA.

The effect of the duration of the transretinal current pulse is shown in Fig. 3. The transient depolarization became larger as the pulse duration was increased up to about 5 msec. Further increase beyond 5 msec did not affect the transient depolarization, but increased the amplitude of the subsequent damped oscillation. Figure 3B shows a duration-response curve for the same cell shown in Fig. 3A. The amplitude of the transient depolarizations increased almost proportionally to the pulse duration up to about 5 msec.

The results shown in Figs. 2 and 3 indicate that the transient depolarization evoked by a transretinal current pulse is a graded, but not an all-or-none response.
Ca\textsuperscript{2+}-dependence of the evoked response

We next examined the effect of extracellular Ca\textsuperscript{2+} concentration on the transient depolarization evoked by a transretinal current pulse (Fig. 4). The retina was first perfused with normal Ringer solution, and the depolarizations were evoked by three successive transretinal stimulations (Fig. 4A). When the perfusate was switched to the low Ca\textsuperscript{2+} (1.0 mM) solution, the membrane potential in the dark shifted towards depolarization, and the amplitude of the light-induced response was increased (FAIN et al., 1977; BROWN and FLAMING, 1978). Under these conditions, however, the electrically evoked responses were reduced in amplitude. The perfusate was then replaced by the high Ca\textsuperscript{2+} (3.0 mM) solution (Fig. 4C). The membrane potential in the dark shifted towards hyperpolarization below the control level, and the light-induced response was reduced (FAIN et al., 1977; BROWN and FLAMING, 1978), but the electrically evoked responses were enhanced. These changes were reversible (Fig. 4D).

The electrically evoked responses were enhanced by Ba\textsuperscript{2+} and were suppressed by Co\textsuperscript{2+}. Figure 5 shows the effect of substituting BaCl\textsubscript{2} for CaCl\textsubscript{2}. In the solution containing 1.8 mM Ba\textsuperscript{2+}, the rod membrane was depolarized, and the light-induced response became much larger in amplitude and longer in dura-

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**Fig. 2.** Effect on electrically evoked response, of intensity of transretinal current pulse with a fixed duration of 5 msec. A: sample records obtained from a single rod in normal Ringer solution with increasing current intensity as indicated. In each record, three current pulses were given as shown by the triangles. B: relative amplitude of electrically evoked response as a function of intensity of pulse current, including the data not shown in A. The amplitudes of the responses elicited by the first current pulses were plotted.
Fig. 3. Effect on electrically evoked response, of duration of transretinal current pulse with a fixed intensity of 10 mA. A: sample records obtained from a single rod in normal Ringer solution with increasing duration as indicated. B: relative amplitude of evoked response plotted as a function of pulse duration. The other explanations are the same as in Fig. 2.

Fig. 4. Effects of extracellular Ca\(^{2+}\) concentration on the electrically evoked and light-induced responses. A, responses in normal Ringer solution; B, in low Ca\(^{2+}\) concentration (1.0 mM); C, in high Ca\(^{2+}\) concentration (3.0 mM); D, recovery in normal Ringer solution. A calibration is shown where 0 mV corresponds to the membrane potential of the control record (A).

When the transretinal stimulations were given, the first and the second electrical stimuli applied at the hyperpolarized level evoked small responses, whereas the third one, given at the slightly depolarized level, evoked a spike-like response almost twice as large as that evoked in normal Ringer solution. Thus the amplitude of the electrically
evoked response depended upon the level of membrane potential.

Figure 6 shows the effect of Co$^{2+}$ on the evoked responses. In the Co$^{2+}$-containing solution (1 mM), the membrane potential hyperpolarized, and the evoked oscillations were markedly suppressed (Fig. 6B). The responses recovered, though not fully, at about 5 min after the solution was replaced by normal Ringer solution (Fig. 6C). If the retina was immersed longer in the Co$^{2+}$-solution, the oscillations were completely eliminated, but the recovery was very poor.

Thus, the amplitude of the evoked response was related to the extracellular Ca$^{2+}$ concentration, enhanced by Ba$^{2+}$, and suppressed by Co$^{2+}$. These observations indicate that the transient depolarization evoked by the current pulse is produced by an increase in Ca$^{2+}$ conductance of the rod membrane.

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Effects of membrane polarization on the evoked responses

We next examined whether the hyperpolarizing rebound following the depolarization is generated by a reverberating interaction from horizontal cells, or it is generated by an oscillatory property of rod membrane. If the late hyperpolarization is an IPSP-like response produced by the feedback input from horizontal cells, it is expected to have a reversal potential which is negative to the dark membrane potential as has been shown in carp cones (Murakami et al. 1982).

As shown in Fig. 7B, the amplitudes of the late hyperpolarization decreased in amplitude when the rod was depolarized (2 top traces). Because of the strong non-linearity of the electrode resistance, it was difficult to determine the membrane potential during current injection, but it was estimated to be close to the reversal potential of the photocurrent (0 mV, Bader et al., 1978) with +0.6 nA, since at this current level the light-induced response became very small (Fig. 7A). The late hyperpolarization increased when the rod was hyperpolarized (-0.2 and -0.4 nA, 2 bottom traces of Fig. 7B). If the current-voltage relationship of the rod membrane does not deviate much from linearity (Bader et al., 1982), the membrane potential during -0.4 nA current injection is estimated to be -65 mV.

It is, therefore, unlikely that the late hyperpolarization is due to an IPSP-

![Figure 7. Effects of membrane polarization on light-induced (A) and electrically evoked (B) responses in normal Ringer solution. The rod was polarized by passing constant current through the recording electrode with the intensities as indicated on the left of each record.](image-url)
like response coming from horizontal cells. Instead, the late hyperpolarization is evoked by some ionic currents generated in the rods themselves following the early transient Ca$^{2+}$ depolarization.

**Perfusion with aspartate and glutamate solutions**

A further test to confirm the above conclusion was to observe the electrically evoked response after the synaptic transmission is blocked by L-aspartate or L-glutamate, both of which have been shown to steadily depolarize horizontal cells (MURAKAMI et al., 1972). Even after the retina was perfused with 2 mM aspartate or glutamate for 3 min, the oscillation was still observed in the rods as it was in normal Ringer solution. Blockade of the transmission was complete, since the b-wave of the electoretinogram (ERG) was totally eliminated in 2 min, leaving only the distal PIII, which corresponded to the extracellular recording of the photoreceptor potential (MURAKAMI and KANEKO, 1966; MURAKAMI et al., 1972). It is therefore unlikely that the late hyperpolarization is due to an IPSP-like response evoked by the negative feedback input from horizontal cells.

**DISCUSSION**

*Origin and electrical properties of the evoked response.* Our present experiment showed that the amplitude of the rod response evoked by the transretinal current pulse varied with extracellular Ca$^{2+}$ concentrations (Fig. 4), and was enhanced by application of Ba$^{2+}$ (Fig. 5), and suppressed by exposure to Co$^{2+}$ (Fig. 6). These observations indicate that the evoked response is attributed to an increase of Ca$^{2+}$ conductance and is identical to the Ca$^{2+}$ response observed by other investigators using other methods (FAIN et al., 1977, 1980).

In the preliminary stage of the present experiment, we tried to evoke the response by applying depolarizing current pulses through intracellular microelectrodes in the hope that this method might also evoke responses in the rod. However, current injection was not effective in producing the response, probably because the applied current leaked through the electrical coupling between rods (SCHWARTZ, 1973; FAIN et al., 1975; COPENHAGEN and OWEN, 1976). On the other hand, the transretinal current pulses, flowing from the receptor side to the vitreous side, could evoke the responses in the rods. Since the rods are elongated cells lying perpendicular to the retinal layers, the current pulse could depolarize the terminals of all the rods exposed to the current flow. Therefore, it is reasonable to assume that the individual terminals were depolarized at nearly equal membrane potential levels and little leakage current flowed between rods. Thus, the transretinal current was effective in stimulating rods (TRIFONOV, 1968; BYZOV and TRIFONOV, 1968).

The transretinal current which evokes the Ca$^{2+}$ response is in the direction with which the current depolarizes the rod terminals. Therefore, it is likely that
the Ca\(^{2+}\) response has its origin at the rod synaptic region or nearby, where Ca\(^{2+}\) channels may be present and contribute to the transmitter release (FAIN et al., 1980; PICCOLINO and GERSCHENFELD, 1980). In fact, the voltage-activated Ca\(^{2+}\) current has been demonstrated in the dissociated solitary inner segments of the tiger salamander (BADER et al., 1982).

Our experiments in Figs. 2 and 3 show that the Ca\(^{2+}\) response normally does not have an all-or-none property, but rather has a graded one depending on the current intensity, in other words, depending on how much the terminals were depolarized. This seems consistent with the commonly accepted view that the Ca\(^{2+}\) activity facilitates transmitter release in the graded manner depending on the level of the rod membrane potential.

Oscillation and negative feedback. In the present experiment, the electrical stimulation frequently evoked a damped oscillation in the frog rods (Figs. 1, 2, 3, and 7). Its frequency was about 2 Hz, being comparable with that of the spontaneously occurring oscillation (for example, 1.5–3.5 Hz, NORMANN and POCHOBRADSKÝ, 1976; 2 Hz, YAMADA and TAUCHI, 1982).

Our experiments demonstrated that the electrically evoked responses, including the subsequent damped oscillation, were diminished when the rods were depolarized by outward current injection (Fig. 7B), indicating that these responses contain no IPSP. However, the current-voltage relation of the rod membrane has been reported to be non-linear, showing an outward rectification at depolarized levels (LASANSKY and MARCHIAFAVA, 1974; WERBLIN, 1978; BADER et al., 1978; FAIN and QUANDT, 1980). Therefore, it is possible that a shunting effect of the rectification would disturb an observation of potential changes. In Fig. 7A (+0.6 nA), however, the effect of rectification was not that strong, since the light-induced response was still elicited, although greatly suppressed by the depolarizing current (TOYODA et al., 1969). More conclusively, the synaptic blocking agents, aspartate and glutamate, did not affect the responses, indicating that the oscillation does not result from reverberating interactions between rods and the postsynaptic neurons. Our conclusion is consistent with those of BROWN and PINTO (1974), COPENHAGEN and OWEN (1976), FAIN et al. (1980), and YAMADA and TAUCHI (1982). Therefore, the frog rod membrane itself contains an oscillatory property.

Our experiment cannot delineate the ionic mechanisms of the oscillation occurring at the rod membrane. However, BADER et al. (1982) have suggested that, in addition to a Ca\(^{2+}\) conductance, the rod membrane contains voltage-dependent and Ca\(^{2+}\)-dependent K\(^{+}\) conductances. These ionic currents have also been found in such nerve cells as molluscan neurons (MEECH, 1979), mammalian central neurons (LLINÁS, 1980), and cultured neuroblastoma cells (MOOLENAAR and SPECTOR, 1979). In these cells, Ca\(^{2+}\) entry activates a Ca\(^{2+}\)-dependent K\(^{+}\) conductance, resulting in an afterhyperpolarization, and inactivation of the K\(^{+}\) conductance produces a rebound depolarization which, in turn,
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can trigger Ca^{2+} entry. Therefore, it is supposed that recurrence of these sequential processes can form a damped oscillation in the rod membrane.

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