Digitonin Effects on Photoreceptor Adenylate Cyclase

Abstract. *Adenylate cyclase is described in a number of photoreceptor membranes. Vertebrate rod outer segments contain light-regulated cyclase, and light regulation is abolished by digitonin. Disruption of microvilli in cone and rhabdom photoreceptors is also associated with loss of light regulation and retention of full enzymic activity. The data suggest that inhibitory constraint provides regulation in cyclase systems and that disruption of membrane structure uncouples catalytic and regulatory elements.*

Adenylate cyclase (cyclase) plays a role in the hormone regulation of cellular processes (*1, 2*). In each tissue, cyclase exhibits unique hormone binding characteristics and specificity (*3*), which suggests that discrete hormone receptors are involved in the molecular sequences which convert hormonal signals into changes in cyclase activity.

We have described a light-regulated cyclase in vertebrate photoreceptor organelles, which has a high specific activity (*2*). Because the fraction of cyclase inactivated is proportional to the fraction of rhodopsin molecules bleached (*4, 5*), and adenosine 3′,5′-monophosphate (cyclic AMP) mimics the physiological effects of light in the *Limulus* photoreceptor, we suggested that cyclase activity is linked to the photochemical state of rhodopsin and to light-induced sodium conductance changes (*6*) in photoreceptor cells.

Frozen dark-adapted bovine retinas (Hormel) were prepared as described. No homogenization steps preceded purification in 47.6 percent sucrose. Rod outer segment suspensions were mixed with one-third volume of distilled water or 2 percent digitonin. Resulting solutions were assayed for cyclase activity with [8-^{14}C]adenosine triphosphate (ATP) and thin-layer chromatography and examined with the electron microscope (*7*).

Suspensions of frog photoreceptors, of ground squirrel cones, and of invertebrate rhabdoms were sonicated in sucrose (47.6 percent) and prepared as above. In all cases a dense paste of purified photoreceptors rose to the air-sucrose interface, and was harvested, diluted with water (one-third its volume), sonicated, and assayed.

Studies with digitonin suspensions of rod outer segments produced the following results: (i) There was a progressive loss of regulation by light in outer segment membrane cyclase which was complete by 2 hours, paralleling an observed progressive destruction of outer segment disk integrity. (ii) There was relative preservation of cyclase activity throughout this time period. (iii) The loss of light regulation occurred whether or not the sample had been illuminated (Table 1 and Fig. 1).

Spectrophotometric measurements of solutions of rhodopsin extracted with digitonin showed both that the absorbancy at 500 nm is constant (in digitonin) for 12 hours in the dark (*8*), and the expected fall in the absorbancy was observed when these solutions were exposed to light. Sucrose suspensions of rod outer segments inactivated by light and then dark-adapted for 2 hours exhibited no regeneration of enzymic activity or increase in the absorbancy at 500 nm. The time-dependent increase in cyclase activity observed in digitonin suspensions of outer segments was not accompanied by a change in the absorbancy and was not reversed by illumination.

Homogenization (glass on glass) of cyclase preparations of bovine rod outer segments produced loss of light regulation and reduction of cyclase activity greater than that caused by digitonin (Table 1).

Table 2 shows cyclase activity in different vertebrate and invertebrate photoreceptors. A number of species including ground squirrel (cones) and three invertebrates (rhabdoms) exhibited no change in photoreceptor cyclase activity in vitro with illumination. Cone cyclase exhibited the highest (specific) activity of all tissues studied.

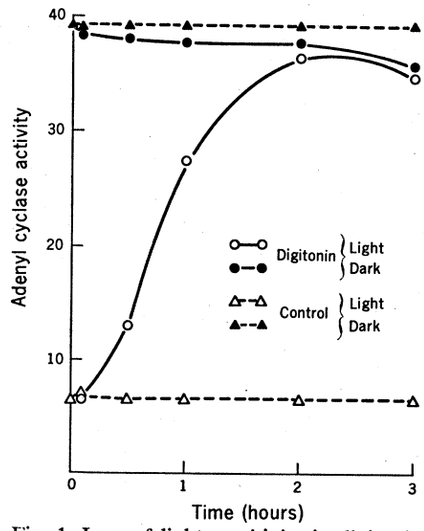

Fig. 1. Loss of light sensitivity in digitonin suspensions of rod outer segments as a function of time. Activities, conditions, and reactants as in Table 1. The times shown are the duration of room temperature incubation in the presence (○) or absence (△) of digitonin.

Electron micrographs of bovine and frog rod preparations sonicated in sucrose showed mostly intact disks. When such suspensions were mixed with digitonin, electron micrographs of material fixed at different times after mixing show a progression from intact disks to linear membrane fragments and vesicles roughly one-fifth of the disk diameter. Purified crayfish rhabdoms sonicated in sucrose showed numerous linear membranous structures with few vesicles.

Our findings favor inhibitory constraint as the regulatory mechanism for cyclase. Digitonin did not destroy photochemical properties of rhodopsin (analogous to the hormone receptor) or the enzymic activity of cyclase, but appears to disrupt coupling between cyclase and regulatory components. Although digitonin-treated enzyme was no longer sensitive to light, its activity was equivalent to dark-adapted cyclase in sucrose. In these experiments, spectrophotochemical properties of rhodopsin provide a unique opportunity for estimating the functional state of a cyclase receptor component. Homogenization resembles digitonin in selectively disrupting regulation and preserving activity.

In brain slices (*9*), where cell structure is preserved, cyclase activity was increased as much as 50-fold by appropriate neurohormones. Activities obtained in hormone-insensitive brain homogenates (*10*) suggest that the homogenate enzyme is locked in the "on" position. Similar comparisons have been found for cyclase activity in slices and homogenates of salmon testes (*11*). Although activities in homogenate and slice preparations are not strictly comparable, the observed differences support the concept that inhibitory constraint is lost in homogenate preparations.

Alternatively, effects of hormones could be mimicked by detergent or homogenization, either of which could nonspecifically activate cyclase. It seems unlikely, however, that detergent or homogenization could mimic the effects of specific endogenous regulators and more likely that either could uncouple regulatory and catalytic elements. Rhabdom and cone cyclase systems resemble brain homogenates and digitonin suspensions of rod disks in being uncon-

Table 1. Effects of digitonin on light sensitivity of bovine photoreceptor adenyl cyclase. Adenyl cyclase activity is expressed as the number of nanomoles of cyclic AMP per milligram of protein per 10 minutes. The averages for six determinations are given. Replicate values agreed within 6 percent. Reaction mixtures included [8-^{14}C]ATP, Mg^{2+}, aminophylline, an ATP regenerating system, and glycylglycine, *p*H 7.4, in a reaction volume of 20 μl as described (*2*). Reactions were performed at 30°C for 5 minutes, and stopped by immersion in a boiling water bath for 30 seconds.

Preliminary treatment		Adenyl cyclase activity	
Illumination*	Incubation at 22°C	Light	Dark
	Sucrose suspension		
None	5 minutes	5.7	36.0
None	2 hours	5.8	34.6
	2 percent digitonin sucrose		
None	5 minutes	6.7	34.2
None	2 hours	31.7	33.0
Full	5 minutes	7.4	7.4
Full	2 hours	34.8	34.8
	Homogenized sucrose suspension		
None	5 minutes	21.8	22.4

* Samples were exposed to light before the addition of digitonin.

strained. Although invertebrate photoreceptor cyclases thus far examined do not exhibit light regulation in broken cell preparations, intracellular recordings from *Limulus* photoreceptor cells show that cyclic AMP and aminophylline mimic the effects of light, suggesting the presence of light-activated cyclase in vivo (*4*).

The anatomy of rhabdom (*12*) and cone photoreceptors (*13*), is different from that of rod outer segments of vertebrates. Although the plasma membrane of the vertebrate rod outer segment is disrupted by sonication in sucrose, the disk membrane is not; therefore, the disks survive as intact organelles in the preparations that show regulation by illumination in vitro. All the preparations that do not show regulation by light in vitro (vertebrate cone and invertebrate rhabdoms), are continuous infoldings of the plasma membrane, an arrangement that results in disruption during sonication and does not permit isolation of intact organelles. That vertebrate rods are anatomically different from cones and rhabdoms, could explain the ease with which constraint is lost in the cones and rhabdoms. In those systems (for example, liver, kidney, melanocyte). (*14*), where hormone regulation survives homogenization, the coupling of cyclase regulatory elements would appear to have greater intrinsic stability.

Table 2. Measurements of photoreceptor adenyl cyclase activity in various species. Enzyme activity, reaction components, and conditions are as described in the legend of Table 1.

Species	Adenyl cyclase activity	
	Dark	Light
	Rod	
Leopard frog	78.4	7.4
Bull frog	47.6	5.8
Cow	33.4	5.7
	Cone	
Ground squirrel	157.0	157.0
	Rhabdom	
Lobster	17.8	17.7
Crayfish	15.8	16.0
Gypsy moth	16.4	16.5

Although isolation of rod outer segments in 35 percent sucrose can be associated with mitochondrial contamination (*15, 16*), mitochondria are an unlikely source of light-regulated cyclase in our preparation for the following reasons. (i) Contamination by inner segments is significant (26 percent total protein) only when the entire retina is homogenized before purification. Our electron micrographs reveal that mechanical shaking of frog or bovine retinas in 47.6 percent sucrose is accompanied by minimal inner segment contamination. Earlier preparations (*15*), used brief periods of low-speed sedimentation. With high-speed centrifugation for longer periods (100,000*g* for 90 minutes), significant purification is obtained (*2, 16*). (ii) Cyclase activity has never been detected in purified mitochondrial membranes; it has only been found in plasma membrane or its derivatives [in synaptosomes (*17*), sarcoplasmic reticulum (*18*), and hepatic plasma membrane envelopes (*19*)]. (iii) It is unreasonable to attribute the very large cyclase activity to a minor contaminant, which would further increase the specific activity by an order of magnitude. (iv) The amount of rhodopsin bleached is directly proportional to the percentage of cyclase inactivated (*4*), linking disk membrane rhodopsin and cyclase. (v) Using McConnell's sucrose gradient without tris buffer (*16*), Heitzmann (*20*) has prepared a (homogenized) membrane fraction entirely free of mitochondrial contamination with more than 80 percent of its protein rhodopsin. This fraction contains more than 40 percent of the cyclase activity found in our homogenized sucrose preparation.

A preferred tentative location for cyclase is the outer surface of rod disk membranes, a location that would permit access of cyclase to both rhodopsin and ATP. Location in the outer segment plasma membrane cannot be excluded.

The model presented is not meant to imply contiguity between rhodopsin and catalytic elements of cyclase. Our data emphasize the possibility that regulation of outer segment cyclase depends on the integrity of photoreceptor membranes and possibly the release of sequestered molecular or ionic intermediates such as Ca^{2+} (*21*).

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References and Notes

1. E. W. Sutherland and T. W. Rall, *J. Amer. Chem. Soc.* **79**, 3608 (1957); M. A. Rizack, *J. Biol. Chem.* **239**, 392 (1964); M. W. Bitensky and S. R. Burstein, *Nature* **208**, 1282 (1965); J. G. Schofield, *ibid.* **215**, 1382 (1967); J. R. Turtle, G. K. Littleton, D. M. Kipins, *ibid.* **213**, 727 (1967); R. C. Haynes, Jr., *J. Biol. Chem.* **233**, 1220 (1958); I. Pastan, *Biochem. Biophys. Res. Commun.* **25**, 14 (1960); A. L. Goldberg and J. J. Singer, *Proc. Nat. Acad. Sci. U.S.* **64**, 134 (1969).
2. M. W. Bitensky, R. E. Gorman, W. H. Miller, *Proc. Nat. Acad. Sci. U.S.* **68**, 561 (1971).
3. L. Birnbaumer, S. L. Pohl, H. Michiel, J. Kraus, M. Rodbell, *Advan. Biochem. Psychopharmacol.* **3**, 185 (1970); R. J. Lefkowitz, J. Roth, I. Pastan, *Nature* **228**, 864 (1970); A. M. Spiegel and M. W. Bitensky, *Endocrinology* **85**, 638 (1969)
4. W. H. Miller, R. E. Gorman, M. W. Bitensky, *Science* **174**, 295 (1971).
5. M. W. Bitensky, W. H. Miller, R. E. Gorman, A. H. Neufeld, R. Robinson, *Advan. Cyclic Nucleotide Res.*, in press.
6. T. Tomita, *Quart. Rev. Biophys.* **3**, 179 (1970); R. D. Penn and W. A. Hagins, *Nature* **223**, 201 (1969).
7. M. W. Bitensky, V. Russell, M. Blanco, *Endocrinology* **86**, 154 (1970).
8. H. J. A. Dartnall, *The Visual Pigments* (Wiley, New York, 1957), p. 13.
9. S. Kakiuchi and T. W. Rall, *Mol. Pharmacol.* **4**, 367 (19[illegible] H. Shimizu, J. W. Daly, C. R. Creveland, *J. Neurochem.* **16**, 1609 (1969).
10. L. M. Klainer, V. M. Chi, S. L. Freidberg, T. W. Rall, E. W. Sutherland, *J. Biol. Chem.* **237**, 1239 (1962); G. Krishna, B. Weiss, B. B. Brodie, *J. Pharmacol. Exp. Ther.* **163**, 379 (1968).
11. K. M. J. Menon and M. Smith, *Biochemistry* **10**, 1186 (1971).
12. W. H. Miller, *J. Biophys. Biochem. Cytol.* **3**, 421 (1957).
13. F. S. Sjöstrand, *Rev. Mod. Phys.* **31**, 301 (1959).
14. K. L. Cohen and M. W. Bitensky, *J. Pharmacol. Exp. Ther.* **169**, 80 (1969); M. W. Bitensky, H. Demopoulos, V. Russell, in *Pigmentation: Its Genesis and Biologic Control*, V. Riley, Ed. (Appleton-Century-Crofts, New York, 1971).
15. Z. Saito, *Tokohu J. Exp. Med.* **32**, 432 (1938).
16. D. G. McConnell, *J. Cell Biol.* **27**, 459 (1965).
17. G. Rodriguez de Lores Arnaiz and E. DeRobertis, *Acta Physiol. Latamer* **16**, 187 (1966).
18. M. Rabinowitz, L. DeSalles, J. Meisler, L. Lorand, *Biochim. Biophys. Acta* **97**, 29 (1965).
19. S. L. Pohl, L. Birnbaumer, M. Rodbell, *Science* **164**, 566 (1969).
20. H. Heitzmann, personal communication.
21. S. Yoshikomi and W. A. Hagins, *Biophys. Soc. Abstr.* **11**, 47a (1971).
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