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TERBIUM BINDING TO NEOPLASTIC GH3 PITUITARY CELLS

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The fluorescent properties of terbium (Tb^{3+}) were used to study the calcium (Ca^{2+}) binding sites on GH3 pituitary tumor cells. The fluorescence emission of Tb^{3+} was enhanced with the binding of GH3 cells, accompanied by a red shift in its excitation maximum to resemble the excitation peak of the native cell fluorescence. The Tb^{3+} fluorescence enhancement increased with increasing concentrations of GH3 cell protein. Scatchard plots revealed at least two classes of Tb^{3+} binding sites on GH3 cells. The low and high affinity binding sites have apparent dissociation constants equal to 0.56 mM and 11 μM, respectively. The high affinity Tb^{3+} binding was displaced by Ca^{2+}, but the more abundant low affinity site was not sensitive to Ca^{2+}. The data suggest that GH3 cells possess a specific Ca^{2+} binding receptor on their plasma membrane.

The importance of Ca^{2+} in stimulus-secretion coupling is unquestioned (1-15). In the presence of extracellular Ca^{2+}, thyrotropin-releasing hormone (TRH) or depolarizing concentrations of K^{+} have been reported to stimulate the release of prolactin from neoplastic GH3 pituitary cells (3-13). A detailed molecular description of the involvement of Ca^{2+} in stimulus-secretion coupling is not easy to obtain because Ca^{2+} has few spectroscopic and physical properties that are suitable for probing its environment (16-18). Fortunately Tb^{3+} possess qualities which makes it an excellent probe for studying the interactions of Ca^{2+} with GH3 pituitary cells (17,19). Tb^{3+} has been successfully used to study Ca^{2+} receptor sites on biological membranes (16-18, 20-25). The fluorescence intensity of Tb^{3+} undergoes a significant enhancement when it binds to biomembranes (16, 25). The enhanced fluorescence emission of the bound Tb^{3+} is sensitized by resonant energy transfer from nearby aromatic residues in the receptor which are directly excited by ultra-

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violet radiation. In this report we describe the binding of Tb^{3+} to the plasma membrane of GH3 pituitary cells.

MATERIALS AND METHODS

Terbium chloride ($TbCl_3.6H_2O$) was obtained from Aldrich Chemical Co., and terbium chloride stock solutions were made with double-distilled water. All other solutions were made with sterile Hepes buffered saline containing 0.87 percent NaCl in 0.01 M Hepes (N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid) (Sigma Chemical Co.) buffer; the pH was adjusted to 7.4 with 12 N NaOH. All chemicals were of analytical quality and were used without further purification.

The GH3 pituitary tumor cells were purchased from American Type Culture Collection, Rockville, Md. The cells were grown as a monolayer culture, on a wet polylysine (20 mg/ml) coated surface, in 75-cm^2 plastic flasks (Falcon) containing Ham's F-10 medium supplemented with 15 percent horse serum and 2.5 percent fetal bovine serum at 37°C in a humidified atmosphere of 90 percent air and 10 percent CO_2. Penicillin and streptomycin were added to the culture medium at a final concentration of 50 U/ml and 50 µg/ml, respectively. All experiments were performed on subcultures having a low number of serial passages from tissue of origin. Four to six hours prior to each experiment, the culture medium was removed from the flasks, the cells were harvested and washed 3 times with cold (4°C) Hepes buffered saline. The final pooled pellet was gently resuspended in Hepes buffered saline, filtered through a mesh and placed on ice. The protein concentrations were determined by the Bio-Rad protein assay, according to the method of Bradford (26), using bovine plasma albumin as standard.

The fluorescence excitation and emission measurements were made on an Aminco-Bowman Spectrophotofluorometer with Ellipsoidal Condensing System, using 1x1-cm quartz cuvettes. The excitation and emission wavelengths were 280 nm and 543 nm, respectively. In order to eliminate error due to light scattering and interferences due to the native fluorescence of the cells, the Tb^{3+} fluorescence was followed in conjunction with a 455 nm long wavelength pass filter. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

Figure 1 shows the excitation and emission spectra of Tb^{3+} bound to GH3 pituitary tumor cells. The binding of Tb^{3+} to the GH3 cells induced a shift in its excitation maximum from 267 nm to 284 nm, which resembles the excitation peak of the natural cell fluorescence (at 285 nm). The value of the 284nm/267nm excitation intensity ratio for the Tb^{3+}-GH3 complex (1.46) was 330 percent greater than that of free Tb^{3+} (0.34), indicating that energy was transferred from nearby aromatic residues to the bound metal ions (25,27). DeJersey et al (27) have reported that the 292nm/276nm ratio, R, may be used to diagnose the aromatic chromophore responsible for transferring energy to Tb^{3+}. The value of R in Figure 1 is equal to 1.03 and is indicative of tryptophan as the energy donor. Since our excitation spectrum of the

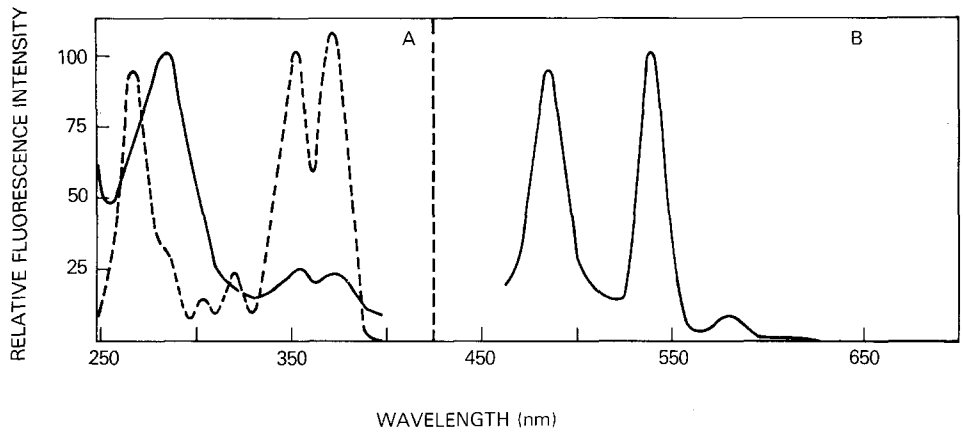

Figure 1 Uncorrected fluorescence excitation (A) and emission (B) spectra of Tb^{3+} bound to GH3 pituitary tumor cells in 0.01M Hepes buffered saline at pH 7.4 (solid lines). Note: In this experiment, the sample solution did not contain any unbound excess Tb^{3+} because the labelled GH3 cells were first centrifuged, then the pellet was resuspended in Tb^{3+} free Hepes buffered saline. Superimposed on the excitation spectrum of the Tb^{3+}-GH3 complex is an excitation spectrum of free Tb^{3+} alone in solution (dashed line). λ_{ex} = 280 nm, λ_{em} = 543 nm. Slit widths: ex = 5.5 nm, em = 11 nm.

Tb^{3+}-GH3 complex was obtained without correction for the wavelength dependence of the xenon arc lamp intensity, the reliability of 1.03 as the correct numerical value for R must be questioned (28). In any case, the wavelength of maximum fluorescence emission for unlabelled GH3 cells (at 340 nm) was similar to that of tryptophan. The emission spectrum of the Tb^{3+}-GH3 complex revealed the characteristic Tb^{3+} fluorescence quartet at approximately 488, 543, 584 and 619 nm.

Figure 2 illustrates the enhancement of the Tb^{3+} fluorescence brought about by its binding to GH3 pituitary cells. The relative fluorescence intensity of free Tb^{3+} at 0 µg/ml GH3 protein was almost non-detectable or very low; but, the addition of GH3 cells to the solution markedly enhanced the fluorescence emission. The relative fluorescence intensity of Tb^{3+} increased as the concentration of GH3 cells increases to 8 and 32 µg/ml protein for 10^{-4}M and 10^{-3}M $TbCl_3$, respectively. The fluorescence intensities for 10^{-3}M $TbCl_3$ were enhanced to a greater net extent than those for 10^{-4}M $TbCl_3$, in that the slope of the line between 0 and 8 µg/ml GH3 protein for 10^{-3}M $TbCl_3$ was approximately five times greater than that for 10^{-4}M

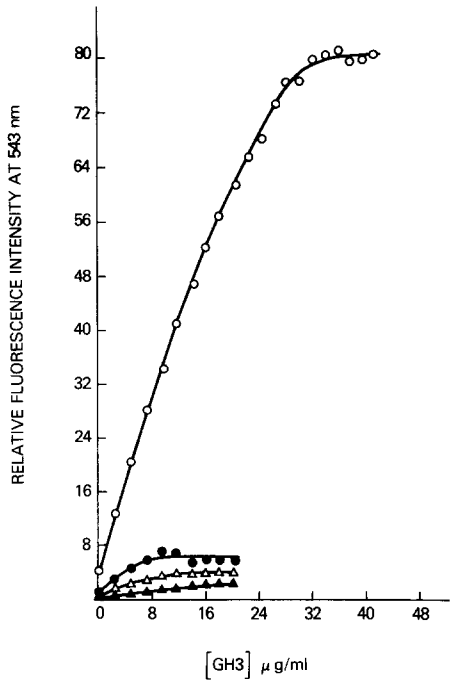

Figure 2 Enhancement of Tb^{3+} fluorescence as a function of GH3 protein concentration. 20 μl aliquots of GH3 cell suspension at 4.9 μg/ml protein were added to 2.0 ml of either $1x10^{-3}$M $TbCl_3$ (opened circles), $1x10^{-4}$M $TbCl_3$ (closed circles) or $1x10^{-5}$M $TbCl_3$ (opened triangles) in 0.01M Hepes buffered saline at pH 7.4; and, a buffer control solution (closed triangles).

$TbCl_3$. Since the magnitude of the Tb^{3+} fluorescence enhancement is dependent on the distance between the energy donor and acceptor pair, the different slopes may reflect structural differences between the various Tb^{3+} binding sites on GH3 cells (29). Alternatively, the slopes may reflect different quantum yields at the various Tb^{3+} binding sites; especially, since the dominant mode of radiationless deactivation of the excited Tb^{3+} ion is via loss of energy to high-frequency hydrogen stretch vibrations in its local environment, and the loss of energy is proportional to the number of hydrogen vibrations which may vary among the different Tb^{3+} binding sites (30). Also, it may be possible that at one type of site more than one tryptophan residue is transferring energy to the bound Tb^{3+}.

The binding characteristics of Tb^{3+} to GH3 pituitary cells were determined by a fluorometric titration procedure. When increasing concentrations

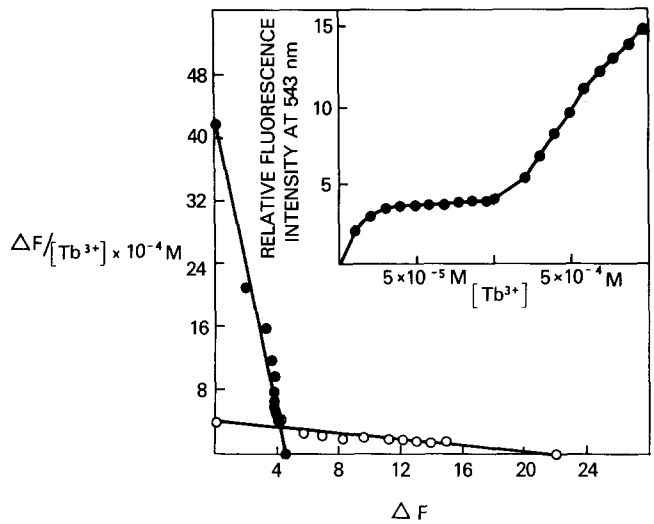

Figure 3 Eadie-Scatchard plot of Tb^{3+} binding to GH3 pituitary cells. Ordinate: the change in fluorescence intensity at 543 nm divided by the total Tb^{3+} concentration, multiplied by 10^{-4} M. Abscissa: the corresponding change in fluorescence intensity at 543 nm. Inset: Tb^{3+}-GH3 fluorescence intensity at 543 nm as a function of the $TbCl_3$ concentration. 10 µl aliquots of $2x10^{-3}$M and $2x10^{-2}$M $TbCl_3$ were added to 2.0 ml of GH3 cells at 5.1 µg/ml protein in 0.01M Hepes buffered saline at pH 7.4 (OD_{280} = 0.05). Throughout the titration the concentration of free Tb^{3+} was assumed to be equal to the total concentration of $TbCl_3$ added to the suspension.

of $TbCl_3$ were added to a GH3 cell suspension, the fluorescence intensity at 543 nm increased, then gradually tapered into a plateau at 4 x 10^{-5}M $TbCl_3$, but above 1 x 10^{-4}M $TbCl_3$ the intensity began to increase rapidly (Fig. 3, inset). The fact that the fluorescence became saturated at a low $TbCl_3$ concentration, is suggestive of Tb^{3+} binding to a high affinity site. It is clear, from the Eadie-Scatchard plot shown in Fig. 3, that the binding of Tb^{3+} to GH3 cells involves at least two classes of binding sites. The apparent dissociation constant, K_d, for Tb^{3+} binding to the high affinity site was estimated to be 1.1 x 10^{-5}M and the K_d for the low affinity site was 5.6 x 10^{-4}M (the slope = $-1/K_d$). The value of K_d for the low affinity site was more reproducible than that of the high affinity site. Possibly, an aromatic residue may be attached in the first coordination sphere of Tb^{3+} at the low affinity site resulting in efficient energy transfer. Whereas, the binding of Tb^{3+} to the high affinity site may only be in

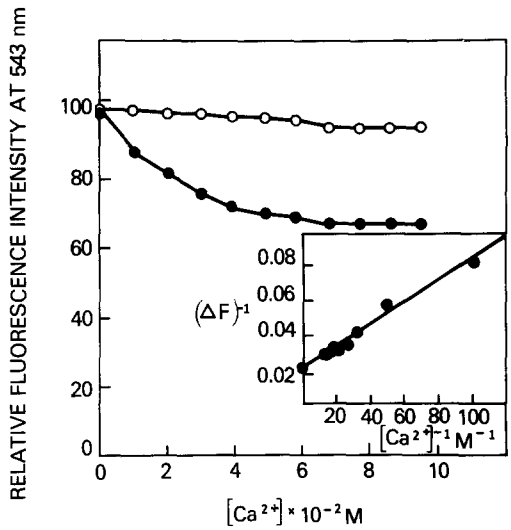

Figure 4 Effects of Ca^{2+} concentration on the fluorescence emission of Tb^{3+} at the low affinity (opened circles) and high affinity (closed circles) binding sites on GH3 cells. 10 µl aliquots of 2.0 M $CaCl_2$ were added to 2.0 ml of either $2x10^{-3}$M or $5x10^{-5}$M $TbCl_3$ with GH3 cells in 0.01M Hepes buffered saline at pH 7.4. The fluorescence intensities are expressed relative to the value in the absence of Ca^{2+}. Inset: The fluorescence intensities of the high affinity Tb^{3+}-GH3 complex plotted against the Ca^{2+} concentration in a double reciprocal Lineweaver-Burk type plot. The change in fluorescence intensity is expressed as the value of (I_0-I_c), where I_0 and I_c are the intensities of the high affinity Tb^{3+} binding site before and after Ca^{2+} titration, respectively.

the neighborhood of an aromatic group which would lower the efficiency of energy transfer (31). The lower relative fluorescence enhancement of the high affinity site presumably reflects a longer distance between the bound Tb^{3+} and the aromatic side chain (29). The maximum Tb^{3+} fluorescence (the x-intercept) for each component suggests that the low affinity sites out number the high affinity sites approximately five to one. In preliminary experiments, depolarizing concentrations of K^+ were found to increase the maximum Tb^{3+} fluorescence intensity of the high affinity component, without changing its K_d, suggesting that K^+ may induce an increase in the number of high affinity Tb^{3+} binding sites available (data not shown). The maximum fluorescence and K_d of the low affinity component were not affected by depolarizing concentrations of K^+. Further, the maximum Tb^{3+} fluorescence and K_d for the high and low affinity components were not affected by TRH. These

results are in agreement with those of Moriarty and Leuschen (9). Using atomic absorption spectrophotometry, they demonstrated that GH3 cells experience a net increase in cellular Ca^{2+} accumulation when exposed to high concentrations of K^{+}, while TRH exposure did not affect the cellular accumulation of Ca^{2+}.

The ability of Ca^{2+} to displace Tb^{3+} was employed to assess the binding of Tb^{3+} to Ca^{2+} receptor sites on the GH3 pituitary cells. The fluorescence intensity of the high affinity Tb^{3+} binding decreased monotonically with increasing Ca^{2+} concentration (Fig.4). In contrast, the fluorescence intensity of Tb^{3+} bound to the low affinity site remained virtually the same throughout the titration. This suggests that the low affinity site is not a specific Ca^{2+} binding site, but a nonspecific negatively charged site. However, the high affinity site is definitely a Ca^{2+} binding site. The K_d for Ca^{2+} binding to the high affinity site was estimated to be about 2.8×10^{-2}M (Fig. 4, inset). The affinity of Ca^{2+} for GH3 cells was less than that of Tb^{3+} due to the more electropositive character of Tb^{3+}; and relatively high concentrations of Ca^{2+} were required to replace Tb^{3+} from its binding sites because of the greater stability of Tb^{3+} chelates compared to Ca^{2+} chelates (18,20,29).

Tan and Tashjian (10) and Gershengorn (11,15) have demonstrated that GH3 cells have a Ca^{2+} storage system associated with their plasma membrane. The biphasic nature of Tb^{3+} binding to GH3 cells may depict specific and nonspecific Ca^{2+} receptor sites, distinguishable by differences in their apparent dissociation constant, concentration and degree of Tb^{3+} fluorescence enhancement. The function of the high affinity Tb^{3+} binding site is not known; but, it is tempting to suggest that this Tb^{3+} binding receptor may be intimately associated with the activation of ion channels. Dufy and Barker (14) have suggested that the activation of the membrane K^{+} conductance of GH3/B6 pituitary cells is dependent on membrane potential and calcium ions.

References

1. Douglas, W.W. (1968) Br. J. Pharmac. 34, 451-474.
2. Taraskevich, P.S. and Douglas, W.W. (1977) Proc. Natl. Acad. Sci. 74, 4064-4067.
3. Ozawa, S. (1980) Biomed. Res. 1, 140-143.
4. Ozawa, S. and Kimura, N. (1979) Proc. Natl. Acad. Sci. 76, 6017-6020.
5. Ozawa, S. (1981) Brain Res. 209, 240-244.
6. Dufy, B., Vincent, J.D., Fleury, H., Du Pasquier, P., Gourdji, D. and Tixier-Vidal, A. (1979) Science 204, 509-511.
7. Gautvik, K.M. and Tashjian, A.H. (1973) Endocrinology 93, 793-799.
8. Tashjian, A.H., Lomedico, M.E. and Maina, D. (1978) Biochem. Biophys. Res. Commun. 81, 798-806.
9. Moriarty, C.M. and Leuschen, M.P. (1981) Am. J. Physiol. 240, E705-E711.
10. Tan, K-N. and Tashjian, A.H. (1981) J. Biol. Chem. 256, 8994-9002.
11. Gershengorn, M.C. (1980) J. Biol. Chem. 255, 1801-1803.
12. Ozawa, S. and Miyazaki, S. (1979) Jap. J. Physiol. 29, 411-426.
13. Drust, D.S. and Martin, T.F.J. (1982) J. Biol. Chem. 257, 7566-7573.
14. Dufy, B. and Barker, J.L. (1982) Life Sciences 30, 1933-1941.
15. Rebecchi, M.J., Kolesnick, R.N. and Gershengorn, M.C. (1982) Clin. Res. 30, 493A.
16. Mikkelsen, R.B. (1976) Biol. Memb. 3, 153-190.
17. Martin, R.B. and Richardson, F.S. (1979) Quart. Rev. Biophys. 12, 181-209.
18. Breer, H. and Jeserich, G. (1981) J. Neurochem. 37, 276-282.
19. Wallace, R.W., Tallant, E.A., Dockter, M.E. and Cheung, W.Y. (1982) J. Biol. Chem. 257, 1845-1854.
20. Mikkelsen, R.B. and Wallach, D.F.H. (1974) Biochim. Biophys. Acta 363, 211-218.
21. Morris, S.J. and Schober, R. (1977) Eur. J. Biochem. 75, 1-12.
22. Ohyashiki, T. Chiba, K. and Mohri, T. (1979) J. Biochem. 86, 1479-1485.
23. Deschenes, R.J., Hilt, D.C., Marquis, J.K. and Mautner, H.G. (1981) Biochim. Biophys. Acta 641, 166-172.
24. Deschenes, R.J., Mautner, H.G. and Marquis, J.K. (1981) Biochim. Biophys. Acta 649, 515-520.
25. Canada, R.G. (1982) Biochim. Biophys. Acta 720, 371-377.
26. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
27. DeJersey, J., Jeffers-Morley, P. and Martin, R.B. (1981) Biophys. Chem. 13, 233-243.
28. Jeffers-Morley, P., Martin, R.B. and Boatman, S. (1981) Biochem. Biophys. Res. Commun. 101, 1123-1130.
29. Canada, R.G. (1981) Biochem. Biophys. Res. Commun. 99, 913-919.
30. Kropp, J.L. and Windsor, M.W. (1965) J. Chem. Phys. 42, 1599-1608.
31. Sherry, A.D. and Cottam, G.L. (1973) Arch. Biochem. Biophys. 156, 665-672.