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Activation of Bovine Photoreceptor Guanylate Cyclase by S100 Proteins

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S100 proteins are acidic calcium-binding proteins present in high concentration in the brain and at lower concentrations in peripheral tissues. Their function remains to be elucidated in many tissues. In this report we show for the first time that S100 proteins stimulate the bovine photoreceptor membrane guanylate cyclase. The extent of stimulation varied between the different S100 proteins with the S100b exhibiting the highest level of influence. The stimulation by all S100 proteins was calcium dependent, with the half-maximal stimulation occurring at about 35–40 μM calcium. These results suggest that in some cells S100 proteins can mediate calcium signals via cyclic GMP.

S100 refers to a family of acidic proteins which were first discovered in brain (1) and subsequently found in lower concentrations in many other tissues (2,3). These proteins are made up of two subunits, α and β, which may exist as monomers, homologous or heterologous dimers, and oligomers. Dimers of the composition $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ are designated as S100 a_0, S100a and S100b respectively (4–6). Both S100-α and S100-β bind calcium and contain two EF-hand calcium-binding sites each (4,7). Further, both subunits bind zinc, though the affinity of the β subunit for zinc is higher than for calcium (8).

S100 proteins were found to have both extracellular and intracellular effects. S100b was shown to have neurite extension and neurotrophic activities in select neurons (9), and mitogenic activity in glial cells (10). Among the intracellular effects are calcium-dependent activation of aldolase by both S100-α and S100-β (11) and calcium-dependent inhibition of the phosphorylation of the microtubule-associated τ protein (12) and other specific proteins in brain by S100b (13). The adenylate cyclase activity of skeletal muscle is inhibited by S100b (14) and stimulated by $S100a_0$ (15). There has been only one report on the effect of S100 proteins on guanylate cyclase: the enzyme in Tetrahymena is unaffected by S100, though it is stimulated by another calcium-binding protein, calmodulin (16).

It was hitherto thought that retinal photoreceptor membrane guanylate cyclase is stimulated by a class of calcium-binding proteins termed guanylate cyclase activating proteins (GCAPs) which stimulate cyclase at calcium concentrations below 250 nM (17,18). However, we observed recently that this enzyme is dual regulated: besides stimulation by GCAPs at low calcium concentrations, it can also be stimulated at micromolar calcium concentrations by a protein (calcium-dependent-guanylate cyclase activator protein or CD-GCAP) isolated from bovine retina (19). The subunit size of CD-GCAP, as determined by SDS-PAGE, is 6–7 kDa, similar to that of S100 protein subunits (9), and like them, it is also a calcium-binding protein. In view of these similarities, we tested the effects of S100 proteins on photoreceptor guanylate cyclase. In this report we show that S100 proteins activate guanylate cyclase and that the activation is calcium-dependent.

MATERIALS AND METHODS

Materials. (^{32}P)GTP was purchased from ICN Biomedicals. $S100a_0$ and S100a were purchased from Sigma and S100b was from Calbiochem. Anti-S100-α and anti-S100-β monoclonal antibodies were obtained from Sigma.

Isolation of rod outer segment membranes enriched in guanylate cyclase activity. All operations were conducted under

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infrared light. Fresh bovine eyes were obtained from Wolverine Packing Co., Detroit, MI, dark adapted for 3–4 hours at room temperature, and intact rod outer segments (ROS) were isolated according to the method of Schnetkamp et al (20). To prepare ROS membranes free of soluble and peripheral proteins, intact ROS were disrupted by freezing and thawing, and suspended in and washed three times with 10 mM Tris-HCl (pH 7.5) at 0.1 mg of protein per milliliter. The washed membranes were monitored for cGMP phosphodiesterase activity (21), and guanylate cyclase activities in the presence of either 2 mM EGTA or 1 mM $CaCl_2$. Only the preparations which did not exhibit phosphodiesterase activity and whose cyclase activity was not influenced by EGTA or Ca were used in this study.

In some experiments guanylate cyclase partially purified from ROS membranes was used instead of the membranes. The purification of cyclase by GTP-agarose chromatography was done according to previously published procedures (21).

S100 proteins. S100 proteins were dissolved (0.5 mg of protein per ml) in 50 mM Tris-HCl, pH 6.8 containing 50 mM NaCl and concentrated to 0.2 ml in Centricon-10 (Amicon). To remove traces of EGTA present in commercial protein preparations the solution was diluted in the above buffer to 2.0 ml and concentrated again to 0.2 ml. After one more dilution and concentration, the preparations were tested for effects on cyclase activity.

Guanylate cyclase assays. The assays were done under infrared light. Ten μl of suitably diluted membranes were assayed in a reaction mixture of 40 μl. The guanylate cyclase reactions were started with the addition of 10 μl of a substrate mixture giving the following final concentrations in the assay: 40 mM Hepes, pH 7.4, 15 mM $MgCl_2$, 2 mM cyclic GMP, 1.25 mM IBMX, 4 mM dithiothreitol, 1 mM GTP, and about 6 μCi of (α-^{32}P)GTP. After 10 min at 30°C, the reactions were terminated with the addition of 20 μl of 150 mM EDTA containing 2 mM each of GTP, cyclic GMP and GMP. The tubes were left in an 90°C water bath for 5 min, cooled, and centrifuged. The (^{32}P)cyclic GMP formed in the assay was separated and measured as described earlier (22).

To measure if guanylate cyclase activity was influenced by the addition of S100 proteins, the assays were supplemented with the desired concentration of test protein and 1 mM $CaCl_2$ or 2 mM EGTA. In some experiments, the assays were run in the presence of the S100 protein at several different concentrations of calcium.

RESULTS AND DISCUSSION

As shown in Figure 1, the basal guanylate cyclase activity in washed ROS membranes was similar in the presence of 1 mM $CaCl_2$ and 2 mM EGTA indicating that the soluble protein which activates the cyclase at low calcium concentrations (17,18) was completely removed by the washing of the membranes. When incubated with 7 μM S100b, the cyclase activity of the membranes increased to 600–800% of the control, and the increase was seen only in the presence of calcium. At the same concentration, S100a_0 increased the activity to 300–350% of control, and S100a increased it to 400–450% of control and again, in both cases, the increase was observed only in the presence of calcium. The calcium-dependent activation of guanylate cyclase by S100b appeared to be direct and not mediated by other proteins because partially purified cyclase was activated as well as the cyclase in ROS membranes (data not shown). Since the activation by S100a_0 was much lower than that by S100b, we tested the possibility that at least a fraction of the activation was due

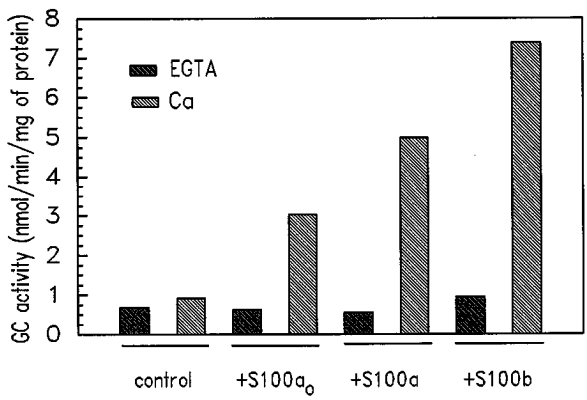

FIG. 1. Influence of S100 proteins on guanylate cyclase activity. Washed ROS membranes were assayed for guanylate cyclase activity in the absence (control) or presence of different S100 proteins. In each case assays were done in the presence of either 1 mM $CaCl_2$ or 2 mM EGTA. The concentration of S100 proteins in the assay was 7 μM. The data presented here are from one of three experiments with similar results.

to contamination by the latter. Western blot analysis of $S100a_0$ using antibodies specific for S100-β revealed no staining (data not shown). Conversely, S100b reacted only with S100-β antibody and not with antibodies specific for S100-α. These results suggest that is spite of the differences in the level of activation, the different S100 proteins are capable of activating the cyclase independently.

Since S100b was the most active of the three proteins, further studies reported here were focused on S100b. Figure 2 shows the dependence of cyclase stimulation on the concentration of S100b in the assay. Half maximal stimulation was observed at about 2 μM concentration and maximal stimulation at about 20 μM. Similar dose-dependence pattern was observed with S100a also. These concentrations are well within the range of concentrations at which S100 proteins were reported to influence enzymatic reactions in other tissues. For example, half-maximal activation of skeletal muscle adenylate cyclase by $S100a_0$ is at 0.2 μM (15). Half-maximal influence of S100 proteins on phosphorylation of specific proteins in brain is at 0.6 μM (23) and 4.0 μM (24). The concentrations at which S100 proteins stimulated cyclase activity were also within the 10–20 μM concentrations at which they are reported to be present in brain (5). The current studies were done on the membranes from retinal photoreceptor cells and the concentration of S100 proteins in retina is still unknown. However, that S100 proteins are present in retina is well established (25,26). Species-specific differences in the distribution of S100 proteins between retinal glial and neuronal cells were reported (26,27). While the experiments discussed here were done on a neuronal cyclase, it is likely that S100 proteins may activate specific cyclases in glial cells also. Further work is required to identify the distribution of S100-sensitive cyclases in other tissues. It may be mentioned that the photoreceptor guanylate cyclase, activated by S100 proteins as shown here, is not activated by calmodulin (19) whereas the Tetrahymena guanylate cyclase was reported to be activated by calmodulin but not by S100 proteins (16).

S100 proteins are known to have two calcium binding sites per subunit with the K_d for calcium of 20–50 μM for one site and about 200–500 μM for the other (4). To test which of these sites might be mediating the calcium-dependent activation of guanylate cyclase, cyclase activity was measured in the presence of 7 μM S100b and different concentrations of calcium. As shown in Figure 3, half maximal activation was observed at about 40–50 μM calcium. Similar results were obtained for calcium dependence of the activation by $S100a_0$ (data not shown). These results suggest that of the two calcium binding sites in S100 proteins, the one with the higher affinity for calcium may be involved in the activation of cyclase. S100b is both a calcium-binding protein and a zinc-binding protein (28). The effects of S100b on myelin ATPase activity (29) and phosphorylation of τ protein (12) were potentiated by zinc. We therefore tested the effect of zinc chloride

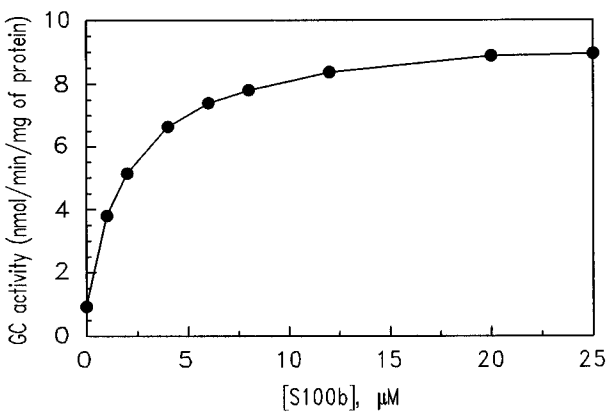

FIG. 2. Dependence of guanylate cyclase activation on the concentration of S100b. Guanylate cyclase activity in washed ROS membranes was assayed in the presence of 1 mM $CaCl_2$ and the specified concentrations of S100b. Data shown are from one of three experiments with similar results.

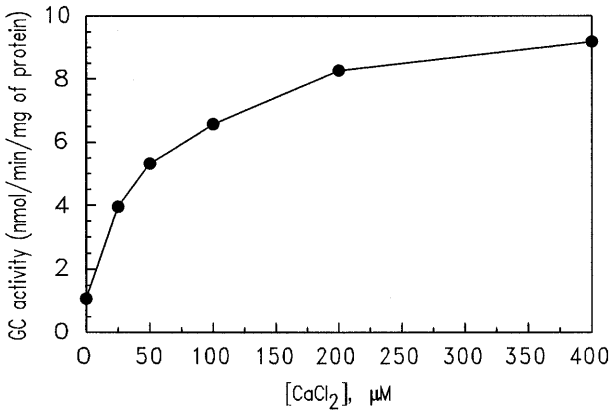

FIG. 3. Dependence of guanylate cyclase activation on calcium concentration. Guanylate cyclase activity in washed ROS membranes was assayed in the presence of 7 μM S100b and the specified concentration of $CaCl_2$. Similar calcium dependence was observed for $S100a_0$ also.

on the activation of guanylate cyclase by S100b. In this case, however, zinc strongly inhibited the activation, but the inhibition was evident only when dithiothreitol was not present in the assays. Half-maximal inhibition was observed at 30–35 μM and full inhibition at about 100 μM zinc (data not shown). Basal cyclase activity was also inhibited by zinc. The inhibition of both basal and stimulated cyclase activities was completely overcome by 10 mM dithiothreitol. Inhibition of lung soluble guanylate cyclase by zinc and its reversal by dithiothreitol were reported earlier (30).

The calcium concentrations at which S100 proteins activated photoreceptor guanylate cyclase are much higher than the concentration at which CD-GCAP activated the enzyme (19) but are well within the calcium concentrations likely to be achieved in localized areas of synapses during transmitter release (31,32). S100 proteins were reported to be present in retinal and brain synaptosomes (33,34), and in retina they were measured in relatively higher concentration in ganglion cell and nerve fiber layers (25). It is therefore possible that S100 proteins activate guanylate cyclase in the retinal synaptic layers in response to a rise in calcium concentration. In this context, it is significant that photoreceptor membrane guanylate cyclase was reported to be present not only in the outer segments but also in retinal synaptic layers (35,36). S100 protein-mediated increase in cyclic GMP may therefore have a role in the modulation of synaptic transmission.

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REFERENCES

1. Moore, B. W. (1965) *Biochem. Biophys. Res. Comm.* **19,** 739–744.
2. Zimmer, D. B., and Van Eldik, L. J. (1987) *Am. J. Physiol.* **252,** c285–c289.
3. Suzuki, F., Nakajima, T., and Kato, K. (1982) *J. Biochem.* **92,** 835–838.
4. Hilt, D. C., and Kligman, D. (1991) *in* Novel Calcium-Binding Proteins (Heizmann, C. W., Ed.), pp. 65–103, Springer-Verlag, New York.
5. Donato, R. (1986) *Cell Calcium* **7,** 123–145.
6. Van Eldik, L. J., and Zimmer, D. B. (1988) *in* Calcium and Calcium Binding Proteins (Gerday, C. L., Gilles, R., and Boils, L., Eds.), pp. 114–127, Springer-Verlag, Berlin, Heidelberg.
7. Szebenyi, D.M. E., Obendorf, S. K., and Moffat, K. (1981) *Nature* **294,** 327–332.
8. Baudier, J., Glasser, N., and Gerard, D. (1986) *J. Biol. Chem.* **261,** 8192–8203.
9. Kligman, D., and Marshak, D. (1985) *Proc. Natl. Acad. Sci. USA,* **82,** 7136–7139.
10. Selinfreud, R. H., Barger, S. W., Pledger, W. J., and Van Eldik, L. J. (1991) *Proc. Natl. Acad. Sci. USA,* **88,** 3554–3558.
11. Zimmer, D. B., and Van Eldik, L. J. (1986) *J. Biol. Chem.* **261,** 11424–11428.
12. Baudier, J., and Cole, R. D. (1988) *J. Biol. Chem.* **263,** 5876–5883.

13. Patel, J., and Marangos, P. J. (1982) *Biochem. Biophys. Res. Commun.* **109,** 1089–1093.
14. Fano, G., Fulle, S., Della Torre, G., Giambanco, I., Aisa, M. C., Donato, R., and Calissano, P. (1988) *FEBS Lett.* **240,** 177–180.
15. Fano, G., Angelella, P., Mariggio, D., Aisa, M. C., Giambanco, I., and Donato, R. (1989) *FEBS Lett.* **248,** 9–12.
16. Kudo, S., Muto, Y., Inagaki, M., Hidaka, H., and Nozawa, Y. (1985) *Comp. Biochem. Physiol.* **80B,** 495–498.
17. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* **12,** 1345–1352.
18. Gorczyca, W. A., Gray-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994) *Proc. Natl. Acad. Sci. USA.* **91,** 4014–4018.
19. Pozdnyakov, N., Yoshida, A., Cooper, N.G. F., Margulis, A., Duda, T., Sharma, R. K., and Sitaramayya, A. (1995) *Biochemistry* **34,** 14279–14283.
20. Schnetkamp, P.P. M., Klompmakers, A. A., and Daemen, F.J. M. (1979) *Biochim. Biophys. Acta* **552,** 379–389.
21. Sitaramayya, A., Duda, T., and Sharma, R. K. (1995) *Molec. Cell. Biochem.* **148,** 139–145.
22. Sitaramayya, A., Lombardi, L., and Margulis, A. (1993) *Visual Neurosci.* **10,** 991–996.
23. Patel, J., Marangos, P. J., Heydorn, W. E., Chang, G., Verma, A., and Jacobowitz, D. (1983) *J. Neurochem.* **41,** 1040–1045.
24. Sheu, F.-S., Azmitia, E. C., Marshak, D. R., Parker, P. J., and Routtenberg, A. (1994) *Molec. Brain Res.* **21,** 62–66.
25. Moore, B. W., , Perez V., and Gehring, M. (1968) *J. Neurochem.* **15,** 265–272.
26. Linser, P., and Moscona, A. A. (1981) *Dev. Neurosci.* **4,** 433–441.
27. Terenghi, G., Cocchia, D., Michetti, F., Diani, A. R., Peterson, T., Cole, D. F., Bloom, S. R., and Polak, J. M. (1983) *Invest. Ophthal. Vis. Sci.* **24,** 976–984.
28. Baudier, J., Haglid, K., Haiech, J., and Gerard, D. (1983) *Biochem. Biophys. Res. Commun.* **114,** 1138–1146.
29. Simonian, A., Baudier, J., and Haglid, K. G. (1989) *Neurochem. Res.* **14,** 761–764.
30. Hardman, J. G., and Sutherland, E. W. (1969) *J. Biol. Chem.* **244,** 6363–6370.
31. Smith, S. J., and Augustine, G. J. (1988) *Trends Neurosci.* **11,** 458–464.
32. Heidelberger, R., Heinemann, C., and Neher, E., and Matthews, G. (1994) *Nature* **371,** 513–515.
33. Haglid, K., Hamberger, A., Hansson, H-A., Hyden, H., Persson, L., and Ronnback, L. (1974) *Nature* **251,** 532–534.
34. Starostina, M. V., Nikolaenkova, A. A., Malup, T. K., Korochkin, L. I., and Sviridov, S. M. (1993) *Cell. Mol. Neurobiol.* **13,** 677–691.
35. Liu, X., Seno, K., Nishizawa, Y., Hayashi, F., Yamazaki, A., Matsumoto, H., Wakabayashi, T., and Usukura, J. (1994) *Exp. Eye Res.* **59,** 761–768.
36. Yoshida, A., Cooper, N.G. F., Liu, L., Pozdnyakov, N., and Sitaramayya, A. (1995) *Invest. Ophtal. Vis. Sci.* **36,** S270.