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RHODOPSIN PHOSPHORYLATION INHIBITED BY ADENOSINE IN FROG RODS: LACK OF EFFECTS ON EXCITATION

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Abstract—1. The rod photocurrent was studied by recording the transretinal voltage from the aspartate-treated isolated frog retina before and after perfusion with 2 mM adenosine, which inhibited 60–80% of the light-induced rhodopsin phosphorylation.

2. Adenosine did not affect the time courses of the flash photoresponses or the OFF responses after a steady light.

3. The introduction of adenosine while the retina was illuminated by a steady background did not enhance the effect of light. Instead, the opposite change, due to PDE inhibition, was observed.

4. The results indicate that rhodopsin phosphorylation does not determine the time course of the decay of excitation.

INTRODUCTION

A rod can respond to changing illumination rapidly enough only if the excitation brought about by a photon absorption is comparatively short-lived. This requires one or several inactivating mechanisms connected to the excitatory process. According to prevalent notions, excitation consists in photoisomerized rhodopsin (Rh*) activating a *disc process*, which through a change in the concentration of *internal transmitter* in the cytoplasm results in the closing of sodium channels in the plasma membrane. There is strong biochemical evidence that the disc process involves the activation of phosphodiesterase (PDE) by Rh*, leading to the hydrolysis of cyclic GMP in the cytoplasm (for reviews, see Pober and Bitensky, 1979; Hubbell and Bownds, 1979). On the other hand, it is known that isomerized rhodopsin is phosphorylated by a rhodopsin kinase (Kühn and Dreyer, 1972; Bownds *et al.*, 1972; Frank *et al.*, 1973) and that this (ATP-consuming) phosphorylation is probably the reaction quenching PDE-activation (Sitaramayya *et al.*, 1977; Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983).

Provided that PDE-activation is an actual link in the excitatory chain, these results naturally give rise to the idea, advanced by Sitaramayya and Liebman (1983), that rhodopsin phosphorylation is the key process effecting the inactivation of the photoresponses (see the Scheme in Fig. 1). This is the particular hypothesis we wanted to put to physiological test in the present work. As such it is fully compatible with either cGMP or Ca^{2+} being the final internal transmitter. (In fact, with slight modifications our simple scheme could just as well relate to some quite different type of excitatory process that would be terminated by rhodopsin phosphorylation, e.g. the opening of a rhodopsin pore.) To test the hypothesis we recorded the changes in mass receptor photoresponses brought about by introducing the rhodopsin kinase inhibitor adenosine into the perfusing fluid. Our conclusion is that rhodopsin phosphorylation cannot be the reaction chiefly responsible for turning off photoresponses.

METHODS

Recording, perfusion and stimulation

Dark-adapted retinas from common frogs (*Rana temporaria* L.) were dissected in cooled Ringer under dim red light and placed receptors upwards in a specimen holder similar to that of Bastian and Fain (1979). The upper (receptor) side was perfused by air-saturated Ringer at 12°C (composition: 95 mM NaCl, 3 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 12 mM Na phosphate buffer (pH 7.5), 10 mM glucose and 2 mM Na-aspartate). The mass receptor potential was recorded with two Ag/AgCl electrodes, one connected to the Ringer space beneath the retina, the other in chloride solution connected to the perfusing fluid through a porous plug. The DC-amplified signal was continuously recorded on a slow pen recorder, while the photoresponses were additionally recorded on a fast recorder using higher speed and greater sensitivity, and monitored on a storage oscilloscope. We shall call the voltage recorded positive when the proximal side of the retina turns more positive compared with the receptor side. Under this convention (as in the ERG), light elicits negative receptor responses.

The gravitation-controlled perfusion system involved two tubes joining at the inlet to the specimen chamber; the flow could be switched from one to the other with valves outside the light-tight box. After a switch there was a delay of about 30 sec before the new solution bathed the retina. This delay had been measured by letting in solutions with varying potassium concentrations, e.g. a decrease in $[K^+]$ at the retina immediately produces a negative voltage and vice versa.

The optical system had two channels, one being used for stimulation (493 nm, 0.1 sec flashes), the other for background light (613 nm). The intensities were independently controlled by neutral density filters and wedges.

Checking currents of non-ROS origin

The voltage measured across the retina is the combined effect of all radial current densities. Even after the blocking of the receptor synapses with asparate or glutamate, this voltage may contain considerable components which, in a

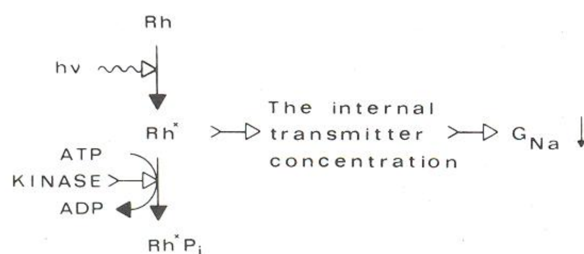

Fig. 1. A scheme for the effect of rhodopsin phosphorylation. Filled arrows signify reactions, open arrows influences.

study of rod outer segment (ROS) mechanism, are highly undesirable. Light-induced changes in the extracellular potassium concentration affect radial glial currents (see Tomita and Yanagida, 1981), and the current loop from the rod inner segment to the synaptic end of the rod reacts to changes in membrane potential or ion concentrations (see e.g. Zuckerman, 1973; Arden, 1977; Fain and Quandt, 1980). Calcium channel blockers and Ba^{2+} have been found effective in reducing these currents (Low, 1978; Bolnick *et al.*, 1979). Therefore we used Ba^{2+} (0.4 mM $BaCl_2$ in Ringer) and the D600 analogue isoptin (Knoll, also known as verapamil; 1 mg/10 ml Ringer; cf. Åkerman, 1981; Åkerman and Nicholls, 1981) to control non-ROS currents. When using Ba^{2+} we substituted for the phosphate buffer by 12 mM Tris (Trizma, Sigma). With isoptin, we used 4 mM glutamate instead of the asparate, as we got the impression that the deterioration of the retina observed in isoptin was more marked in the presence of asparate. In drug-free Ringer there seemed to be no difference between asparate and glutamate.

A slow change in the light-sensitive ROS current can be distinguished from other currents, except by the pharmacological means mentioned above, also by the use of saturating flashes of light. The reason is that the light-sensitive current can always be brought to zero by a sufficiently strong flash, which means that the amplitude of the maximal response gives a measure of the strength of the current. Then, if, for example, that amplitude remains constant all through a slow voltage transient, this implies that the transient is not caused by any change in the light-sensitive ROS current.

Inhibiting the rhodopsin kinase

Our means of achieving kinase inhibition was to add adenosine to the perfusing medium (generally 2 mM). The reason for this choice is as follows. Studies by Miller *et al.* (1975) and Frank and Buzney (1977) show β,γ-methylene-ATP and guanosine, in addition to adenosine, to be effective kinase inhibitors; all of them, however, simultaneously inhibit PDE to a higher or lower degree. With guanosine, the findings of Hemilä (1981, 1983), who used it in electrophysiological studies, suggest PDE inhibition to be the dominant effect. This conclusion is strengthened by the similarity to the action of isobutylmethyl-xanthine (IBMX), which is known as a powerful PDE-inhibitor. β,γ-Methylene-ATP, on the other hand, had practically no effect when added to the perfusion fluid, and indeed it is likely that it has difficulty entering the rods because of its hydrophilic characteristics. For adenosine, however, active transport into the cells of the outer nuclear layer has been demonstrated (Schaeffer and Anderson, 1981). Furthermore, adenosine should have a much more advantagenous kinase inhibition/PDE inhibition ratio than guanosine (Frank and Buzney, 1977).

By the following precautions we tried to filter out possible effects not due to the inhibition of rhodopsin kinase:

(1) We chose experimental conditions such that, for the parameters measured, kinase and PDE inhibition should as far as possible have different effects (see the Results section).

(2) We carried out parallel experiments using IBMX (0.1 mM) and compared the results with those obtained in adenosine in order to spot similarities. Although the use of IBMX as a PDE-inhibitor is highly problematic (see e.g. Wells and Kramer, 1981), it would suffice for our present purpose, as our comparative experiments were needed only to reveal effects due to causes other than kinase inhibition, not to identify them unequivocally with PDE-inhibition.

(3) After initial experiments employing adenosine concentrations in the range 0.1–10 mM we settled for the concentration 2 mM as about the lowest one still producing slight "PDE-inhibition-like" effects and therefore thought to be just sufficient for powerful kinase inhibition.

The degree of inhibition brought about by adenosine

Still, we had to ascertain that a sufficient degree of inhibition was obtained in the exact conditions of our experiments. Therefore, we performed two experiments where we measured the incorporation of ^{32}P-labelled phosphate into the rhodopsin of intact retinas, which had been treated exactly as in the physiological experiments. They were pre-loaded with radioactive phosphate, perfused with normal Ringer either containing or lacking 2 mM adenosine, and either illuminated or not. Rhodopsin-bound radioactivities were then measured from digitonin-extracts, from which relative amounts of rhodopsin could also be obtained spectrophotometrically.

Six frogs were dark-adapted overnight. In the morning, the twelve retinas were isolated as usual in deep red light in phosphate-free Tris Ringer. Each retina was mounted receptors downwards on a nylon net in a small specimen holder. These were then transferred to the first incubation chamber, where the receptor side bathed in slowly circulating Tris Ringer (12°C, pH 7.5) containing inorganic ^{32}P without inactive carrier (0.3 mCi/ml in the first experiment, 0.025 mCi/ml in the second).

The frogs were killed two at a time and the left retinas dissected first to form sample one, the right retinas forming sample two. Assuming that the two eyes of one frog are essentially similar, this yielded three pairs of fully comparable samples, each sample consisting of two retinas.

The retinas were loaded with radioactive phosphate for *ca* 4 hr in the completely dark incubation chamber. In that time, even in the isolated retina, the small amount of rhodopsin bleached during preparation is regenerated (Donner and Hemilä, 1975) and phosphorylated rhodopsin molecules dephosphorylated (Kühn and Bader, 1976). Still in darkness, the retinas were transferred in strict order at 17 min intervals to another perfusion chamber, where normal phosphate Ringer either with or without 2 mM adenosine was circulating. After five minutes in this "phosphorylation chamber" eight of the twelve retinas were given a 10 sec light exposure bleaching 30% of the rhodopsin, then left to phosphorylate for 12 min. After 12 min phosphorylation is nearly saturated, but dephosphorylation is still insignificant (see Kühn and Bader, 1976). Every retina spent a total of 17 min in the phosphorylation chamber and was then moved to a cup containing 0.7 ml Ringer +30 mM hydroxylamine. There the rod outer segments were removed by shaking and and scratching in deep red light. Each ROS suspension was immediately frozen in liquid air, the suspensions from two retinas being joined. The result was six samples, pairwise stemming from the same frogs (both left eyes or both right eyes).

As the suspensions were thawed and 1.5 ml distilled water added to each, the outer segments were disrupted, which stops both phosphorylation and dephosphorylation (Kühn and Bader, 1976). This was done in bright roomlight, bleaching all rhodopsin, which then reacted with the hydroxylamine to form stable oxime molecules. We may safely

Table 1. Relative levels of rhodopsin phosphorylation under different conditions

			Experiment I			Experiment II		
No.	Ringer	Exposure	A	N	N/A(%)	A	N	N/A(%)
1	Standard	Dark	0.185	359	100	0.32	97.8	100
2	Standard	Bleach	0.198	789	205	0.31	192	200
3	Standard	Bleach	0.172	795	239	0.36	199	177
4	+ Adenosine	Bleach	0.131	402	158	0.33	111.5	109
5	+ Adenosine	Dark	0.155	254	85	—	78.5	—
6	+ Adenosine	Bleach	0.161	461	148	0.29	113	126

Adenosine concentration 2 mM, bleaches *ca* 30% A, oxime absorbances at 360 nm; N, measured beta activities in 10^3 counts per min; N/A, *relative phosphorylation per amount of oxime*, in per cents of the standard dark value. For sample 5 of the second experiment no N/A value could be calculated, because the absorbance measurement suffered from some strong background absorption.

assume that oxime formation does not affect earlier rhodopsin phosphorylation, because these reactions occur in different parts of the opsin molecule and both lead to covalent bonds,

Each suspension was centrifuged for 30 min at 25,000 rpm, the supernatant pipetted off and the outer segments suspended in 0.3 ml 2% digitonin solution. After 3 hr of pigment extraction the samples were centrifuged again so that a clear oxime extract was obtained. The transmission spectrum (wavelength range 600–250 nm; Beckman DB spectrophotometer) and radioactivity (in 10 ml Packard Insta-gel II scintillation cocktail; Wallac 1211 Minibeta counter) were measured from each of the six extracts.

In hydroxylamine solution, all rhodopsin photoproducts which have not yet reached the retinol stage end up as oxime. As only a negligible fraction of the original pigment could have escaped to retinol before immersion into hydroxylamine, the absorbances at the oxime peak (360 nm) could be used as measures of the relative total amounts of rhodopsin, both the 30% bleached in part of the intact retinas and the rest bleached in the ROS suspension after thawing. The protein peak at 275 nm does not significantly affect the absorbance at 360 nm (see Collins *et al.*, 1952), but we used it to estimate impurities, i.e. protein content other than rhodopsin.

In this biochemical study the amount of rhodopsin bleached and thus the substrate concentration of the kinase was several orders of magnitude larger than in the physiological experiments where photoresponses to light flashes were recorded. We accepted this, because there are no grounds for believing that the per cent inhibition would be any less with smaller bleaches. In fact, competitive enzyme inhibition leads to an increase in the apparent Michaelis constant K_M implying an increase in the percent inhibition when the substrate concentration is decreased at fixed inhibitor concentration.

RESULTS

We have divided the results into three sections, of which the first one describes the outcome of the experiments done to measure the degree of inhibition brought about by 2 mM adenosine. The second section is dedicated to observations on the effects of Na^+ and isoptin, intended to suppress currents not associated with the rod outer segment. The final section then brings the central results on the physiological effects of adenosine.

The degree of inhibition from 2 mM *adenosine*

Table 1 gives the results of the two phosphorylation experiments with phosphorylation N/A given as percentages of the background (dark) phosphorylation. The difference between samples 3 and 4 (from the same frogs) gives the inhibition due to 2 mM adenosine; if it is divided by the uninhibited light-induced phosphorylation (i.e. sample 3 minus background), the fraction of light-induced phosphorylation inhibited by adenosine is obtained. In the first experiment this gives

$$(239\text{–}158)/(239\text{–}100) = 0.58 \text{ or } 58\%$$

and in the second experiment:

$$(177\text{–}109)/(177\text{–}100) = 0.88 \text{ or } 88\%.$$

If all four bleached samples are used for calculating inhibition (2 and 3 vs 4 and 6), the result is 56% in the first and 80% in the second experiment. Note, however, that adenosine also inhibits "dark" phosphorylation somewhat (compare samples 1 and 5).

The protein absorption peak at 275 nm was used to test the purity of our rhodopsin solutions. Combining data of Collins *et al.* (1952) and Reuter *et al.* (1971), we find that in a digitonin solution of pure rhodopsin oxime the ratio of absorbances at 275 and 360 nm is $A_{275}/A_{360} \approx 1.6$. Thus we may calculate the amount of impurities expressed as a ratio ρ to the amount of rhodopsin:

$$\rho = \frac{A(\text{impurities})_{275}}{A(\text{rhodopsin})_{275}} = \frac{A_{275}/A_{360} - 1.6}{1.6}.$$

As our purification procedures were rather crude, these ratios were rather high. For example, in the first experiment they were, in order, 1.5, 1.4, 1.4, 1.9, 1.9 and 3.1 for the six samples. The important point is that the impurities did not much affect the measured phosphorylation: e.g. in sample 6 phosphorylation is slightly less than in sample 4, although ρ is 63% higher.

Photoresponses in standard Ringer and the effects of barium and isoptin

First compare in Fig. 2 the sets of DC-recorded flash responses seen in standard Ringer with those obtained after the addition of Ba^{2+} (first and second row). Ba^{2+} always caused an uncomfortably great reduction of all response amplitudes (to a fifth of those in standard Ringer on an average). Let us, however, instead consider the kinetics of the responses as characterized by their times to maximum

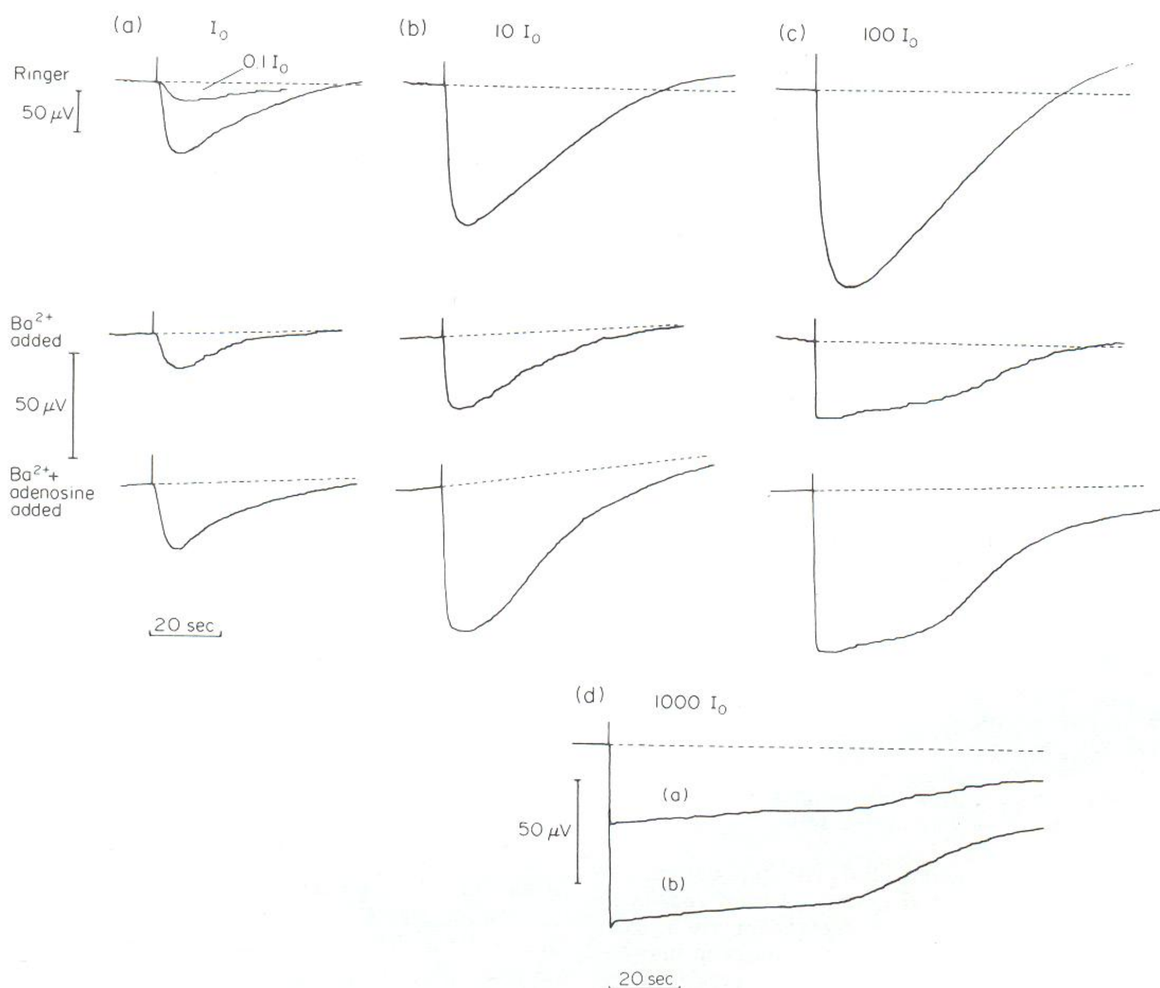

Fig. 2. Flash responses in Tris-Ringer in the dark-adapted state: the effects of barium and the subsequent addition of adenosine. Top row: responses in drug-free Tris-Tinger, just before the addition of barium. Second row: *ca* 1.5 hr after the addition of 0.4 mM Ba^{2+}, just before the addition of adenosine. Third row: *ca* 1 hr 20 min after the application of 2 mM adenosine in the presence of 0.4 mM Ba^{2+}. The columns A–C show responses to progressively increasing flash intensities I_0, 10 I_0, and 100 I_0, I_0 being approximately 10 Rh* per rod. In the top row of column A a response to the intensity 0.1 I_0 is also shown. D shows two responses to the high intensity 1000 I_0, a in Ba^{2+} and b in Ba^{2+} plus adenosine.

(TTM) and the times they take to decay to half-maximum (t_D). At this temperature (12°C) they are and remain slow with a TTM of some 7 sec and the only change is that t_D decreases by some 15%. It should be noted that, assuming a Q_{10} of *ca* 2 for the reciprocal of the TTM (cf. Baylor *et al.*, 1974), these asparate-isolated mass responses are not all that different from those recorded intracellularly from toad rods by Baylor *et al.* (1979); at room temperature their TTMs ranged from 0.6 to 2.5 sec.

The effects of Ba^{2+} on the shape of near-saturated responses, however, are pronounced (Fig. 2C). The high dome-shaped top of the response is cut off, leaving only a steep rise, sometimes terminating in a "nose" (Fig. 2D), followed by a long plateau. This is consistent with the notion that there is a prominent Ba^{2+}-sensitive glial component in the saturated responses. In contrast to Bolnick *et al.* (1979), however, we should like to emphasize that the length of the response (e.g. as measured by t_D) remains essentially unaffected, indicating that it is actually determined by the ROS photocurrent. The general effect of Ba^{2+} on the stimulus–response function is to make it steeper, so that it approaches the curve corresponding to the Michaelis equation (cf. Hemilä, 1977).

When we switched from standard Ringer to Ba^{2+}-containing Ringer, a large positive deflection always appeared (typically about four times the maximal photoresponse). It was not accompanied by any growth of the maximal flash response, and as it was very similar to the potential we observed when increasing the potassium concentration of the perfusing fluid, we think it is reasonable to interpret it as a change in glial currents (Müller cells act as potassium electrodes, see Tomita and Yanagida, 1981).

Isoptin also initially produced a positive, light-insensitive deflection, but it was much smaller (less than the maximal photoresponse). Likewise, the effects of isoptin on flash responses were modest compared with those of barium. Only the t_D values of both small and big responses were somewhat reduced.

It seems likely that the dramatic effects of barium on high-intensity responses are due to the suppression of glial currents. However, at least part of the general reduction of the photoresponses in barium may in fact be due to action on other currents, notably the loop of the sodium and potassium currents from the inner segment to the proximal part of the rod. Up to a certain intensity this current is approximately pro-

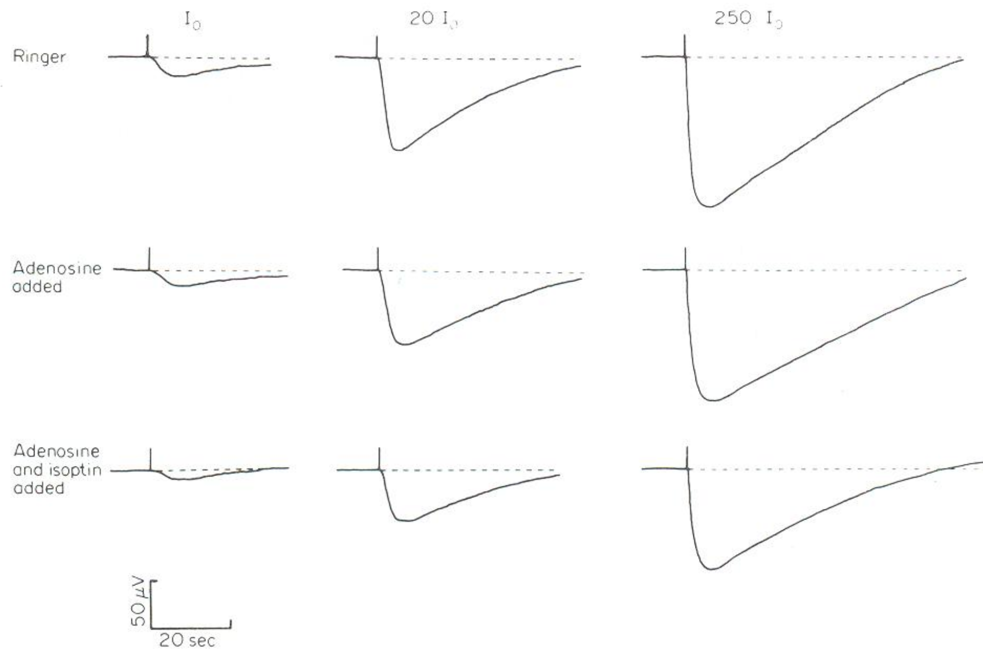

Fig. 3. Flash responses in phosphate Ringer in the dark-adapted state; The effects of adding first adenosine and subsequently isoptin. Top row: drug-free Ringer. Second row: 50 min later, 30 min after the addition of adenosine. Third row: 70 min after top row, 15 min after the addition of isoptin in the presence of adenosine. The columns again show responses to increasing flash intensities I_0 (2 Rh*/flash), 10 I_0 and 100 I_0.

portional to the outer segment current (Zuckerman, 1973) and the reduction of its contribution to the photoresponse would be seen only as an overall scaling-down of response amplitudes.

The effects of adenosine and IBMX

As already mentioned in the Methods section, PDE inhibition is an unavoidable side effect when adenosine is used for kinase inhibition. Therefore we selected three specific experimental situations where, according to the model we were testing (Fig. 1), kinase inhibition would be expected to have effects different from, or even opposite to, those of PDE inhibition. The effects we regarded as "expected" from PDE-inhibition were such as found by other authors using PDE inhibitors (e.g. Lipton *et al.*, 1977; Hemilä, 1981, 1983; Capovilla *et al.*, 1983). Most of them are in a simple way compatible with the idea that PDE inhibitors increase the levels of cGMP and thereby the dark current.

I. When the kinase is inhibited, the photoresponse elicited by a flash of light in the dark-adapted state should decay more slowly than normally, because Rh* maintains its excitatory activity longer. The rising phase, however, should be little affected. PDE inhibitors, on the other hand, should produce an initial delay and a slow rise, and in fact also a slower decay. (The slow decay is an experimental observation which cannot be simply explained on the basis of increased cGMP-levels.)

II. When the kinase is inhibited, the positive-going OFF-response after a period in steady light should be less steep than normal for the same reason as explained in case I. Its amplitude might also be expected to grow somewhat, as the longer life-time of Rh* serves to exaggerate background intensity, increasing the difference between the ROS current in the dark and in the presence of the background. However, this small amplitude increase would probably be hard to identify, as it will be masked by a much greater one due to PDE inhibition. The inhibition of PDE, resulting in an abnormally strong dark current, should be accompanied by a greatly magnified OFF-transient, during which abnormally big but slow photoresponses can be elicited.

III. If the retina is first exposed to the kinase inhibitor while illuminated by a steady background light, a negative-going potential (hyperpolarization) should be observed, accompanied by a corresponding reduction of the *maximal* responses to flashes of light. This is again because inhibition of phosphorylation should prolong the life-time of the excitatory form of Rh*, thus, in effect, enhancing the background (i.e. bringing the rod nearer to saturation). By contrast, PDE inhibition should lead to the opposite results: a positive-going transient (depolarization) combined with bigger responses to saturating flashes.

We shall now deal with these experiments one by one. As we wanted to make the comparison of kinase and PDE inhibition in the identical conditions of illumination, glial suppression, etc., we repeated all our adenosine experiments (on different retinae) using IBMX instead, which in spite of its weaknesses (see Wells and Kramer, 1981) has been the most widely used agent for producing "PDE inhibition".

The shape of dark-adapted flash responses

A comparison of the first and second row in Fig. 3 and of the second and third row in Fig. 2 brings out what adenosine does—or fails to do—to flash responses in the dark-adapted retina. As expected, the rate of rise was not affected. However, the change in

Table 2. Characteristic times of low-intensity photoresponses in the dark-adapted state

	t_1 (sec) range		TTM (sec) range		t_D (sec) range		N
Phosphate Ringer	3.0	3.6	6.0	8.9	17	22	4
+ adenosine	3.2	4.3	7.4	11.0	22	29	
Ratio of means	1.1		1.3		1.3		
Phosphate Ringer	2.8	3.2	8.0	8.9	15	23	2
+ IBMX	12	20	36	50	70	85	
Ratio of means	5.3		5.1		4.1		
Phosph. Ring. + Isoptin	1.8	2.4	5.4	5.5	11	14	2
+ adenosine	2.5	2.6	7.5	7.7	19	21	
Ratio of means	1.2		1.4		1.6		
Tris-Ringer + Ba^{2+}	2.7		6.5		18		1
+ adenosine	2.2		7.5		21		
Ratio	0.8		1.2		1.2		
Tris-Ringer + Ba^{2+}	3.0		5.0		13		1
+ IBMX	12		20		84		
Ratio	4		4		6.5		
Tris-Ringer	3.0	3.4	7.2	8.0	21	22	2
+ adenosine	2.9	3.6	7.2	8.3	23	31	
+ adenosine + Ba^{2+}	4.0	4.1	8.5	9.2	19	21	
Ratio (first two)	1.0		1.0		1.3		
Ratio (last two)	1.2		1.1		0.74		

t_1 = time to half-maximum, TTM = time to maximum, t_d = the time when the response has decayed to half-maximum.

decay time t_D, although qualitatively conforming to predictions (I), was small (about 30% for small responses and 40% for saturated responses). Barium (Fig. 2, rows two and three) and isoptin (Fig. 3, last two rows) had little effect on these results. They shortened responses with and without adenosine more or less equally.

Table 2 gives the average values of the decay characteristics in different solutions. As seen, by comparison, the IBMX-induced changes make the adenosine effects seem very modest indeed. In IBMX, response latency grew to 12–20 sec and the whole response was slowed down so that t_D could be up to 85 sec even for small responses. This refers to results obtained in the presence of Ba^{2+}, proving them not to be glial artifacts.

The response to turning off a steady background

Our second specific test concerned the return of the voltage towards the dark level after the offset of a steady background light. Again, the effect of 2 mM adenosine was very weak, as seen from Fig. 4A. In standard Ringer the positive OFF-response reached half-maximum in 13.5 sec on average (range 10–17 sec, three experiments), in adenosine Ringer the corresponding time was 15 sec (range 14–17 sec, three experiments). The amplitude of the response remained roughly the same as in standard Ringer (at the chosen background intensity about 60% of the maximal response in dark). In IBMX-Ringer, on the other hand, the OFF-response grew conspicuously, becoming much bigger than the maximal dark response (cf. Hemilä, 1983). But there was also increases in latency and time to half-maximum qualitatively resembling the small effects of adenosine (Fig. 4B).

Initial transients due to application of drug

Figure 5 shows an example of the initial voltage transients we recorded when first switching from standard Ringer to adenosine-containing Ringer in the presence of a steady background light. (Turning on a background light always elicits a fast negative ON-response not shown in Fig. 5, followed by a slow return towards the original baseline. With weak backgrounds such as used here, light-adaptation is near-complete within half a minute (Hemilä, 1977), and the slow return bears no relation to outer segment current.)

It first appeared that adenosine does bring about an initial negative deflection consistent with the scheme in Fig. 1. However, the maximal flash responses elicited before and on the summit of this transient turned out to be practically equal, showing that the light-sensitive current had not changed. Furthermore, the transient varied a lot in amplitude and was occasionally even negligible, especially so in the few experiments carried out in the presence of barium. We conclude that it was not associated with changes in the sodium conductance of the outer segment, but rather due to some side effect of adenosine on other currents, probably glial.

The negative transient was followed by a slower positive-going deflection (see Fig. 5). Although generally smaller in amplitude, it had a time-course quite similar to the initial transient invariably obtained when IBMX-containing Ringer first reached the retina. Both these transients were actually accompanied by an increase in the amplitude of saturated flash responses fully compensating for the positive deflection from the original baseline, and neither of them was abolished by Ba^{2+} or isoptin. Accordingly, we interpret them as reflecting real changes in the light-sensitive current of the ROS, probably due to PDE inhibition. Much bigger but otherwise similar transients obtained by introducing IBMX (or guanosine) in darkness have been reported earlier and given a similar interpretation (e.g. Hemilä, 1983).

DISCUSSION

All our results either clearly disagree with, or at least fail to support, the hypothesis that phosphorylation curtails excitation. On the one hand, we

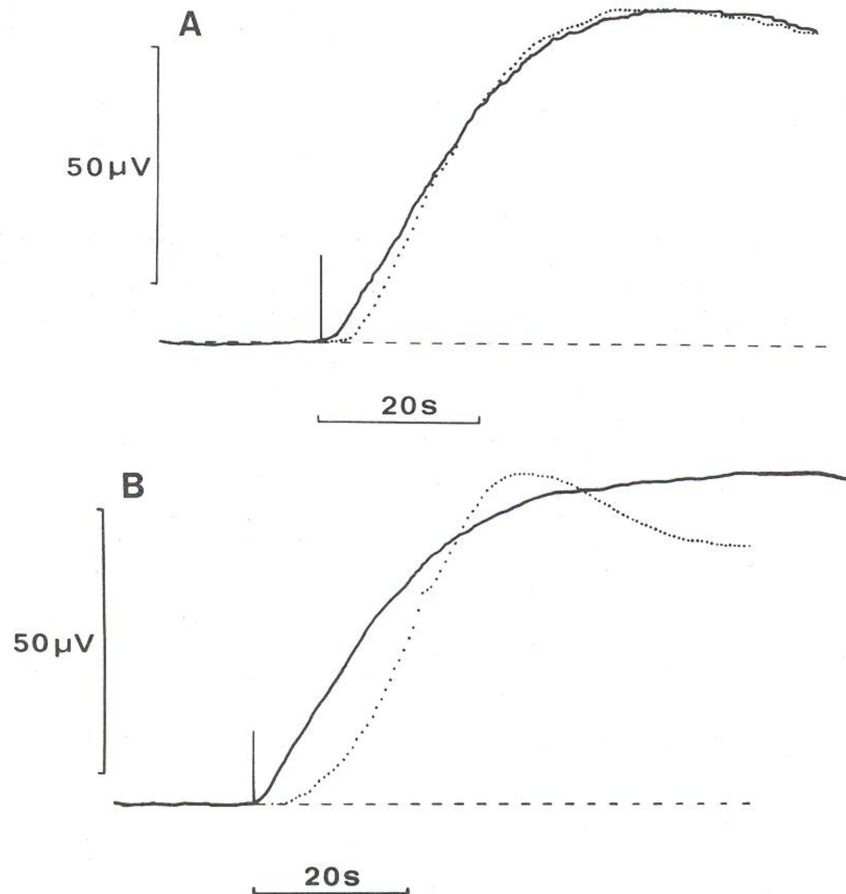

Fig. 4. OFF-responses (obtained after turning off a steady background light): the effect of adenosine (A) and IBMX (B) respectively. In both cases the continuous line shows the response in standard Ringer, before the application of the drug, and the dotted line shows the response in the presence of the drug, normalized to equal amplitude to facilitate the comparison of time-courses. Therefore, in (A) the adenosine voltages have been divided by a factor 1.28 and in (B) the IBMX voltages have been divided by 3.6.

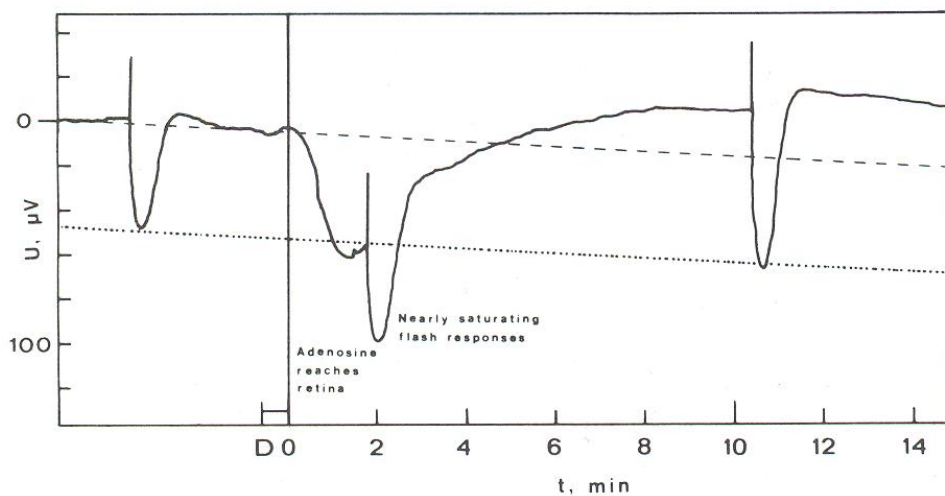

Fig. 5. Initial transients obtained when 2 mM adenosine was first applied to the retina in the presence of a background light. The background was turned on 8 min before the change to adenosine Ringer, which was done at time D. After a delay of about 35 sec the drug entered the retina at time $t = 0$. The dashed line is the interpolated long-term baseline; the dotted line has been drawn parallel to this through the peak of the first near-saturated flash response (far left). All the flash responses shown (at times -3, 2 and 10 min) were elicited by the same, near-saturating intensity; note that the last one peaks at the same level as the first one. Both the negative transient of non-ROS origin (peaking at about $t = 2$ min) and the slow positive "PDE-inhibition like" transient (peaking at about $t = 12$ min) are seen.

have directly shown that adenosine does produce significant inhibition of phosphorylation in intact retinae. The importance of this is hardly lessened by the fact that rhodopsin is known to be multiply phosphorylated, as there is no reason to think that adenosine selectively inhibits "unimportant" phosphorylation. On the other hand, the evidence that the phosphorylation of Rh* is the reaction that stops PDE activation seems convincing. Then, although the lowering of the cyclic GMP concentration may well be the first step in the excitatory chain (Miller and Nichol, 1979; Clack *et al.*, 1983), the conclusion is that the concentration of cGMP does not directly control the time-course of the photoresponse. This agrees with recent studies by Woodruff and Fain (1982) and Waloga (1983).

So, although the hypothesis that rhodopsin phosphorylation determines the decay of photoresponses may still be saved by various accessory assumptions, the present results do make such a role unlikely. Instead, it seems natural to envisage a role for rhodopsin phosphorylation in adaptation. One need only assume, for example, that Rh*, after triggering the excitation sequence, keeps some compartment (e.g. disc), where it is present, saturated. Then that compartment would regain its sensitivity to light only after the removal of the Rh*-action, for instance by phosphorylation. This type of local (non-spreading) light–dark-adaptation would become important only at comparatively high levels of steady illumination and would not be noticed in our experimental conditions, with weak backgrounds and practically non-bleaching flashes.

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