Biochimica et Biophysica Acta, 732 (1983) 276–281
Elsevier

BBA 71750

INVOLVEMENT OF ATP IN ACTIVATION AND INACTIVATION SEQUENCE OF PHOSPHODIESTERASE IN FROG ROD OUTER SEGMENTS

SATORU KAWAMURA *

Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, WI 53706, (U.S.A.)

(Received December 28th, 1982)
(Revised manuscript received April 26th, 1983)

Key words: cyclic GMP phosphodiesterase activation; ATP; Light intensity; (Frog rod outer segment)

Cyclic GMP phosphodiesterase in frog rod outer segments is activated after flash illumination and is inactivated when left in the dark. ATP reduces the initial peak activity caused by dim flashes (with 50 μM ATP being required for a half-maximal effect) and also accelerates inactivation (with 2 μM ATP being required for a half-maximal effect). An acceleration of inactivation caused by ATP addition is 3–7-fold, depending on the preparation, and ATP effect can be observed even 1 min after a dim flash is given. The accelerated inactivation is also flash intensity-dependent. A low intensity of light causes more rapid inactivation than does a high intensity of light. ATP appears to control phosphodiesterase activity in various ways.

Introduction

The first event in vision is photon capture by visual pigments. In rod outer segments, the pigments are located on the disk membrane which is morphologically separated from the plasma membrane where electrical response generates in the light. Therefore, an intracellular transmitter is postulated between the two sites. Recent work reveals that cyclic GMP and/or calcium are highly suitable as the candidates (see Ref. 1).

Light introduces hyperpolarization of photoreceptor cells by closing sodium channels [2,3]. Light is also known to decrease rapidly the cyclic GMP level in frog rod outer segments [4,5]. It has been shown that intracellular injection of cyclic GMP into toad rods causes depolarization [6]. Therefore, cyclic GMP in photoreceptor cells is postulated to open sodium channels, possibly through phosphorylation [7,8]. The enzyme responsible for the rapid decrease of cyclic GMP is phosphodiesterase, which hydrolyzes cyclic GMP to 5′-GMP and is activated in the light [9–12].

There are many factors reported which control phosphodiesterase activity in vitro. They are calcium [11,12], K_m shift [11], GTP [13–16] and ATP [12,17,18]. Among them, the calcium effect and the K_m shift can be detected only in minimally disrupted rod outer segments. This implies that some factors are easily eluted. Therefore, by using conventional methods for the isolation of rod outer segments, one may lose essential factor(s) for biochemical reactions underlying phototransduction in rod outer segments [11]. In the present work, the effect of ATP on phosphodiesterase activity was investigated using the minimally disrupted rod outer segment preparations.

Previous work has shown that ATP accelerates phosphodiesterase inactivation after a brief exposure to light [12,17]. The effect is pronounced at

* Present address: Department of Physiology, Keio University School of Medicine, Shinjuku, Tokyo 160, Japan.

low concentrations of calcium [12]. It has been proposed that the phosphodiesterase inactivation mechanism together with the calcium effect underlies light adaptation of phosphodiesterase under low intensity of light [12]. The present work concentrated on further analysis of the inactivation process at a low concentration of calcium. The procedure used for continuous monitoring of phosphodiesterase activity was the pH assay method used in the previous study [12]. It becomes evident that ATP has a physiological role in regulating phosphodiesterase activity; however, the mechanism is rather more complicated than has been proposed by other investigators [17].

Materials and Methods

In each experiment the retinas were prepared from one or two dark-adapted bullfrogs (*Rana catesbeiana*). Gently shaking with forceps for 1 min, the rod outer segments were isolated from the retinas in 1–2 ml of 10^{-9} M calcium-Ringer's solution (115 mM NaCl, 2.5 mM KCl, 10 mM *N*-2-hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid (Hepes), 2mM $MgCl_2$, 2.78 mM EGTA, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, pH 7.8). The suspension was diluted 5–8-fold to bring the rhodopsin concentration to 7–15 μM. No further purification was carried out because elutable factors may be lost during the purification process [11]. As the ATP effect on phosphodiesterase inactivation is pronounced at low calcium concentrations [12], the calcium level was kept at 10^{-9} M. All manipulations were carried out under infrared illumination.

Phosphodiesterase activity was measured by the pH assay method described previously [12]. A 200 μl portion of the outer segment suspension was made 1 mM in GTP by adding 5 μl of 40 mM GTP, since GTP is required for the activation of phosphodiesterase [13]. 10 μl of 80 mM cyclic GMP were added just before the light flash. The cyclic GMP concentration was chosen to be 4 mM so that is was over the K_m of the light-activated phosphodiesterase (0.5–1.5 mM [11]). The conditions of ATP addition are specified in the legend to each figure. Proton release accompanied by the light-induced hydrolysis of cyclic GMP to 5'-GMP was monitored continuously by a pH microelectrode (MI 410, Microelectrodes Inc., Londonderry, NH) and recorded on a pen chart recorder. The pH was allowed to drop from 7.8 to 7.7. The amount of hydrolysis was calibrated by back-titrating a known amount of 0.1 N NaOH. Phosphodiesterase activity in unit time was calculated from the tangent of the pH profile and expressed as mol of cyclic GMP hydrolyzed/mol of rhodopsin present per min. When the time course of phosphodiesterase inactivation was determined (Figs. 1 and 3), the tangent was determined at various time intervals after the flash. The calibrated light source (Sunpak Auto 411, Berkey Marketing Co., Woodside, NY) used an orange filter and the duration was 0.36 ms. Assuming first-order kinetics, the inactivation time constant was determined with the aid of Hewlett Packard Model 97 Computer. Optical measurement was done with a Cary 14 Spectrophotometer. When ATP concentration was changed (Fig. 2), the total nucleoside triphosphate concentration (GTP and ATP) was kept at nearly 1 mM to ensure that the free Mg^{2+} concentration would be constant. Magnesium is known to be essential for phosphodiesterase activation [9,12], and nucleoside triphosphate chelates Mg^{2+}.

10^{-9} M calcium-Ringer's solution was made with ultra-pure NaCl and KCl from Alfa Inorganics (Beverly, MA) and $MgCl_2$ was purchased from Johnson Matthey Chemicals (Royston, U.K.). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Results and Discussion

Phosphodiesterase is activated by a brief exposure to light and inactivated when left in the dark. This inactivation process is accelerated by the addition of 10 μM ATP (Fig. 1). The time of ATP addition after the flash was varied. The reaction maintained its potential ability to reduce phosphodiesterase activity rapidly for more than 1 min. The rate of inactivation caused by the addition of ATP at different time intervals was not significantly different. Assuming single first-order kinetics in Fig. 1, the inactivation time constant of the fast decay caused by the addition of ATP was calculated to be 0.098 ± 0.007 s^{-1} (a mean of four fast decays in Fig. 1). The decay observed when ATP was not added was found to consist of two

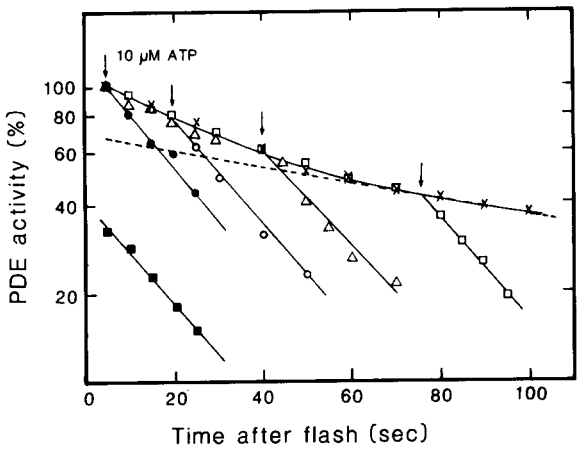

Fig. 1. Phosphodiesterase inactivation process maintains its ability for more than 1 min. A rod outer segment suspension in 10^{-9} M calcium and 1 mM GTP was divided into five portions. Each portion was illuminated by a flash bleaching 0.001% of rhodopsin. The phosphodiesterase (PDE) activity was measured continuously by the pH assay method. Dark activity was subtracted and the net activity elicited by the flash is shown as a function of time after the flash. 10 μM ATP was added before (●) and at various time intervals after the flash: 20 (○), 40 (△) and 75 s (□). As a control no ATP was added (×). Each data was normalized to the peak activity measured in each portion at 5 s after the flash. Assuming first-order kinetics, the best-fitted lines were calculated and these are shown by solid lines. The time constants are 0.091 s^{-1} (●), 0.106 s^{-1} (○), 0.102 s^{-1} (△) and 0.092 s^{-1} (□). The data points without ATP (×) consisted of two different first-order reactions (see text). The time constant for the slow decay (dashed line) is 0.0146 s^{-1} and that for the fast decay is 0.104 s^{-1} (■). In five portions, the average dark activity was 32.6 ± 2.2 cyclic GMP/rhodopsin per min and that of the initial peak activity after the flash was 190.0 ± 17.4 cyclic GMP/rhodopsin per min.

processes. One was a slow decay and the other was a fast decay caused by ATP. When one exponential decay was assumed only using data points at 80, 90 and 100 s after the flash (broken line in Fig. 1), we got an inactivation time constant of 0.0146 s^{-1}. When contributions of this slow decay was subtracted from the observed decay, we obtained a decay of first-order with an inactivation time constant of 0.104 s^{-1} (Fig. 1, ■). This inactivation time constant was comparable with that of 0.098 ± 0.007 s^{-1} observed when exogenous ATP was added. The fast decay without exogenous ATP could be attributed to the presence of a residual level of ATP which was known to exist in our preparation [12]. The slow decay could be an ATP-independent process and possibly a spontaneous decay. Therefore, in this preparation ATP increased the inactivation rate about 7-fold. The magnitude of acceleration of the inactivation varied from preparation to preparation. However, it was in a range of 3–7-fold. Since neither ADP nor β,γ-methylene ATP substitutes for ATP (data not shown), the latter observation agrees with that of Liebman and Pugh [18], the process involves phosphorylation with γ-phosphate of ATP.

In the isolated rod outer segments, the cyclic GMP level rapidly decreases after the onset of light, but when a low intensity of light is used, the level returns to the original dark level [4]. For this to be possible, phosphodiesterase should return to the dark level sometime after the flash. The time required for this return in the cyclic GMP level after 1 s of a flash bleaching 0.0017% of rhodopsin is about 1 min [4]. In the absence of ATP, at 1 min after the flash phosphodiesterase activity is about half of the maximal value elicited by a flash bleaching 0.001% of rhodopsin (Fig. 1). However, with ATP the reaction of inactivation is accelerated and at 1 min after the flash bleaching 0.001% of rhodopsin, phosphodiesterase activity is almost 10% of the peak activity (Fig. 1 and Ref. 12). Now the return of phosphodiesterase activity is comparable in time scale with that of cyclic GMP in the isolated rod outer segments.

In a previous report (Fig. 11 in Ref. 12), it was found that ATP not only increases the inactivation time constant but also reduces the initial peak activity after a sub-saturating low intensity of light. To examine the relation between these two ATP effects, the concentration function on the effects was determined (Fig. 2a and b). The half-maximal inactivation effect by ATP took place at a concentration of about 2 μM (Fig. 2a) when the light bleaching 0.003% of rhodopsin was used. However, the half-maximal effect on the initial peak activity was observed at a concentration of about 50 μM, which was clearly different from the half-maximal value for the inactivation time constant. Therefore, it is clear that ATP has two different effects on the phosphodiesterase activity. One is related to the inactivation time constant and the other to the initial peak activity. The inactivation time constant and the peak activity slightly varied

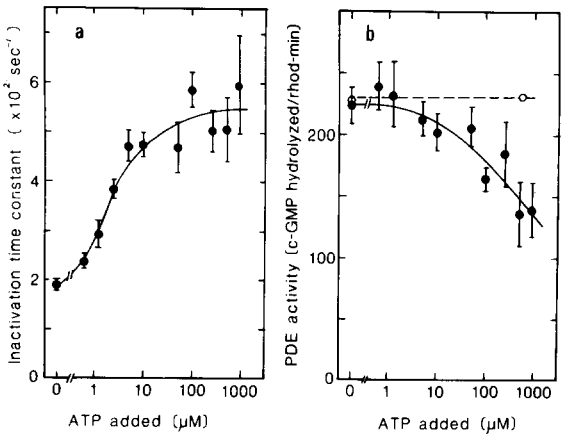

Fig. 2. Concentration effect of ATP on inactivation (a) and activation (b) of phosphodiesterase (PDE) activity. Rod outer segments were illuminated with a sub-saturating light flash bleaching 0.003% of rhodopsin in the presence of 4 mM cyclic GMP and in various concentrations of GTP and ATP. The initial peak activities were measured and the inactivation time constants were determined as in Fig. 1. The total nucleoside triphosphate concentration was kept at nearly 1 mM. The data shown are the average (±S.E.) of five to ten separate experiments. Open circles in b are the maximum phosphodiesterase activities elicited by the saturating intensity of the light flash.

from preparation to preparation. The data shown in Fig. 2 are the average of 5–10 separate experiments and the inactivation time constant was measured together with the peak activity in the same preparation. In the preparations shown in Fig. 2, ATP increased the inactivation time constant about 3-fold and reduced the initial peak activity to about 60% of the maximum.

In Fig. 2 the total nucleoside triphosphate (GTP and ATP) concentration was kept at nearly 1 mM (see Materials and Methods). One may expect that the effects of ATP come from the decrease in the GTP concentration. However, when the ATP concentration was below 50 μM, the GTP concentration was always 1 mM. With 50 μM ATP, the inactivation acceleration is almost complete (Fig. 2a) and the effect on the peak activity is about 50% (Fig. 2b). This clearly illustrates that the effect shown is ATP-specific.

Since it has been shown that ATP does not affect the maximal total phosphodiesterase activity under intense light (Ref. 12 and open circles in Fig. 2b), the result in Fig. 2b could be interpreted as the sensitivity control of phosphodiesterase by ATP. That is, at a mmol level of ATP, phosphodiesterase is more desensitized than at a μmol level of ATP. This agrees with Liebman and Pugh [17]. The actual ATP level in frog rod outer segment has been reported to be about 1 mM [19,20].

The inactivation time constant is a function of the intensity of the light flash. In the experiment shown in Fig. 3, where an ND 2.0 filter (bleaching 0.01% of rhodopsin) was used, the time constant was 0.034 s^{-1} whereas with an ND 3.5 filter it was 0.077 s^{-1}. The constants varied from preparation to preparation. For example, the constant with an ND 2.0 filter varied from 0.021 to 0.041 s^{-1} in three different preparations. However, in one single preparation they were stable and always increased with the decrease in light intensity.

We can assume that at certain time after a flash the phosphodiesterase activation and inactivation are still continuing and the amount of the active phosphodiesterase is a net product of these com-

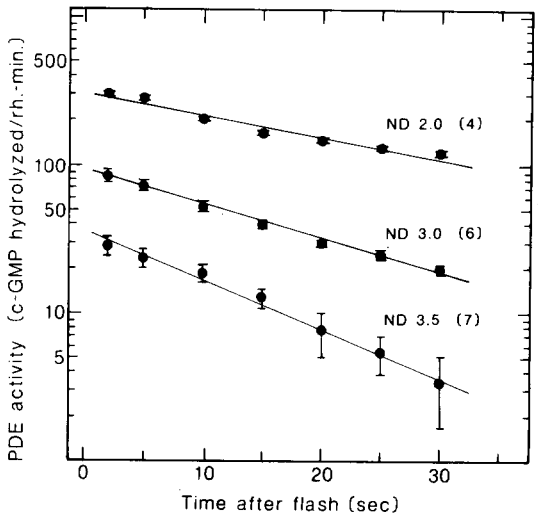

Fig. 3. Phosphodiesterase inactivation rate depends on light intensity. In the presence of 4 mM cyclic GMP, rod outer segment suspensions in 10^{-9} M calcium-Ringer's solution containing 0.5 mM GTP and 0.5 mM ATP were exposed to three different intensities of light flash, which was attenuated by neutral density filters. Assuming first-order kinetics, the time constants of the inactivation were calculated. The best fitted values are 0.034 s^{-1} (ND 2.0), 0.053 s^{-1} (ND 3.0) and 0.077 s^{-1} (ND 3.5). Each point is an average (±S.E.) of several experiments (the number of the experiments are shown in parentheses in the figure).

peting reactions. When flash intensity is increased, the amount of active phosphodiesterase increases. If the activation and inactivation processes are independent of flash intensity, the inactivation time constant should not change, even if the phosphodiesterase activity increases. This does not agree with the present work. Therefore, the results in Fig. 3 indicate that the activation process is enhanced in time base and/or the inactivation process is inhibited, when a higher light flash intensity is given.

When a series of light flashes are given, the subsequent flashes after the first flash give only a slight increase in phosphodiesterase activity compared with the activity elicited by the first flash [12]. Therefore, the first flash makes the activating reaction less sensitive to the subsequent flashes, while the inactivation process itself has a tendency to become ineffective when an intense light flash is given. Whatever the mechanism is, the findings shown in Fig. 3 may underlie the mechanism of the prolonged photoresponse in photoreceptor cells usually found when an intense light flash is given (for example, see Ref. 21), if cyclic GMP is involved in phototransduction mechanism.

It has been suggested that a bleaching intermediate, presumably metarhodopsin II [18,22,23], interacts with a protein called G-protein and forms a G-protein-GTP complex [15,24–26]. The complex is found to activate phosphodiesterase [16]. One suggestion for the mechanism of phosphodiesterase inactivation has been that metarhodopsin II is quenched by phosphorylation [17]. However, this mechanism has been questioned (see Ref. 8). Another possibility is that phosphorylated substance(s) quenches active phosphodiesterase. In order to clarify this possibility, rod outer segments were exposed to the first flash in the presence of ATP and washed twice by centrifugation with 10^{-9} M calcium-Ringer's solution without ATP. When the second flash was given 6.5 min after the first flash in the absence of ATP, the rod outer segments did not show the accelerated inactivation. In control experiments, the washed preparation showed the inactivation again in the presence of ATP 9 min after the first flash. Therefore, if a phosphorylated quencher is involved in the inactivation process, it is soluble or it has a lifetime shorter than 6.5 min.

Though the entire mechanism of phosphodiesterase inactivation by ATP is not clear at this point, it is possible that ATP shortens the lifetime of the G-protein-GTP complex. Since the initial peak activity is a function of the ATP concentration (Fig. 2b), the complex formation could also be reduced by ATP.

From the present study, and the recent findings by Hermolin et al. [8], it can be said that further work is needed before the entire mechanism of the phosphodiesterase activation and inactivation sequence can be understood. It is evident that ATP has at least two distinctive roles in controlling phosphodiesterase activity (Fig. 2).

Acknowledgements

The author is grateful to Professor M.D. Bownds for his encouragement and financial support of this study. Part of the travelling expenses were paid by a Naito Research Grant.

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