Effect of Isobutylmethylxanthine and Related Drugs on the Receptor Response (ERG; a-Wave) of the Frog Retina at Various Extracellular Calcium Concentrations

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Various drugs known or expected to increase the levels of cyclic nucleotides in cells were applied to isolated superfused frog retinae, and their influence on the aspartate-isolated a-wave was studied.

Isobutylmethylxanthine (IBMX), triacetylguanosine (TAG), and dimethylaminopurine (DAMP) strongly influenced the responses elicited from dark-adapted retinae by flashes of light: With all three drugs the response amplitude was increased, and latency and time to peak were prolonged.

If, on the other hand, the retinae were light-adapted by background light of various intensities, the drugs showed different effects on the response amplitude: IBMX either did not influence the amplitude at all or even caused a decrease (4 of 6 experiments), DAMP decreased the amplitude and TAG caused an increase of the amplitude in 2 of 3 experiments. But latency and time to peak were still prolonged by all three drugs.

When dark-adapted retinae were superfused with IBMX or TAG Ringer solution and simultaneously calcium concentration was raised, different effects of calcium on the three measured parameters of the a-wave were observed: By increasing the extracellular calcium concentration the increase of the amplitude caused by the drugs was reversed, down or even below the control level, whereas latency and time to peak remained prolonged.

Thus, both an increased calcium level and light adaptation had the same effect, namely to reverse only that part of the drug effect concerning the amplitude but not latency or time to peak of the response. The data suggest that calcium and cyclic nucleotides act through different ways in the rod cells.

Introduction

Photon absorption by rhodopsin in the vertebrate rod outer segment disc membrane causes a decrease of ion permeability in the plasma membrane leading to hyperpolarization of the rod cell. The mechanism by which bleaching of rhodopsin is transduced into a permeability change of the plasma membrane is still not understood. Both Ca^{2+} ions (Yoshikami and Hagins [1]; Hagins [2]; and others) and cyclic GMP (Bitensky *et al.* [3]; Lipton *et al.* [4]; Woodruff *et al.* [5]; a. o.) are believed to be involved in the control of the plasma membrane permeability of the rod cell. Light activates a phosphodiesterase (PDE) (Bitensky [3]) which reduces the concentration of cyclic GMP in the rod outer segments to about half of its dark value (Goridis *et al.* [6]; Woodruff and Bownds [7]). If the intracellular cGMP level is artificially increased by injection of cGMP (Miller and Nichol [8]) the rod cell is depolarized, and both the amplitude and latency of the light response are highly increased. The same physiological effects are obtained by incubating retinae with the phosphodiesterase inhibitor isobutylmethylxanthine (= IBMX; Lipton *et al.* [4]; Pinto *et al.* [9]). Cohen *et al.* [10] have demonstrated that extracellularly applied IBMX in fact raises the intracellular cGMP level.

Increasing the Ca^{2+} level, on the other hand, appears to affect the rod cell physiology in an opposite direction as compared to the effects of cGMP: Increased intracellular and/or extracellular Ca^{2+} leads to hyperpolarization of the cell and to a decrease of the response amplitude (Brown and Pinto [11]; Lipton *et al.* [12]). This raises the question whether Ca^{2+} and cGMP may be "antagonists" regulating the permeability of the plasma membrane through similar pathways but in opposite direction.

In the present report, the effect of IBMX and related drugs on the photoreceptor mass response

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Abbreviations: IBMX, isobutylmethylxanthine; TAG, triacetylguanosine; DAMP, dimethylaminopurine; cGMP, cyclic guanosine 3′,5′-monophosphate; PDE, phosphodiesterase.

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were studied both in dark- and light-adapted retinae, and simultaneously the Ca^{2+} level in the same Ringer solution was varied. It is shown that some of the changes (response amplitude) caused by phosphodiesterase inhibition can be compensated by raising the extracellular Ca^{2+} level as well as by light-adapting the retinae, whereas other changes cannot be reversed (latency, time to peak).

Methods

Preparation

Adult frogs (*R. esculenta*) were dark-adapted for 12 h. Frogs were decapitated and the eyes enucleated under dim red light. Retinae were isolated without pigment epithelium by gentle shaking the posterior half of the eyecups in Ringer's solution. The isolated retina was placed in a perfusion chamber (Sickel [14]) suited for ERG measurements.

Solutions

For recording the mass receptor potential an aspartate Ringer's solution was used to isolate the photoreceptor response from other retinal neuron activity (Furukawa and Hanawa [15]). The aspartate Ringer's solution (75 mmol NaCl/l, 20 mmol Na-aspartate/l, 0.1 mmol $MgCl_2$/l, 0.5 mmol $CaCl_2$/l, 2 mmol KCl/l, 2 mmol KH_2PO_4/l, 24 mmol $NaHCO_3$/l 5–10 mmol Glucose/l) was adjusted to pH 7.6–7.8 with CO_2-gas. The retinae were superfused with aspartate Ringer's solution at a flow rate of 2 to 4 ml/min, and the solution was gased during the experiment with 95% O_2/5% CO_2 to maintain the pH constant. All pharmacological reagents were dissolved in this solution. The experiments were done at 21 ± 0.5 °C.

Stimulation

A dual beam photostimulator was used, each beam originating from a 150 W halogen lamp. Electromagnetic shutters interrupted, and neutral density filters or neutral wedges attenuated each beam. Interference filters with peak transmittance at 503 nm were placed in the path of both beams. One beam provided diffuse, 1 s test flashes, the other diffuse background illumination. The absolute light intensity of each beam was calibrated with a photodiode (PIN 020 A, United Detector Technology). At the light intensities and wavelenghts used, the responses recorded originated exclusively from rod cells (Hood *et al.* [16]).

Recording

A-waves were recorded between two Ag/AgCl wires on opposite sides of the retina. The electrodes were connected to an AC-coupled amplifier with a pass band of 0.03 to 200 Hz. Each electroretinogram was displayed on a storage oszilloscope along with an electrical analog of the light stimulus and was photographed with a Polaroid camera.

Results

Drug effects on dark-adapted retinae – Three purine derivatives were tested for their effects on the light-evoked a-wave of the electroretinogram: isobutylmethylxanthine (= IBMX) which is known to be a potent phosphodiesterase inhibitor (Cohen *et al.* [10]), triacetylguanosine (= TAG), and dimethylaminopurine (= DAMP). All three of them were found to increase the response amplitude of the dark-adapted retinae elicited by a test flash of constant light intensity, and to change the waveform of the response.

IBMX was tested at three concentrations (10, 100 and 1000 µmol/l) and increased the response amplitude during the first 30 min. The changes were maximal at about 3 to 5 min after drug addition with amplitudes up to 6 times higher than the control amplitudes. Thereafter the response amplitude decreased and 1–2 h after addition of IBMX the retinae got desensitized below the original level when 100 or 1000 µmol IBMX/l were used. 10 µmol IBMX/l, on the other hand, caused a stable, 2-fold increase of the amplitude for at least 2 h (but see Lipton [4]). Furthermore only when 10 µmol IBMX/l was used, the drug effect on the response amplitude was reversible by replacing the IBMX solution with control Ringer's within 1–2 h after drug addition. Therefore 10 µmol IBMX/l was used in all further experiments.

TAG and DAMP were tested always at a concentration of 1 mmol/l. This concentration caused a 2–3-fold increase in response amplitude and altered the waveform of the response too. The effect of both drugs remained stable for at least 2 h and were reversible by washing the retinae with control Ringer's solution within 1–2 h after drug addition.

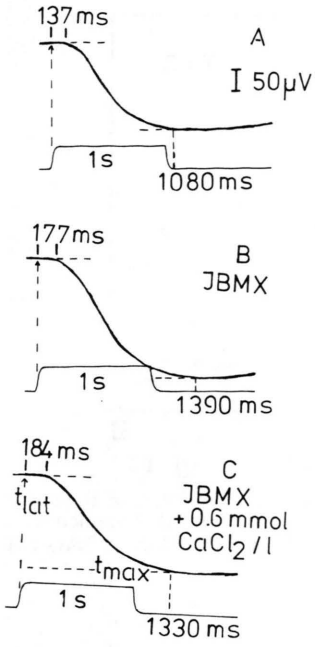

Fig. 1. Three a-waves from a dark-adapted isolated frog retina. The recording in the upper figure was obtained in control Ringer's (A) and that in the middle figure was obtained 20 min after addition of 10 µmol/l IBMX (B). The recording in the lower figure was obtained 30 min after increasing the calcium concentration in the IBMX test solution from 0.5 to 1.1 mmol/l $CaCl_2$ (C; see below). In each figure the lower trace represents the electrical analog of the 1 s test flash (light intensity of all three test stimuli: 10^{-2} erg cm^{-2} s^{-1} = 1.4×10^3 photons rod^{-1} s^{-1}). The vertical bar in Fig. 1 A indicates 50 µV calibration. The dashed lines in all three figures indicate how latency (t_{lat}) and time to peak (t_{max}) were measured.

Fig. 1 shows three original recordings from one retina superfused with control Ringer's (A) and after 20 min superfusion with 10 µmol IBMX/l (B). In the presence of IBMX a third recording was taken after the calcium concentration in the test solution was increased from 0.5 to 1.1 mmol $CaCl_2$/l (see below). All three a-waves were elicited by a 1 s test flash of identical light intensity (10^{-2} erg cm^{-2} s^{-1}). In this experiment the superfusion with the IBMX test solution caused an increase of the response amplitude from 199 µV (A) to 270 µV (B), 203 µV (C).

Fig. 2. shows two voltage-intensity curves recorded from different retinae before and after addition of 10 µmol IBMX/l (A) or 1 mmol DAMP/l (B), respectively. In both experiments the response amplitude is significantly increased at all flash intensities used as a result of drug addition. Similar results were obtained in 20 experiments with IBMX, 13 with TAG and 4 experiments with DAMP.

In the later part of the experiments described above the recordings along with an electrical analog of the stimulus were photographed (see Fig. 1 A–B) so that the time course of each a-wave could be analyzed. When dark-adapted retinae were exposed to 10 µmol IBMX/l, 1 mmol TAG/l or 1 mmol DAMP/l, respectively, then the light-evoked response showed in addition to the increase in the response amplitude also a significant prolongation in latency and time to peak at all flash intensities used. Fig. 3 shows the decrease of latency and time to peak with increasing light intensity of the test flash.

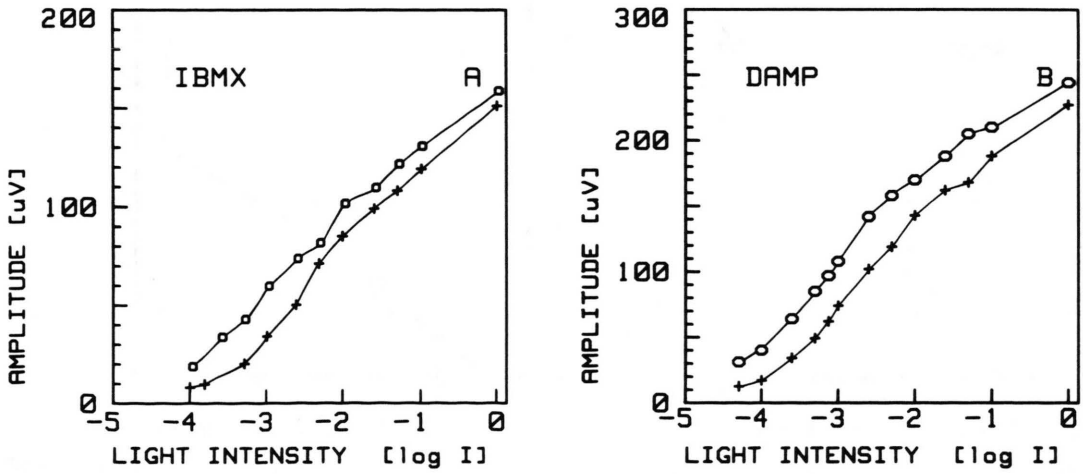

Fig. 2. Voltage-intensity curves from two dark-adapted retinae before (+) and after addition of 10 µmol/l IBMX (A, ○) or 1 mmol/l DAMP (B, ○), respectively. Test flashes were delievered with increasing light intensity and with a period of 3 min. At all flash intensities the response amplitude is increased by both drugs compared with the responses obtained in control Ringer's.

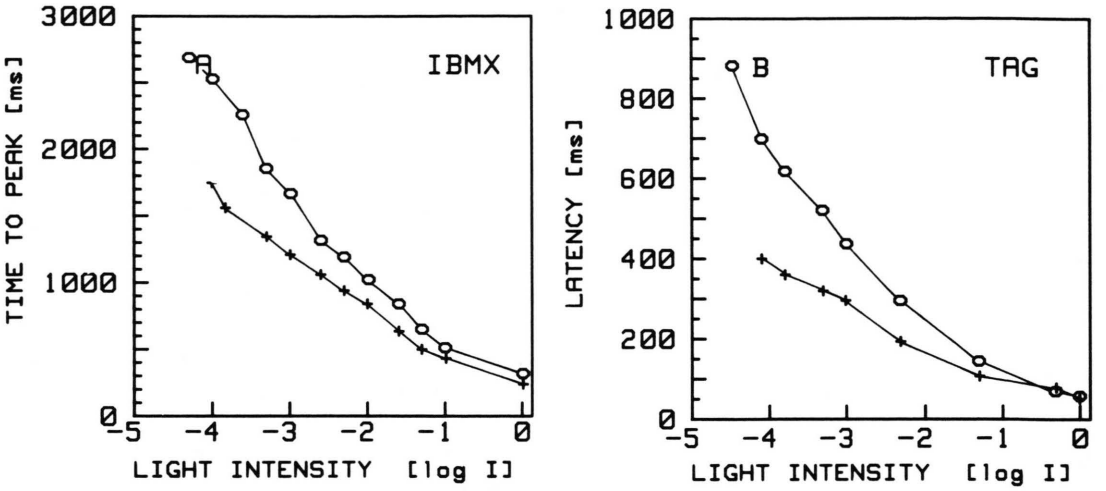

Fig. 3. Decrease of time to peak (t_{max}) and latency (t_{lat}) with increasing test flash intensity. After addition of 10 µmol/l IBMX or 1 mmol/l TAG to dark-adapted retinae both, t_{max} and t_{lat} are prolonged in comparison to the data obtained with control Ringer's. (+): responses obtained with control Ringer's. Open circles: responses obtained with IBMX or TAG test solutions.

In the presence of IBMX and TAG both latency and time to peak are prolonged.

Drug effects on light-adapted retinae – Light-adaptation of a retina with constant background light reduces the sensitivity of the retina to light. That means, the amplitude of a response is reduced compared with the amplitude elicited by a test flash of the same light intensity but from the dark-adapted retina. Because light-adaptation of a retina acts antagonistic to IBMX, TAG and DAMP which increase the amplitude of the dark-adapted retina (see above) all three drugs were tested if they could compensate the reduction of the amplitude caused by light-adaptation.

14 retinae were light-adapted with constant background light in the range of log −4 to log −2. With

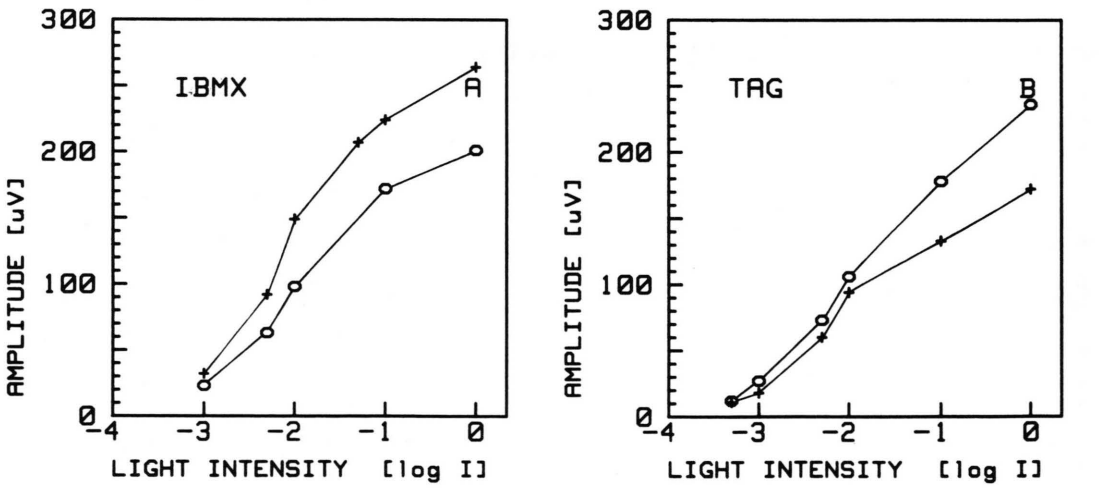

Fig. 4. Voltage-intensity curves from two light-adapted retinae, one treated with 10 µmol/l IBMX (A) and the other with 1 mmol/l TAG (B). Test flashes were delivered with increasing light intensity and a period of 3 min. In both experiments the light intensity of the background was 5×10^{-4} erg cm^{-2} s^{-1} (log − 3.3). At all flash intensities the amplitude is decreased by IBMX (○) and increased by TAG (○) compared with the responses (+) obtained from the light-adapted retinae superfused with control Ringer's.

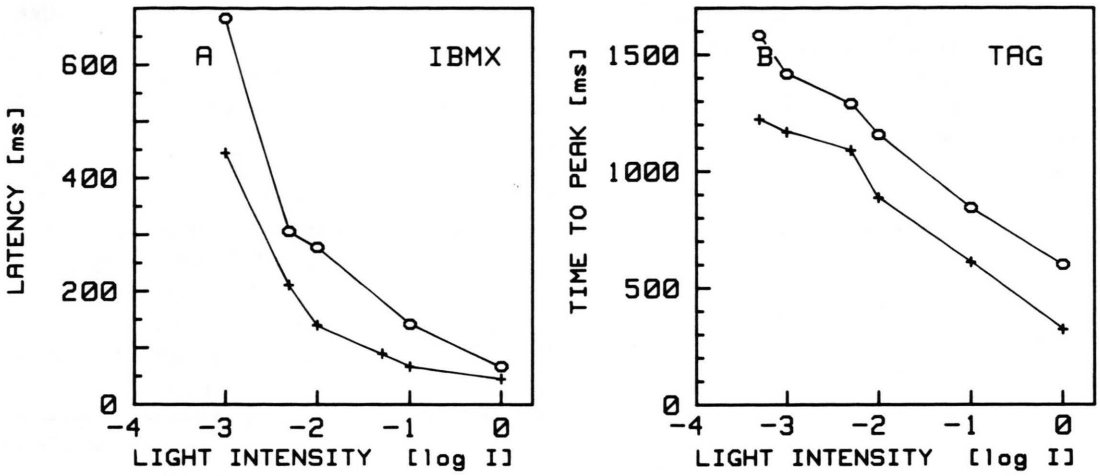

Fig. 5. Decrease of latency and time to peak of two light-adapted retinae, one treated with 10 µmol/l IBMX (A) the other with 1 mmol/l TAG (B). Latency and time to peak remained still prolonged at all flash intensities after the addition of both drugs. (+): responses obtained from light-adapted retinae with control Ringer's. Open circles: responses obtained from the same light-adapted retinae after the addition of IBMX respectively TAG.

the light intensities used no detectable bleaching of rhodopsin occured within 60 min.

Independent of the adapting light intensity 10 µmol IBMX/l either did not influence the amplitude of the light adapted retina at all or even caused a decrease of the amplitude (4 of 6 experiments). 1 mmol DAMP/l caused a decrease of the amplitude in all experiments. But 1 mmol TAG/l caused an *increase* (4 of 6 expr.) of the amplitude. Fig. 4 shows the voltage-intensity curves of two light-adapted retinae before and during treatment with IBMX (A) or TAG (B).

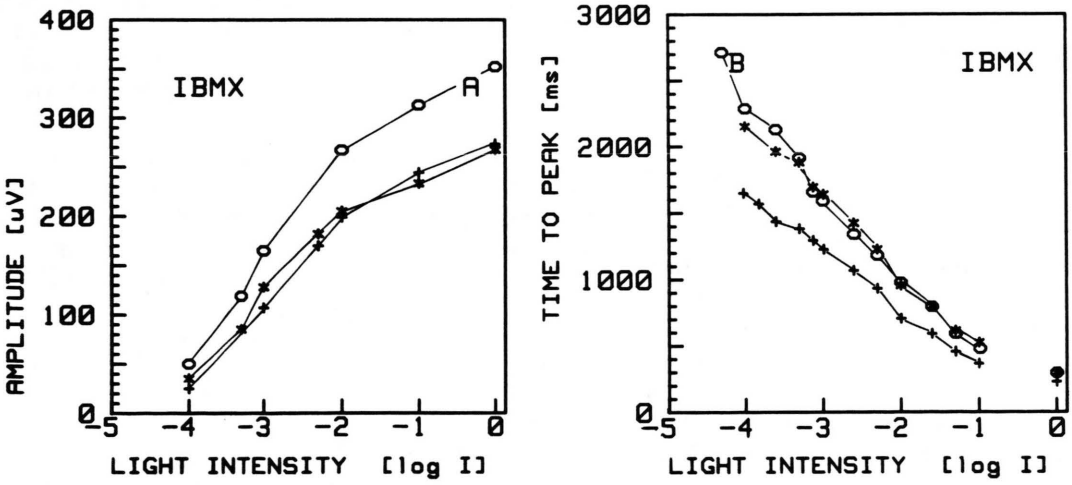

Fig. 6. Effect of increased extracellular calcium concentration on the IBMX-increased amplitude (A) respectively prolonged time to peak (B). In both experiments the extracellular calcium concentration was raised from to 0.5 mmol/l (control and IBMX-Ringer's; +, ○) to 1.08 mmol/l (A); respectively to 1.38 mmol/l (B); after the IBMX (10 µmol/l) influenced responses had been monitored. While the amplitude previously increased by IBMX is reduced by the increased calcium concentration the prolonged time to peak remained still prolonged after the increase of the Ca^{2+} level. (+): responses obtained in control Ringer's (0.5 mmol/l $CaCl_2$; (○): responses obtained with IBMX-Ringer's (0.5 mmol/l $CaCl_2$; (*): responses obtained with IBMX-Ringer's after the calcium concentration was increased to 1.08 respectively 1.38 mmol/l (B) $CaCl_2$.

However, latency and time to peak remained prolonged in all experiments by these drugs. Fig. 5 shows the decrease of both latency and time to peak with increasing test flash intensity before and during superfusion of two light-adapted retinae with IBMX (A) or TAG (B) test solution.

Effect of increased extracellular calcium concentration

Increased extracellular calcium concentration reduces the height of the response amplitude and causes a shift of the voltage-intensity curve (Waloga *et al.* [9]; but see Lipton *et al.* [4]).

In 5 experiments, the calcium concentration in the aspartate Ringer's solution was increased (from 0.5 mmol $CaCl_2$/l to a level between 1.1 and 2.7 mmol $CaCl_2$/l) in the presence of IBMX or TAG after the effect of both drugs on the dark-adapted retina had been monitored. In all experiments, increased calcium reduced the response amplitude previously increased by IBMX or TAG down or even below the control level. For example, an increase of extracellular Ca^{2+} from 0.5 to 1.4 mmol $CaCl_2$/l was much more than sufficient to reverse the effect of 10 µmol IBMX/l on the amplitude (Fig. 6A). But in all experiments the increased extracellular calcium concentration had only minor effects on the prolonged latency and time to peak (Fig. 6B).

Thus, increased calcium concentration used acts "antagonistic" to IBMX or TAG only as far as the response amplitude is concerned.

Discussion

Several effects of IBMX reported on the light-evoked response (Lipton *et al.* [4]; Waloga *et al.* [9]; Nicol and Miller [8]) of dark-adapted retinae have been confirmed for IBMX and two additional drugs, TAG and DAMP, in the present report: An increase in the response amplitude, and a prolonged latency and time to peak. In the experimental system used here, the retinae were more stable than in other systems reported (Lipton *et al.* [4]). The drug – influenced respones remained stable for several hours when 10 µmol IBMX/l was used, and the effect on the amplitude caused by IBMX were reversible by removal of IBMX from the bathing solution within 1–2 h after drug addition.

Most likely, IBMX acts by increasing the intracellular cGMP level through inhibition of the PDE (Cohen *et al.* [10]), but this is not necessarily the only action of IBMX. In this report two additional drugs (TAG, DAMP), both being slightly hydrophobic purine derivatives like IBMX, were tested. Both of them induced effects like IBMX but a higher concentration was required. Furthermore it remains to be determined if both are also PDE inhibitors.

The increase in the response amplitude caused by IBMX or TAG could be reversed by simultaneous increase in the extracellular Ca^{2+} level or suppressed in most of the experiments by light-adapting the retinae. This suggests that both increased Ca^{2+} level and light-adaptation act in the same way and "antagonistic" to the increased cGMP level (presumably raised by the action of the drugs). This is consistant with the finding that light sensitivity of the retina – measured by amplitude criterion – is decreased with increasing Ca^{2+} levels (Waloga *et al.* [9]). On the other hand, the effects of the drugs on the time course of the response – the prolongation of latency and time to peak – are not influenced by increased Ca^{2+} levels nor by light adaptation. This is consistant with the hypothesis of Nicol and Miller [8] who had injected cGMP iontophoretically into rod cells. Based on their experiments they concluded that the effect of increased cGMP on the time course of the response may reflect a direct link of the light-induced enzymatic breakdown of cGMP in the rise of excitation.

The results presented indicate that calcium may control rod cell sensitivity to light (height of the response amplitude) and probably that cGMP is a direct link in the rise of excitation. However, further work needs to be done especially on a possible calcium-cyclic nucleotide interaction such as was observed in other cells (Rasmussen *et al.* [17]).

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