Vol. 83, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1978 Pages 313-320

# STRUCTURE:FUNCTION STUDIES OF RECEPTORS FOR THYROTROPIN AND TETANUS TOXIN: LIPID MODULATION OF EFFECTOR BINDING TO THE GLYCOPROTEIN RECEPTOR COMPONENT

George Lee[1], Eduardo Consiglio[1], William Habig[2], Sherry Dyer[1], Carolyn Hardegree[2], and Leonard D. Kohn[1]

[1] Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

[2] The Bacterial Toxins Division, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20014, U.S.A.

Received May 31, 1978

SUMMARY: $^{125}$I-labeled tetanus toxin interacts with the glycoprotein component of the thyroid thyrotropin receptor when this component is in solution or when it is incorporated into a liposome. Binding can be inhibited by both unlabeled thyrotropin and tetanus toxin but not by unlabeled prolactin, glucagon, insulin, ACTH, or growth hormone; binding can also be inhibited by a purified fragment of the glycoprotein component of the receptor. Changing the phospholipid of the liposome matrix from dipalmitoyl phosphatidylcholine to dioleoyl phosphatidylcholine significantly increases the binding of $^{125}$I-TSH to the glycoprotein component of the receptor but does not affect $^{125}$I-tetanus toxin binding.

Recent studies suggest that the TSH[1/] receptor on thyroid plasma membranes is composed of two components, a glycoprotein and a ganglioside (1-11) and that both components are necessary for message transmission. Other studies show that tetanus toxin interacts with thyroid plasma membranes (12) and that the properties of this interaction closely resemble the properties of TSH interacting with the TSH receptor. In addition TSH inhibits tetanus toxin binding to thyroid membranes (11); tetanus toxin does not bind to membranes of a thyroid tumor with a TSH receptor defect (13); and tetanus toxin stimulates thyroid hyperfunction in mice (13). The implication of these results is that tetanus toxin has a receptor structure analogous to that of TSH.

In the present report we show that tetanus toxin interacts with the glyco-

[1/] Abbreviations: TSH, thyrotropin; LIS, lithium diiodosalicylate; $G_{M3}$, N-acetylneuraminylgalactosylglucosylceramide; $G_{D1b}$, galactosyl-N-acetylgalactosaminyl-[N-acetyl-neuraminyl-N-acetylneuraminyl]-galactosylglucosylceramide; DNS, 1-dimethylaminonaphthalene-5-sulfonate; PC, phosphatidylcholine; ACTH, adrenocorticotrophic hormone.

0006-291X/78/0831-0313$01.00/0

Copyright © 1978 by Academic Press, Inc.
All rights of reproduction in any form reserved.

protein component of the bovine thyroid TSH receptor when it is incorporated within an artificial bilayer (liposome) or when direct interactions are measured using solubilized preparations of the glycoprotein component. In addition, we show that alteration of the fatty acid side chains in the major phospholipid component of the lipid bilayer, phosphatidylcholine, can alter the expression of the binding activity exhibited by this glycoprotein component. The implications of these results are discussed insofar as they concern the structure and function of the tetanus toxin receptor and the ability of lipid components within the membrane to modulate receptor expression.

## MATERIALS AND METHODS

Purified preparations of bovine TSH and $^{125}$I-TSH were obtained as previously described (14, 15) as were the bovine thyroid plasma membranes (5, 15), tetanus toxin and $^{125}$I-labeled tetanus toxin. (12, 13).

Sialic acid residues on the glycoprotein component of the thyroid plasma membranes were tritiated by sequentially exposing the membranes to a mild periodate oxidation procedure and to sodium borotritide (9). Solubilization of the plasma membranes with lithium diiodosalicylate (LIS) and tryptic digestion of the solubilized receptor preparation was as previously described (5-7, 11). Purification of the fragment of the glycoprotein component of the TSH receptor used a modified procedure[2/] from that previously detailed (5, 6) but yielded a preparation with identical properties and characteristics, i.e. a singly banded component on disc gels with specific TSH binding activity.

Liposomes were prepared by a procedure similar to that previously described (9, 16, 17).with the exception that the LIS solubilized receptor preparation or gangliosides were added in 0.02 M trisacetate at pH 7.0; control liposome preparations were prepared by adding buffer alone. The final pellet was suspended in 500 µl of 0.02 M trisacetate, pH 7.0, and stored at 4°. Liposomes were quantitated using a Coulter counter, Model ZB1, with lower and upper thresholds set at 10 µm and 100 µm, respectively; liposomes were diluted prior to the assay to yield 5 X $10^5$ particles per 50 µl. The gangliosides used in this report were obtained, characterized and quantitated, as previously reported (7, 9). Synthetic dipalmitoyl DL-α-phosphatidylcholine, synthetic dioleoyl L-α-phosphatidlycholine and cholesterol were obtained from the Sigma Chemical Company.

$^{125}$I-labeled TSH and $^{125}$I-labeled tetanus toxin binding to plasma membranes was assayed using a filtration technique previously described (9-16). In addition to the agents tested for their ability to influence binding, the standard incubations contained in a 100-µl volume 20 mM Tris-acetate at pH 6.0, 0.6% bovine serum albumin, 50,000-100,000 cpm of $^{125}$I-labeled tetanus toxin or TSH (5-10nM), and either the specially noted concentration of liposomes or a concentration previously described to be within the linear phase of binding. To insure the specificity of binding and the specificity of inhibition, control incubations contained unlabeled TSH (10 µM) or tetanus toxin (7 µM) with or without membranes.

Dansyl-labeled TSH was prepared as previously described (18); dansyl-labeled

2/ Lee, G. (1978) Fed. Proc. 37, 1829.

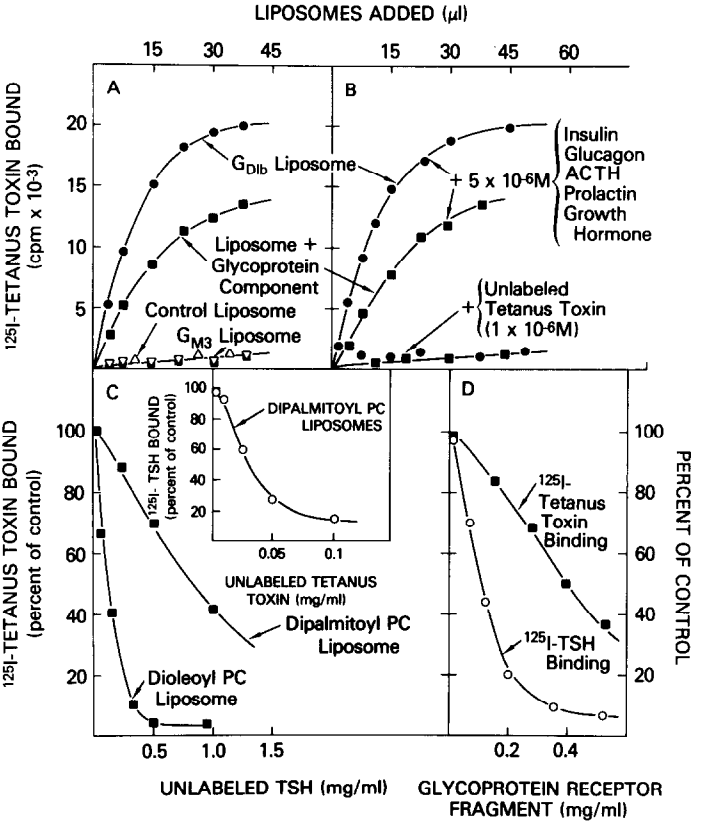


Fig. 1A Binding of $^{125}$I-tetanus toxin to control liposomes (Δ-Δ) or to liposomes containing $G_{D1b}$ (●-●); the glycoprotein component of the bovine thyroid TSH receptor (■-■); or $G_{M3}$ (▼-▼). Liposomes were formed as detailed in Materials and Methods. The glycoprotein component was obtained by solubilizing bovine thyroid membranes with 0.1M lithium diiodosalicylate (LIS) (5-7, 11); the LIS supernate was dialyzed against 3 changes of 0.02M tris acetate, pH 7.0 before incorporation. This preparation contains less than 0.5 nanamole of gangliosides per mg protein (11). The liposome preparation is analogous to that described in Table 1 for dipalmitoyl PC liposomes, i.e. contained approximately 110 μg of the LIS preparation per 9.5 μmoles of phospholipid; the liposomes contained no detectable gangliosides even when analyzed using sialic acid labeled preparations (See Table 1). The $G_{D1b}$ and $G_{M3}$ liposomes contained approximately 1.0 μmole of ganglioside per 9.5 μmoles of phospholipid (16). Binding was assayed as detailed in Materials and Methods; 50 μl of liposomes contain 5 X $10^5$ particles.

Fig. 1B Binding of $^{125}$I-tetanus toxin to the same liposomes as in Fig. 1A but in the presence of the noted unlabeled hormones or toxin.

Fig. 1C Effect of unlabeled TSH on $^{125}$I-tetanus toxin binding to liposomes containing the glycoprotein receptor component (See Fig. 1A). Liposomes were made using dipalmitoyl phosphatidylcholine or dioleoyl phosphatidylcholine as the phospholipid "matrix" (see also Table 1 and Fig. 2). The dipalmatoyl PC liposomes would be analogous to those in Fig. 1A, 1B, and 1D. The insert demonstrates the effect of unlabeled tetanus toxin on $^{125}$I-TSH binding. All experiments used 30 μl of the liposome preparation containing 3 X $10^5$ particles.

Fig. 1D Effect of a purified fragment of the glycoprotein component of the receptor on the binding of $^{125}$I-tetanus toxin (■-■) or $^{125}$I-TSH (o-o) to liposomes containing the intact glycoprotein component (see Fig. 1A). The fragment was prepared as described in Materials and Methods and references 5 and 6. Conditions were the same as in Fig. 1C.

tetanus toxin was prepared by the same procedure. As previously noted (17), dansyl-labeled TSH behaved in a fashion effectively identical to unlabeled TSH insofar as binding to membranes was concerned. Dansyl tetanus toxin also behaved like its native counterpart insofar as binding was concerned and had the same neurotoxic activity as the native toxin (unpublished data). Fluorescence measurements were made as previously described (18).

## RESULTS

Tetanus toxin, like TSH, interacts with gangliosides when they are imbedded in liposomes (Fig. 1A). Tetanus toxin can also interact with the glycoprotein component of the TSH receptor when it is incorporated into a liposome (Fig. 1A). In both cases, the binding is specific in that it is not inhibited by insulin, glucagon, ACTH, prolactin or growth hormone (Fig. 1B) whereas binding can be prevented by unlabeled tetanus toxin. Unlabeled TSH inhibits $^{125}$I-labeled tetanus toxin binding to the glycoprotein component of the TSH receptor (Fig. 1C) and unlabeled tetanus toxin inhibits $^{125}$I-TSH binding (Fig. 1C insert).

Tryptic digestion of the glycoprotein component of the TSH receptor yields a 24,000 molecular weight receptor fragment which retains specific TSH binding activity, (5, 6). As noted in Figure 1D, a purified preparation of this fragment can inhibit tetanus binding to the glycoprotein receptor component of the thyroid TSH receptor when the component is incorporated into a liposome.

When dipalmitoyl phosphatidylcholine is the phospholipid of the liposome, tetanus toxin binding is equal to or better than TSH binding (Fig. 2A). In contrast, when dioleoyl phosphatidylcholine is the phospholipid component of the liposome, TSH binding is 2-3 fold better than tetanus binding (Fig. 2B). This apparent selectivity cannot be accounted for on the basis of a difference in the amount of the glycoprotein component incorporated in the liposome nor on the basis of any difference in the size of the liposome or the number of liposomes added to the assay (Table 1 and Fig. 2). In each case, binding exhibits the same specificity as noted in Figure 1B and 1C above.

The interaction of tetanus toxin and TSH with the glycoprotein component of the bovine TSH receptor can also be monitored directly using dansyl-labeled derivatives of the toxin and hormone (Fig. 3). The interaction of dansyl-TSH with the glycoprotein component is specific in that it is not prevented by the presence

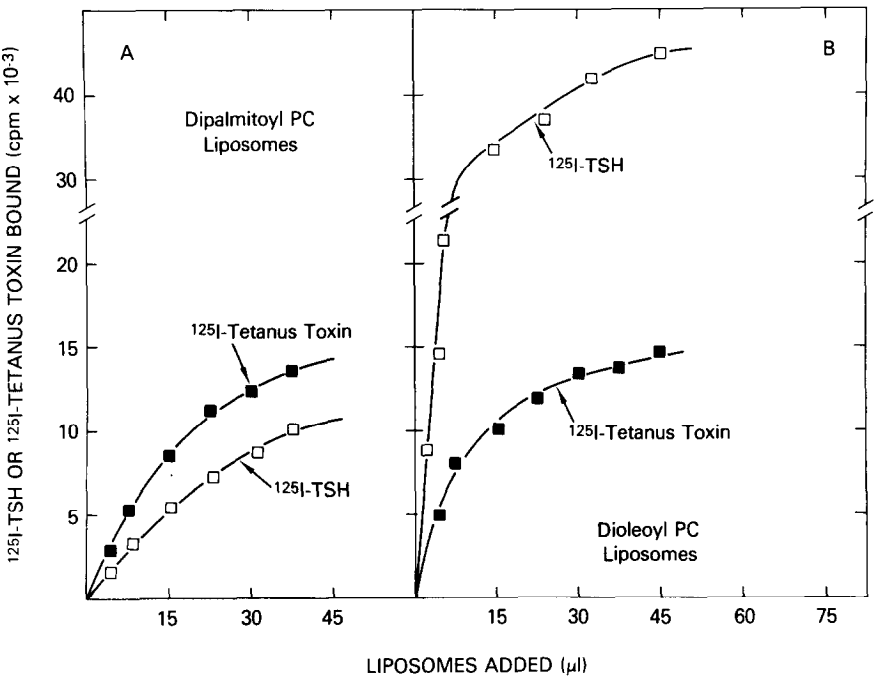


Fig. 2 $^{125}$I-TSH and $^{125}$I-tetanus toxin binding to liposomes containing the glycoprotein component of the bovine TSH receptor (See Fig. 1A for details). In A, liposomes were made with dipalmitoyl phosphatidylcholine as the phospholipid matrix; in B, it was dioleoyl phosphatidylcholine. In each case, 50 µl of the liposome preparation contained 5 X $10^5$ particles; further characterization of each liposome is summarized in Table 1.

of nondansyl-labeled prolactin, growth hormone, or insulin but can be prevented by nondansylated TSH. Of note, however, is that the dansyl TSH fluorescence change can be prevented by unlabeled tetanus toxin; that the dansyl-tetanus toxin change can be prevented by nondansyl-labeled TSH; and that nondansyl-labeled TSH can reverse the dansyl-tetanus toxin perturbation. In short, the interaction of the glycoprotein component of the TSH receptor with TSH can be prevented by tetanus toxin and, inversely, the interaction of the glycoprotein component with tetanus toxin can be prevented by TSH.

## DISCUSSION

The present report shows that tetanus toxin can interact with a glycoprotein component of the thyroid membrane whether in solution (Fig. 3) or in a lipid bilayer akin to a membrane (Fig. 1). In addition, the data indicate that the

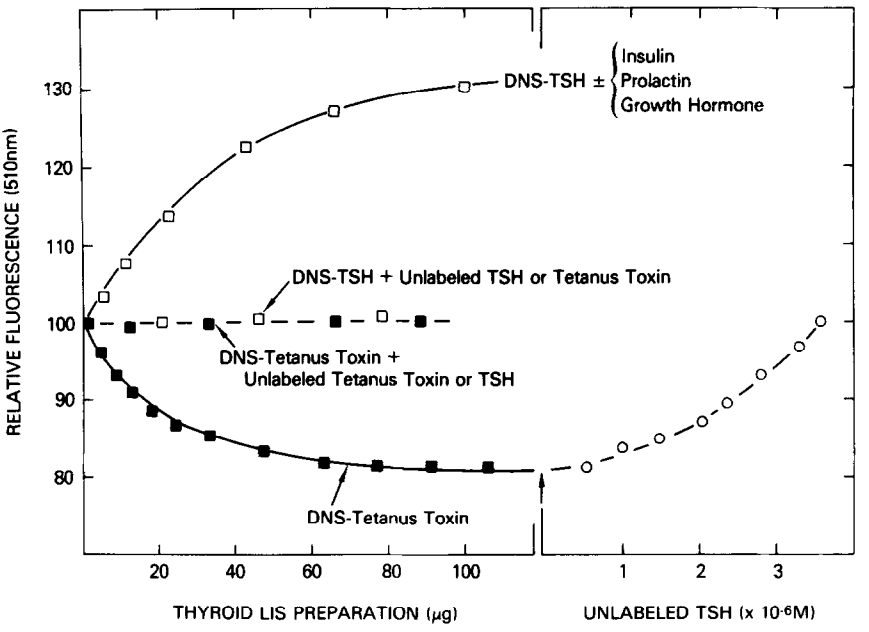


Fig. 3 Effect of the glycoprotein component of the receptor (thryoid LIS preparation) on the relative fluorescence of Dansyl(DNS)-TSH (0.6 μM) or dansyl(DNS)-tetanus toxin (1.3 μm) in the presence or absence of the noted nondansylated (unlabeled) hormones or toxin. Unless specified, the nondansylated effectors were present at 5 X $10^{-6}$ M concentrations. Conditions are detailed in References 16-18.

TABLE I. Incorporation of protein and carbohydrate from the LIS solubilized TSH receptor into dipalmitoyl PC and dioleoyl PC liposomes correlated with the ability of the liposomes to bind $^{125}$I-TSH and $^{125}$I-tetanus toxin

| Phospholipid in liposomes | LIS protein added to lipid film [a] | Tritiated sialic acid added to lipid film [b] | Protein incorporated | Tritiated sialic acid incorporated | $^{125}$I-TSH bound (per 3 x $10^5$ particles)[c] | $^{125}$I-tetanus toxin bound |
|---|---|---|---|---|---|---|
| | μg | cpm | μg | cpm | cpm | cpm |
| Dipalmitoyl PC | 1,000 | 1,000,000 | 109 | 127,000 | 9,000 | 12,500 |
| Dioleoyl PC | 1,000 | 1,000,000 | 111 | 125,000 | 37,000 | 8,500 |

[a] See legend to Fig. 1 and "Materials and Methods" for procedural details.

[b] Sialic acid-containing components in the membrane were labeled with tritium as detailed in "Materials and Methods." The tritiated membranes were solubilized with lithium diiodosalicylate as described; radioactivity was both TCA-precipitable and neuraminidase-sensitive. The labeling procedure did not affect interactions with TSH or tetanus toxin.

[c] Particles determined by Coulter counter analysis. Size distribution was the same, independent of the protein content.

tetanus toxin interaction is with the same glycoprotein component as is the TSH interaction since the two agents are mutually inhibitory in both liposome and fluorescence studies. Our conclusion, that tetanus toxin is interacting with the glycoprotein component of the TSH receptor, is supported by the observation that a purified fragment of the glycoprotein component of the TSH receptor is capable of inhibiting tetanus toxin binding. Last, the current report presents an important observation by demonstrating that receptor expression may be regulated by the phospholipid components of the membrane bilayer and even by the fatty acid component of these phospholipids.

The implication of these observations is threefold. First, although current studies of tetanus toxin interactions with neural tissue implicate gangliosides (19-21) as a component of the tetanus toxin receptor, the possibility is raised that the tetanus receptor on neural tissue might also involve a glycoprotein receptor component. Second, membrane phospholipid content could regulate tetanus toxin sensitivity. Third, despite structural similarities between receptors for glycoprotein hormones, toxins, and interferon (1-4), the phospholipid composition of a membrane could modulate its sensitivity to a particular one of these agents. The possibility that the tetanus toxin receptor contains a glycoprotein component is not inconsistent with recent literature observations; i.e. membrane sialoglycoproteins with oligosaccharide moieties similar to gangliosides have been observed (22, 23). The possibility that a glycoprotein component is involved in the tetanus receptor in neural tissue is under current investigation.

## REFERENCES

1. Kohn, L. D. (1977) in Horizons in Biochemistry and Biophysics, (Quagliariello, E., ed), Vol. 3, pp. 123-163, Addision-Wesley Publishing Company, Reading, Massachusetts.
2. Kohn, L. D., Lee, G., Grollman, E. F., Friedman, R. M., Ledley, F. D., Meldolesi, M. F., Mullin, B. R., and Aloj, S. M. (1978) in Symposium on Cell Surface Carbohydrates, 1977 American Chemical Society Meeting, Academic Press, New York.
3. Kohn, L. D. (1977) in Annual Reports in Medicinal Chemistry, (Clarke, F. H., ed), Vol. 12, pp. 211-222, Academic Press, New York.
4. Kohn, L. D. (1978) in Receptors and Recognition, (Cuatrecasas, P., and Greaves, M. F., eds), Series A, Vol. 5, pp. 134-212, Chapman and Hall, London, England.
5. Tate, R. L. , Holmes, J. M., Kohn, L. D. and Winand, R. J. (1975) J. Biol. Chem. 250, 6527-6533.

6. Tate, R. L., Holmes, J. M., Kohn, L. D. (1976) in Thyroid Research: Proceedings of the 7th International Thyroid Conference, Boston, Massachusetts, June 19-11, 1975 (Robbins, J., and Braverman, L., eds) International Congress Series No. 378, pp. 57-60, Excerpta Medica, Amsterdam, The Netherlands.
7. Meldolesi, M. F., Lee, G., Macchia, V., Aloj, S. M., and Kohn, L. D. (1978) J. Biol. Chem. 253, J. Biol. Chem. in press.
8. Winand, R. J., and Kohn, L. D. (1975) J. Biol. Chem. 250. 6534-6540.
9. Grollman, E. F., Chang, E. H., Lee, G., Lazo, P., Friedman, R. M., and Kohn, L. D. (1978) J. Biol. Chem. 253, in press.
10. Meldolesi, M. F., Fishman, P. H., Aloj, S. M., Kohn, L. D., and Brady, R. O. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4060-4064.
11. Meldolesi, M. F., Fishman, P. H., Aloj, S. M., Ledley, F. D., Lee, G., Brady, R. O., and Kohn, L. D. (1977) Biochem. Biophys. Res. Commun. 75, 581-587.
12. Ledley, F. D., Lee, G., Kohn, L. D., Habig, W. H., and Hardegree, M. C. (1977) J. Biol. Chem. 252, 4049-4055.
13. Habig, W. H., Hardegree, M. C., Grollman, E. F., Ledley, F. D., Meldolesi, M. F., Aloj, S. M., and Kohn, L. D. (1978) Endocrinology, 102, 844-851.
14. Winand, R. J., and Kohn, L. D. (1970) J. Biol. Chem. 245, 967-975.
15. Amir, S.M., Carraway, T. F., Jr., Kohn, L. D., and Winand, R. J. (1973) J. Biol. Chem. 248, 4092-4100.
16. Aloj, S. M., Kohn, L. D., Lee, G., and Meldolesi, M. F. (1977) Biochem. Biophys. Res. Commun. 74, 1053-1059.
17. Aloj, S. M., Lee, G., Grollman, E. F., Bequinot, F., Consiglio, E., and Kohn, L. D. (1978) J. Biol. Chem., in press.
18. Aloj, S. M., Lee, G., Consiglio, E., Formisano, S., Minton, A. P., and Kohn, L. D. (1978) J. Biol. Chem. 253, in press.
19. van Heyningen, W. E., and Miller, P. A. (1961) J. Gen. Microbiol. 24, 107-119.
20. van Heyningen, W. E. (1974) Nature 249, 415-417.
21. van Heyningen, W. E., and Mellanby, J. (1971) in Microbial Toxins (Kades, S., Montie, T. C., and Aji, S. J., eds) Vol. IIa, Chapter 2, Academic Press, New York, pp. 69-108.
22. Finne, J., Krusius, T., and Rauvala, H. (1977) Biochem. Biophys. Res. Commun. 74, 405-410.
23. Tonegawa, Y. and Hakomori, S. (1977) Biochem. Biophys. Res. Commun. 76, 9-17.