Communication

Photoreceptor GTP Binding Protein Mediates Fluoride Activation of Phosphodiesterase*

(Received for publication, July 12, 1984)

Peter J. Stein, Karen R. Halliday‡, and Mark M. Rasenick§

From the Yale University School of Medicine, Department of Ophthalmology and Visual Science, New Haven, Connecticut 06510, the §Life Science Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and the §University of Illinois at Chicago, College of Medicine, Department of Physiology and Biophysics, Chicago, Illinois 60680

In this report, we show that fluoride activates dark-adapted rod outer segment phosphodiesterase, and that this activation is mediated, in analogy with adenylyl cyclase, through a GTP binding protein. The GTP binding protein is released from dark-adapted rod outer segment membranes by exposure to fluoride and subsequent centrifugation. The 39-kilodalton subunit of the GTP binding protein, released from the membrane by this procedure, exhibits altered susceptibility to limited trypsin proteolysis, identical to that seen when hydrolysis-resistant GTP analogs are bound to that subunit. Repeated exposure of dark-adapted rod outer segment membranes to fluoride and subsequent centrifugation results in maximal activation of the membrane-bound phosphodiesterase. Thus, activation of phosphodiesterase by fluoride in the dark appears similar to fluoride activation of adenylyl cyclase.

Adenylyl cyclase (EC 4.6.1.1.) and photoreceptor phosphodiesterase I (EC 3.1.4.1.) have been shown to be remarkably similar enzyme complexes (1–3). In both systems, activation of the catalytic moiety through light (for phosphodiesterase) and hormone or neurotransmitter (for adenylyl cyclase) requires receptor activation, a GTP binding protein (also called G protein, N protein, transducin, etc.), and GTP (or analogs). Activation of the enzyme by fluoride ion has been demonstrated in both the adenylyl cyclase and phosphodiesterase systems as well (4, 5). However, other than demonstrations that GTP binding protein mediates adenylyl cyclase activation by fluoride, the molecular events responsible for this process remain unclear. The single report of fluoride activation of photoreceptor phosphodiesterase shows only that the enzyme can be activated in dark-adapted rod outer segment (ROS) membranes (5).

Guanine nucleotide (GTP or hydrolysis-resistant GTP analog) is required for light activation of ROS phosphodiesterase (6). After illumination and the addition of GTP, the GTP binding protein complex is released from ROS membranes after centrifugation (7, 8). In order to understand the effects of fluoride on the photoreceptor phosphodiesterase cascade, we examined the release of the GTP binding protein from ROS membranes after incubation with fluoride and centrifugation. We also measured the effects of fluoride on the activation of phosphodiesterase. The data show that, in the dark, KF mimics the effects of guanyl nucleotide and light in the activation of phosphodiesterase and the release of the GTP binding protein from ROS membranes. Furthermore, limited trypsin proteolysis of the KF-released GTP binding protein indicates that KF, in the absence of light, causes a conformational change in the GTP binding protein similar to that induced by light and hydrolysis-resistant GTP analogs (9, 10).

MATERIALS AND METHODS

Preparation of ROS—Retinas were dissected from dark-adapted (12–18 h) Bufo marinus and ROS were prepared as previously described (10). The harvested ROS were resuspended in 100 mM Tris (pH 7.3), 1 mM MgCl₂, and 5 mM dithiothreitol (Buffer A). An aliquot of the ROS suspension was solubilized in Emulphogene BC-720 and the absorption spectrum of rhodopsin was measured from 660–250 nm using a Shimadzu UV-3000 spectrophotometer. The rhodopsin concentration was calculated using 406 X 10³ cm²/mol as the molar extinction coefficient.

Release of GTP Binding Protein from ROS—We assessed the association of the GTP binding protein with both bleached and dark adapted ROS by monitoring the appearance of the GTP binding protein into solution after incubation of the ROS membranes (25–30 µm; 100-µl reaction volume) with KF or guanyl nucleotide followed by centrifugation. At the end of their incubation time, the membranes were centrifuged at 12,000 X g for 30 min. The supernatant solutions were recentrifuged for additional 30 min under the same conditions to remove any traces of ROS membrane. Aliquots were removed, solubilized in 3% SDS Laemml sample buffer (11) with 0.1 mM dithiothreitol. Samples were heated to 90 °C for 5 min and subjected to electrophoresis on 8–20% gradient gels or 12.5% gels (as previously described (10)). Gels or gel photographs were scanned with a Shimadzu Instruments scanning densitometer and peak areas were determined by a Shimadzu CI-A Integrator.

Trypsin Proteolysis of the GTP Binding Protein—ROS membranes were prepared as above and split into three aliquots. One aliquot was washed (in the dark) four times with Buffer A and then three times with Buffer A containing 2.5 mM KF. The KF extracts containing the GTP binding protein were pooled. The two remaining aliquots were washed and washed four times with Buffer A and three times with Buffer A containing either GTP or GTP-γ-S. The respective extracts were pooled. Aliquots of each GTP binding protein preparation containing approximately 10 µg of GTP binding protein were incubated with trypsin (14 µg/ml, final concentration). At 1, 5, 10, and 30 min, the proteolysis was stopped by the addition of soybean trypsin inhibitor (56 µg/ml, final concentration). After the addition of 3% SDS Laemml sample buffer, the samples were analyzed on 12.5% polyacrylamide gels.

KF Effects on Phosphodiesterase Activity—To assess the effects of KF washing on membrane-bound phosphodiesterase activity, ROS membranes (prepared as above) were washed four times with Buffer

* This work was supported by Air Force Office of Scientific Research Contract F49620-83-C-0050 (to P. J. S.), AFOSR Grant 83-0249 (to M. M. R.), National Institutes of Health Grant EY00785, the Connecticut Lions, Research to Prevent Blindness, and the Chicago Community Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The term GTP binding protein refers to the three subunits (α, β, γ of molecular mass 32-, 36-, and 6 kilodaltons) which together are necessary for GTP binding and GTPase activity.
Fluoride Activation of GTP Binding Protein

**Fig. 1. Release of GTP binding protein from ROS membranes.** A, dark-adapted ROS were incubated in the dark with Buffer A (lane a), or Buffer A containing 0.25 (lane b), 0.50 (lane c), 1.0 (lane d), 2.5 (lane e), 5.0 (lane f), 10.0 (lane g) mM KF, 10 μM Gpp(NH)p (lane h), or 10 μM GTP (lane i) for 6.5 min before centrifugation and electrophoresis of the supernatant solutions. The supernatant solutions were solubilized in 3% SDS sample buffer and analyzed on an 8-20% gradient gel. B, bleached ROS were incubated with Buffer A (lane a), or Buffer A containing 0.25 (lane b), 0.5 (lane c), 1.0 (lane d), 2.5 (lane e), 5.0 (lane f), 10.0 (lane g) mM KF, 10 μM Gpp(NH)p (lane h), or 10 μM GTP (lane i) before centrifugation and electrophoresis of the supernatant solutions. The amount of protein released from a ROS suspension containing 0.5 nmol of rhodopsin was applied to each lane except B, lane f, which received one-half that amount.

A containing 2.5 mM KCl or KF, and twice with Buffer A. The membranes were diluted to a final rhodopsin concentration of 1-10 μM (400-μl reaction volume) and assayed in the light for cyclic GMP hydrolysis. In the radiochemical assay employed, cyclic GMP was the 125 mM KCl, 1 mM MgCl₂, 5 mM dithiothreitol, and 5 mM diphosphodiesterase (12).

To determine the effect of KF incubation on phosphodiesterase activity, ROS membranes (1-10 μM; 40-μl reaction volume) were preincubated in the dark or bleached and preincubated for 4 min with KF or GTPγS in 125 mM KCl, 1 mM MgCl₂, 5 mM dithiothreitol, and 2.5 mM Tris, pH 8.0. At the end of the preincubation period, 5 mM cyclic GMP was added and the rate of proton evolution (a direct indication of cyclic GMP hydrolysis (13)) was monitored using a pH electrode (MI 410, Microelectrodes Inc., Londonderry, NH) whose output was fed into a voltage follower (WP Instruments, New Haven, CT) and amplifier (WP Instruments, New Haven, CT) and recorded on a Model 220 Brush Recorder (Gould Instruments, Cleveland, OH).

**RESULTS**

**Fluoride Releasess GTP Binding Protein from Dark-adapted ROS Membranes—**The effects of KF on the association of the GTP binding protein with the ROS membrane are shown in Fig. 1. A and B. Fig. 1A demonstrates that incubation of dark-adapted ROS membranes with buffer containing KF at various concentrations, followed by centrifugation, results in a concentration-dependent release (lanes b-g) of GTP binding protein. By contrast, buffer alone, 10 μM Gpp(NH)p and 10 μM GTP (lanes a, h, i) are relatively ineffective in releasing GTP binding protein from the dark membrane. When bleached membranes are incubated with fluoride, the results are strikingly different. Fig. 1B shows that incubation of bleached ROS membranes with fluoride followed by centrifugation releases only small amounts of GTP binding protein into the supernatant solution (lanes b-g) compared with Gpp(NH)p or GTP (lanes h and i). The amounts of GTP binding protein released under these experimental conditions are illustrated in Fig. 2. Incubation of bleached ROS membranes with fluoride releases at most 14% of the amount of GTP binding protein that can be released by Gpp(NH)p. Incubation of dark-adapted ROS membranes with fluoride releases almost 80% of the amount of protein released by Gpp(NH)p in bleached membranes. It appears that half-maximal release occurs at 1.0 mM fluoride.

**Trypsin Proteolysis of the GTP Binding Protein—**Several previous studies (9, 10) showed that the active (capable of activating phosphodiesterase) and inactive conformations of GTP binding protein may be distinguished by their digestion patterns during limited trypsin proteolysis. We employed this technique to analyze the conformation of GTP binding protein released with KF. Fig. 3 (Panels A and C) shows that trypsin digestion of the KF-released protein is similar to that generated for the protein with GTPγS bound. After release from the membrane with either KF or GTPγS, trypsin digestion eventually generates a 32-kilodalton fragment stable to further digestion. By contrast, when GTP binding protein is extracted with GTP and then subjected to trypsin proteolysis the digestion proceeds past the 32-kilodalton stage to generate fragments of 23 and 12 kilodaltons (Fig. 3, Panel B). Thus, limited trypsin proteolysis indicates that the conformation of GTP binding protein, when released by KF in the dark, is...
Fluoride Activation of GTP Binding Protein

Fig. 3. Trypsin digestion of GTP binding protein extracted with KF of GTP\(\gamma\)S. Panel A shows the fragments generated by proteolysis of the GTP binding protein released by KF after 0, 1, 5, 10, and 30 min of incubation with trypsin. Panel B shows the fragments generated by proteolysis of the GTP binding protein released by KF after similar incubation with trypsin. Panel C shows the fragments generated after trypsin digestion of the GTP binding protein released with GTP\(\gamma\)S under the same conditions. The arrowheads indicate the position of the fragments derived from the 39-kilodalton subunit.

Fig. 4. The effect of KF on phosphodiesterase activity. Dark (○) or bleached (○) ROS membranes were preincubated for 4 min with the indicated concentration of KF. Cyclic GMP (40 μl of 50 mM) was added and the rate of proton evolution was recorded. The pH change was calibrated with known aliquots of HCl. Maximum phosphodiesterase activity in this experiment was 273 m cGMP/m rhodopsin/min.

similar to the “active” conformation obtained with hydrolysis-resistant GTP analogs.

Fluoride Activation of Phosphodiesterase—To assess the influence of fluoride on phosphodiesterase activity, we measured cGMP hydrolysis in dark-adapted ROS membranes exposed to buffers containing different KF concentrations. Fig. 4 shows that phosphodiesterase activity is optimally stimulated, in the dark, at 5.0 mM KF. Half-maximal stimulation appears to occur at about 1 mM KF. It is interesting to note that, at 10.0 mM KF, enzyme activity is reduced. Inhibition of the catalytic moiety by fluoride\(^3\) may be responsible for this effect. Similarly, adenylyl cyclase from rat cerebral cortex synaptic membranes is maximally stimulated by 18 mM NaF while concentrations above 25 mM are inhibitory (15).

To analyze the mechanism by which phosphodiesterase is activated by fluoride, we washed dark-adapted ROS membranes with Buffer A containing either 2.5 mM KCl or KF. The activity of phosphodiesterase which (unlike the GTP binding protein) remains membrane-bound was then assayed in the light. Table I shows that KCl-washed ROS membranes (which contain GTP binding protein) require the addition of GTP\(\gamma\)S to obtain maximal hydrolytic activity. By contrast, KF-washed ROS (depleted of GTP binding protein) are maximally active without addition of GTP\(\gamma\)S. Phosphodiesterase activity in KF-washed ROS membranes is not altered by GTP\(\gamma\)S (data not shown). Phosphodiesterase activity in KF-washed ROS was 95–100% of the activity measured in KCl-washed membranes in the presence of light and GTP\(\gamma\)S (4 experiments). Washing dark ROS membranes with KF appears to remove an inhibitory constraint normally present on the catalytic moiety of phosphodiesterase. Reconstitution experiments\(^3\) support the idea that this restraint is due to the phosphodiesterase inhibitor molecule.

DISCUSSION

Incubation of dark-adapted ROS membranes with KF containing buffer results in a change in the conformation of the GTP binding protein such that it is capable of activating phosphodiesterase. This change is also indicated both by the KF concentration-dependent release of the protein from the ROS membrane and by the fact that the trypsin digestion pattern resembles that of the GTP binding protein with hydrolysis-resistant guanine nucleotide analog bound. Since, in this state, the protein is in a conformation capable of activating phosphodiesterase, it appears that fluoride activation of phosphodiesterase is mediated by the GTP binding protein. This idea is supported by reconstitution experiments which show that the fluoride-solubilized GTP binding protein can be added back to dark-adapted ROS membranes and activate the enzyme.\(^3\)

Both fluoride and guanine nucleotides release the GTP binding protein from ROS membranes. However, fluoride releases the protein most efficiently in dark ROS membranes while guanine nucleotides are most efficient in bleached ROS membranes. Washing dark-adapted ROS membranes after exposure to fluoride activates phosphodiesterase. The mechanism of activation appears to involve release of an inhibitory

---

TABLE I

| Additions          | Activity m cGMP/m rhodopsin/min |
|--------------------|-------------------------------|
| KCl-washed ROS     | None                          | 24                           |
| KCl-washed ROS     | GTP\(\gamma\)S                | 355                          |
| KF-washed ROS      | None                          | 350                          |

---

\(^3\) E. Bignetti, R. Tirendelli, and R. T. Sorbi, personal communication.
protein from the catalytic moiety, since a similar mechanism was recently demonstrated to account for light-dependent guanyl nucleotide activation of phosphodiesterase (12). However, fluoride activation occurs in dark-adapted membranes while guanyl nucleotide activation requires bleaching. Thus, the light-dependent rhodopsin-GTP binding protein interaction required for guanyl nucleotide activation of phosphodiesterase is apparently not required for fluoride activation of this enzyme. This parallels observations made for adenylate cyclase where hormone (and hormone receptor) is not necessary for fluoride activation of the enzyme (14, 15). Furthermore, fluoride activation of the adenylate cyclase stimulatory GTP binding protein (Nt) has been shown to resemble activation by GTP analogs (17). The trypsin digestion experiments reported above demonstrate that the GTP binding protein released by fluoride or GTPγS generate identical proteolytic fragments and therefore have similar conformations. Since photoreceptor GTP binding protein can substitute for adenylate cyclase GTP binding protein in the activation of the catalytic moiety (16), it appears likely that a similar conformation change must occur in the adenylate cyclase GTP binding protein during the activation process.

Acknowledgment—We thank Dr. Robert L. Perlman for his critical reading of the manuscript and helpful comments.

REFERENCES
1. Shinozawa, T., Sen, I., Wheeler, G. L., and Bitensky, M. M. (1979) J. Supramol. Struct. 10, 185-190
2. Bitensky, M. M., Wheeler, M. A., Rasenick, M. M., Yamazaki, A., Stein, P. J., Halliday, K. R., and Wheeler, G. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3408-3412
3. Stein, P. J., Rasenick, M. M., and Bitensky, M. W. (1982) Prog. Retinal Res. 1, 222-238
4. Spiegel, A. M., and Downs, R. W. (1981) Endocr. Rev. 2, 275-305
5. Sitaramayya, A., Virmaux, N., and Mandel, P. (1977) Exp. Eye Res. 25, 163-169
6. Wheeler, G. L., and Bitensky, M. W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3408-3412
7. Godchaux, W., and Zimmerman, W. F. (1979) J. Biol. Chem. 254, 7874-7884
8. Kuhn, H. (1980) Nature (Lond.) 283, 587-589
9. Fung, B. K.-K., and Nash, C. R. (1983) J. Biol. Chem. 258, 10503-10510
10. Halliday, K. R., Stein, P. J., Chernoff, N., Wheeler, G. L., and Bitensky, M. W. (1984) J. Biol. Chem. 259, 516-525
11. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685
12. Yamazaki, A., Stein, P. J., Chernoff, N., and Bitensky, M. W. (1983) J. Biol. Chem. 258, 8188-8194
13. Liebman, P. A., and Ewanczuk, A. T. (1982) Methods Enzymol. 81, 532-542
14. Downs, R. W., Spiegel, A. M., Singer, M., Reen, S., and Aurbach, G. D. (1980) J. Biol. Chem. 255, 949-954
15. Rasenick, M. M., and Bitensky, M. W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4628-4632
16. Rasenick, M. M., Stein, P. J., and Bitensky, M. W. (1981) Nature (Lond.) 294, 560-562
17. Sternweis, P., Northup, J. K., Smigel, M., and Gilman, A. G. (1981) J. Biol. Chem. 256, 11517-11526