Constitutive Activation of Photoreceptor Guanylate Cyclase by Y99C Mutant of GCAP-1

POSSIBLE ROLE IN CAUSING HUMAN AUTOSOMAL DOMINANT CONE DEGENERATION*

(Received for publication, April 13, 1998, and in revised form, May 12, 1998)

Alexander M. Dizhoor‡§, Sergei G. Boikov‡, and Elena V. Olshesvskaya‡

From the ‡Department of Ophthalmology/Kresge Eye Institute and §Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201

Photoreceptor membrane guanylate cyclases (RetGC) are regulated by calcium-binding proteins, GCAP-1 and GCAP-2. At Ca\(^{2+}\) concentrations below 100 nm, characteristic of light-adapted photoreceptors, guanylate cyclase-activating protein (GCAPs) activate RetGC, and at free Ca\(^{2+}\) concentrations above 500 nm, characteristic of dark-adapted photoreceptors, GCAPs inhibit RetGC. A mutation, Y99C, in human GCAP-1 was recently found to be linked to autosomal dominant cone dystrophy in a British family (Payne, A. M., Downes, S. M., Bessant, D. A. R., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattacharya, S. S. (1998) Hum. Mol. Genet. 7, 273–277). We produced recombinant Y99C GCAP-1 mutant and tested its ability to activate RetGC in vitro at various free Ca\(^{2+}\) concentrations. The Y99C mutation does not decrease the ability of GCAP-1 to activate RetGC. However, RetGC stimulated by the Y99C GCAP-1 remains active even at Ca\(^{2+}\) concentration above 1 \(\mu\)M. Hence, the cyclase becomes constitutively active within the whole physiologically relevant range of free Ca\(^{2+}\) concentrations. We have also found that the Y99C GCAP-1 can activate RetGC even in the presence of Ca\(^{2+}\)-loaded nonmutant GCAPs. This is consistent with the fact that cone degeneration was dominant in human patients who carried such mutation (Payne, A. M., Downes, S. M., Bessant, D. A. R., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattacharya, S. S. (1998) Hum. Mol. Genet. 7, 273–277). A similar mutation, Y104C, in GCAP-2 results in a different phenotype. This mutation apparently does not affect Ca\(^{2+}\) sensitivity of GCAP-2. Instead, the Y104C GCAP-2 stimulates RetGC less efficiently than the wild-type GCAP-2. Our data indicate that cone degeneration associated with the Y99C mutation in GCAP-1 can be a result of constitutive activation of cGMP synthesis.

The second messenger of phototransduction, cGMP, is synthesized in photoreceptors by two retinal guanylate cyclases, RetGC-1 and RetGC-2 (also referred to as ROGC-1 and -2 or GC-E and GC-F, respectively) (2–10). RetGC1 are regulated by two homologous Ca\(^{2+}\)-binding proteins, GCAP-1 and GCAP-2 (3, 4, 10–13). Ca\(^{2+}\) enters outer segments (OS) of vertebrate photoreceptors through cGMP-gated Na\(^{+}/Ca\(^{2+}\) channels in the plasma membranes. These channels are open in the dark, but they become closed in the light, because illumination stimulates cGMP hydrolysis by phosphodiesterase. Ca\(^{2+}\) is constantly extruded from the OS by a light-independent Na\(^{+}/K\(^{+}\) exchanger, therefore interruption of Ca\(^{2+}\) influx through the channels decreases the intracellular free Ca\(^{2+}\) concentration (10, 14, 15), and that stimulates cGMP resynthesis in photoreceptors (10, 16). This Ca\(^{2+}\) feedback mechanism is essential for the recovery and light adaptation of photoreceptors (10).

RetGC itself is not sensitive to Ca\(^{2+}\), but it can interact with Ca\(^{2+}\) sensor proteins, GCAP-1 and GCAP-2 (3, 4, 11–13). A unique property of GCAPs is that they can be either activators or inhibitors of RetGC (17): at Ca\(^{2+}\) concentrations below 100 nm, characteristic of light-adapted photoreceptors, GCAPs activate the cyclase, and at free Ca\(^{2+}\) concentrations above 500 nm, characteristic of dark-adapted photoreceptors, GCAPs inhibit RetGC. GCAP-1 and GCAP-2 have four EF-hand Ca\(^{2+}\)-binding domains, and GCAPs can be turned into constitutive activators of RetGC by mutations that inactivate the ability of their EF-hands to bind Ca\(^{2+}\) (17–18).

The intracellular level of cGMP may be important not only for the phototransduction, but also for the viability of photoreceptors. Several types of rod or cone degeneration have been linked to the mutations in those photoreceptor proteins that regulate either synthesis or hydrolysis of cGMP (19–23). Recently Payne et al. (1) described a new case of human autosomal dominant cone dystrophy associated with a point mutation in GCAP-1 gene. In this paper we present the evidence that this mutation, Y99C, causes a dramatic change in Ca\(^{2+}\) sensitivity of GCAP-1. As a result, RetGC stimulated by the Y99C GCAP-1 remains active even at high free Ca\(^{2+}\) concentrations. We also demonstrate that the corresponding mutation in GCAP-2 produces a different effect. Our data indicate that dominant cone degeneration associated with the Y99C substitution in GCAP-1 can be caused by permanent activation of cGMP synthesis.

EXPERIMENTAL PROCEDURES

Recombinant GCAP-1 and GCAP-2—Recombinant GCAP-1 and GCAP-2 were expressed in Escherichia coli according to the procedure described previously in detail (17, 24), except that we used BLR(DE3)pLysS E. coli strain (Novagen) instead of BL21(DE3)pLysS. Myristoylated GCAP-2 was expressed as described previously (24). The N terminus of GCAP-1 is a poor substrate for yeast N-myristoyltransf erase (NMT; Ref. 25). Substitution D6S makes it a better substrate (25), that allows us to produce GCAP-1, which is >90% myristoylated and is fully capable of regulating RetGC (Fig. 1). To make the GCAP-1 expression system, a cDNA encoding GCAP-1 was isolated from a bovine retinal cDNA library (a gift from D. Oprian, Brandeis University), amplified by polymerase chain reaction using forward primer

* This work was supported by National Institutes of Health Grant EY11522 and by a Career Development Award from Research to Prevent Blindness (to A. M. D.). 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.

‡ To whom correspondence should be addressed: Dept. of Ophthalmology/Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine, Detroit, MI 48201. Tel.: 313-577-1573; Fax: 313-577-7635; E-mail: adizhoor@med.wayne.edu.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org
A AAAAACCATGAGGGAACATTATGAGCGGTAAGTTGTTG and reverse primer ATATATTGTCCGTTAAAGGATGGAGGTTCGAC. The resulting 0.65-kilobase pair fragment was inserted into the NcoI/BamHI restriction endonuclease sites of pET11d vector (Novagen) and expressed under the lac-controlled T7 promoter in the BL21(DE3)pLysS E. coli strain (Novagen) that harbored a plasmid encoding yeast NMT (a gift from Dr. J. Gordon, Washington University) as described previously (26). To produce Y→C substitutions fragments of GCAPs cDNAs were amplified by polymerase chain reaction using Pfu polymerase (Stratagene) and spliced by “splicing by overlap extension” (26). Pairs of primers encoding the base substitutions were: GTAATTTCACTCTTGAGCAGTGACCAG (for making Y99C GCAP-1) and AGTGGACCTGTCAGGCTGAGAGTGAAGTACC for making Y104C GCAP-2. Mutant GCAP-1 and GCAP-2 were expressed using the same method (24). Expressed proteins were purified as described previously (24) using chromatography on Sephacryl S-100 column. Positions of the mutations were verified by automated DNA sequencing (ABI Prism, Perkin-Elmer). Calculated average isoelectric mass for the myristoylated Y99C used in this study is 23,500.00. The actual average isotopic mass of purified Y99C Y99C found by electrospray mass-spectrometry was 23,500.0.

RESULTS AND DISCUSSION

Mutation Y99C Affects Ca2+ Sensitivity of GCAP-1—GCAPs are highly conserved proteins (28). Human, mouse, and bovine GCAP-1 are virtually identical within their EF-hands regions, and this is also true for GCAP-2 (Ref. 19; also, see Fig. 1, top panel). When expressed as recombinant proteins, both GCAP-1 and GCAP-2 stimulate RetGC in a Ca2+-sensitive manner as it is shown in Fig. 1. It is also important to notice that GCAPs regulate RetGC within the submicromolar range of free Ca2+ concentrations. The exact free Ca2+ concentrations in rods and cones of mammals and humans have yet to be determined, but in dark-adapted resting rods of lower vertebrate, the free Ca2+ concentration is near 550 nM, and it decreases to near 50 nM (10) after strong illumination. Therefore we consider the submicromolar range of free Ca2+ as “physiologically relevant.”

To evaluate the potential functional significance of Y99C substitution reported by Payne et al. (1), we replicated this mutation in recombinant GCAP-1. The mutant protein was expressed in E. coli and purified as described under “Experimental procedures.” We have found that the Y99C substitution does not hamper the ability of GCAP-1 to stimulate RetGC (Fig. 1A). Instead, the Y99C GCAP-1 fails to inhibit the cyclase at high Ca2+ , so that RetGC remains equally active within the whole range of free Ca2+ concentrations between 6 nM and 1 µM. The Ca2+ sensitivity of RetGC in the presence of the mutant GCAP-1 is decreased to such extent that the cyclase remains at near 50% of its maximal activity at Ca2+ concentrations higher than 10 µM.

GCAP-1 and GCAP-2 are nearly 40% identical to each other (12, 28). However, despite the overall functional and structural similarity between GCAP-2 and GCAP-1, a similar substitution, Y104C, in GCAP-2 results in different biochemical phenotype than the Y99C mutation in GCAP-1 (Fig. LB). The Y104C GCAP-2 inhibits activation of RetGC at low Ca2+, but its Ca2+ sensitivity remains practically unaffected. Hence, the
region adjacent to the EF-3 in GCAP-1 apparently plays a different role in RetGC regulation than the same region in GCAP-2. The difference between the Y99C GCAP-1 and the Y104C GCAP-2 in our experiments is consistent with other recent observations. First, unlike GCAP-1, inactivation of EF-3 in GCAP-2 has relatively minor effect on the regulatory properties of GCAP-2 (17, 18). Second, EF-2 is very important for the Ca\(^{2+}\) sensitivity of GCAP-2 (17), although it was postulated not to be essential for the activity of GCAP-1 (18). Third, a calcium-myristoylation switch has been postulated to be critical for the GCAP-1 activity (29); however myristoylation has only a minor significance for the general regulatory properties of GCAP-2 (24).

The Y99C GCAP-1 Competes with the Wild Type GCAP-1 and GCAP-2—Payne et al. (1) reported that the Y99C mutation in GCAP-1 gene had a dominant phenotype. The question is why does the presence of the normal allele(s) of GCAP(s) not protect cone cells from degeneration?

Even though the exact level of GCAP-1 and GCAP-2 expression in cones and rods has not been unambiguously defined, it has been well established that both GCAP-1 and GCAP-2 are expressed in photoreceptors (11–13, 29–32). Several antibodies were raised in different laboratories that could detect both GCAP-1 and GCAP-2 in rods (12, 30) and in cones (30, 31) (some conclusions about the distribution of GCAP-1 and GCAP-2 in rods versus cones (29) were at variance apparently because of the different masking of GCAP-2 epitopes in animal species (30)). Both immunocytochemical (13, 30–32) and in situ hybridization analyses (11) indicate that GCAP-1 is strongly expressed in cones. At the same time, Y99C mutation in GCAP-1 results only in cone dystrophy, and rods appear to be unaffected (1). This fact suggests that GCAP-1 is either not functioning in rods, or its concentration in rods is insignificant for RetGC regulation. It also strongly argues that GCAP-1 plays an important role in RetGC regulation in cones. On the other hand, GCAP-2 was initially found in rod outer segments (12). This localization of GCAP-2 in rods has been confirmed by other groups (30, 32). However, a lower level of GCAP-2 expression in cones has also been detected (30, 32). It is therefore possible that the normal alleles of GCAP-1 and GCAP-2 can both be present in the affected human cones along with the Y99C GCAP-1. Based on that assumption, we tested whether Y99C GCAP-1 could activate RetGC in the presence of both Ca\(^{2+}\)-loaded GCAP-1 and GCAP-2 in vitro.

We have found that the Y99C GCAP-1 efficiently competes with Ca\(^{2+}\)-loaded GCAP-1 and GCAP-2 and prevents their inhibitory effect at free Ca\(^{2+}\) as high as 1 \(\mu\text{M}\) (Fig. 2). The addition of nonmutant GCAP-1 and GCAP-2 increases the EC\(_{50}\) for the RetGC activation by the Y99C GCAP-1, but it does not prevent RetGC from being activated by the mutant protein (Fig. 2A). The Y99C GCAP-1 stimulates RetGC in the presence of equimolar concentrations of either wild type GCAP-1 or GCAP-2 (Fig. 2B). The normal GCAP-1 and GCAP-2 are able to only partially decrease RetGC activity stimulated by the Y99C GCAP-1, at free Ca\(^{2+}\) above 1 \(\mu\text{M}\). Therefore, given that the intracellular free Ca\(^{2+}\) in human photoreceptors in the dark is within the micromolar range (10), the Y99C mutation should be able to cause an excessive synthesis of cGMP in resting photoreceptors, even in the presence of normal GCAPs. That could explain the dominant phenotype of Y99C mutation in GCAP-1 found in vivo.

The steady-state dark/resting level of free cGMP in photoreceptors is maintained at the level of 3–4 \(\mu\text{M}\), and that keeps a few percent of cGMP gated ion channels in the open state (10). It is not immediately apparent why and how the Y99C GCAP-1 effect on RetGC activity would cause photoreceptors to degenerate. So, we can only suggest various scenarios that could potentially lead to the cell death. It is likely that constitutive synthesis of cGMP, especially when it is not balanced by phosphodiesterase activity in the resting photoreceptors, may increase the steady-state level of the free cGMP in the cytoplasm. In such case, high cGMP level, for example, would be able to alter the activity of the cyclic nucleotide-regulated protein kinase(s). Also, higher than normal cGMP concentrations can keep tonoic cGMP gated channels open in the dark and create excessive influx of both Na\(^+\) and Ca\(^{2+}\). Because the activity of RetGC in the presence of the Y99C GCAP-1 is not completely inhibited even by [Ca\(^{2+}\)]\(_{\text{free}}\) above 10 \(\mu\text{M}\) (Figs. 1A and 2B), cGMP synthesis may continue until the free concentration of Ca\(^{2+}\) (and perhaps Na\(^+\)) in the cell dramatically exceeds the normal resting level. That may affect cellular metabolism in general. For example, more ATP will be constantly...
utilized to extrude both Na\(^+\) and Ca\(^{2+}\) from the resting cell. The elevated intracellular Ca\(^{2+}\) concentrations could be also toxic for other vital cell functions.

Acknowledgments—We thank Dr. James Hurley and Greg Niemi (University of Washington) for the electrospray mass-spectrometry analysis of the recombinant GCAPs. We are also grateful to the anonymous reviewer for stimulating criticism.

REFERENCES

1. Payne, A. M., Downes, S. M., Bessant, D. A. R., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattachraya, S. S. (1998) *Hum. Mol. Genet.* 7, 273–277

2. Shyjan, A. W., DeSauvage, F. J., Gilett, N. A., and Lowe, D. G. (1992) *Neuron* 9, 727–737

3. Dizhoor, A. M., Lowe, D. G., Oslehevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* 12, 1345–1352

4. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, O., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 5535–5539

5. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) *Biochem. J.* 302, 455–461

6. Sitaramayya, A., Duda, T., and Sharma, R. K. (1995) *Mol. Cell. Biochem.* 148, 139–145

7. Yang, R. B., Foster, D. C., Garbers, D. L., and Fulle, H. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 602–606

8. Hayashi, F., and Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 4746–4750

9. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol. Chem.* 269, 30741–30744

10. Pugh, E. N., Jr., Duda, T., Sitaramayya, A., and Sharma, R. K. (1997) *Bioess. Rep.* 17, 429–472

11. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C., Oguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and Baehr, W. (1994) *Nature* 13, 395–404

12. Dizhoor, A. M., Oslehevskaya, E. Y., Hensel, W. J., Wong, S. C., Stults, J. T., Ankondevina, I., and Hurley, J. B. (1995) *J. Biol. Chem.* 270, 25200–25206

13. Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995) *J. Biol. Chem.* 270, 22029–22036

14. Baylor, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 560–565

15. Koutalos, Y., and Yau, K.-W. (1996) *Trends Neurosci.* 19, 73–81

16. Koch, K.-W., and Stryer, L. (1988) *Nature* 334, 64–66

17. Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* 271, 19346–19350

18. Rudnicka-Nawrot, M., Surgucheva, I., Holmes, J. D., Haeseleer, F., Sokal, I., Crabb, J. W., Baehr, W., and Palczewski, K. (1998) *Biochemistry* 37, 248–257

19. Farber, D. B., Danciger, J. S., and Aquirre, G. (1992) *Neuron* 9, 349–356

20. Pittler, S. J., and Baehr, W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 322–326

21. Perrault, I., Rozet, J. M., Calvus, P., Gerber, S., Camuzat, A., Dolfias, H., Chatelin, S., Souied, E., Ghazi, I., Leoswaki, C., Bonnemaison, M., Le Paolier, D., Prezelj, J., Dufier, J. L., Pittler, S., Munnich, A., and Kaplan, J. (1996) *Nature* 14, 461–464

22. Semple-Rowland, S. L., Gorczyca, W. A., Buczylko, J., Helekar, B. S., Ruiz, C. C., Subbaraya, I., Palczewski, K., and Baehr, W. (1996) *FEBS Lett.* 383, 47–52

23. Frins, S., Bonick, W., Muller, F., Kellner, R., and Koch, K.-W. (1996) *J. Biol. Chem.* 271, 8022–8027

24. Oslehevskaya, E. V., Hughes, R. E., Hurley, J. B., and Dizhoor, A. M. (1997) *J. Biol. Chem.* 272, 14327–14333

25. Rocque, W. J., McWherter, C. A., Wood, D. C., and Gordon, J. I. (1993) *J. Biol. Chem.* 268, 9964–9971

26. Horton, R. M, Pease, L. R. (1991) in *Directed Mutagenesis: A Practical Approach* (McPherson, M. J., ed) pp. 217–250, Oxford University Press, Oxford

27. Tsien, R., and Poxan, T. (1989) *Methods Enzymol.* 172, 230–262

28. Surguchev, A., Bronson, J. D., Banerjee, P., Knowles, J. A., Ruiz, C., Subbaraya, I., Palczewski, K., and Baehr, W. (1997) *Genomics* 39, 312–322

29. Otto-Bruc, A., Faris, R. H., Haeseleer, F., Huang, J., Buczylko, J., Surgucheva, I., Subbaraya, I., Milam, A. H., and Palczewski, K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 4727–4732

30. Howes, K., Bronson, J. D., Dang, Y. L., Li, N., Zhang, K., Rouz, C., Helekar, B., Lee, M., Subbaraya, I., Kolb, H., Chen, J., and Baehr, W. (1998) *Invest. Ophthal. Vis. Sci.* 39, 867–875

31. Cuenc, N., Wall, S., Lopez, S., Howes, K., Baehr, W., and Kolb, H. (1997) *Invest. Ophthal. Vis. Sci.* 38, S21

32. Kachi, S., Dizhoor, A., Nishizawa, Y., Watanabe, N., Yamazaki, A., and Usukura, J. (1998) *Invest. Ophthal. Vis. Sci.* 39, S1051