Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 308-313

LABELING OF THE ACTIVE SUBUNIT OF CHOLERA TOXIN FROM WITHIN THE MEMBRANE BILAYER

Bernadine J. Wisnieski and John S. Bramhall

The Department of Microbiology and

The Molecular Biology Institute

University of California, Los Angeles, CA 90024

Received January 2,1979

SUMMARY

Using the photoreactive glycolipid probe 12-(4-azido-2-nitrophenoxy)-stearoylglucosamine-[1-^{14}C], we have effected the radiolabeling of the active A_1 subunit of cholera toxin from within the membrane bilayer. The membrane employed as a target was the envelope of Newcastle disease virus which contained the photoreactive probe. Radiolabeling of the A_1 subunit occurred after cholera toxin and virus were incubated together for 15 min at 37° and then irradiated at 366 nm for 1 min. Labeling of A_1 did not occur when cholera toxin was irradiated in a solution of probe without virus or when the 15 min incubation with virus was performed at 0° instead of at 37°.

INTRODUCTION

The purpose of this communication is to describe a protocol to elucidate the dynamics of molecules which insert into or cross the membrane bilayer. The protocol involves the use of photoreactive lipid analogs (1) which studies with spin-label counterparts suggest are restricted to the surface monolayer of the membrane (2). The particular probe employed in the study described here is 12-(4-azido-2-nitrophenoxy)stearoylglucosamine-[1-^{14}C] (Figure 1), λ_{max} = 245 and 366 nm. It is related to photoreactive molecules described by Khorana (3,4), Stoffel (5,6) and their coworkers. A reactive nitrene is generated upon the absorption of light energy ($RN_3 \rightarrow R\ddot{N}$). The nitrene is resident within the membrane bilayer, approximately 13 Å from surface carboxyl groups.

The membrane system in these studies is the membrane envelope of Newcastle disease virus (NDV), an animal virus. The envelope of NDV is relatively

0006-291X/79/050308-06$01.00/0

well defined with respect to protein location, and the successful attachment of the photoreactive probe to viral membrane proteins has been demonstrated (1). It is the objective of this report to assess the ability of the photoreactive probe to attach covalently to cholera toxin components after toxin binds to the surface of NDV. We chose cholera toxin for preliminary studies since the requisite receptor, ganglioside GM_1, is known to occur in avian membranes and would presumably occur in egg-grown NDV since the viral envelope is representative of host plasma membrane in lipid and glycolipid composition (7). Moreover, NDV possesses neuraminidase activity (8) and consequently might be especially rich in GM_1 (9).

The obvious advantage of the technique we describe is that it enables us to follow the movement of proteins across specified zones within the membrane bilayer where the azido groups reside. Cholera toxin is ideal for these studies because it is a relatively simple toxin, antitoxin as well as receptor is readily available, and the basic system lends itself to a variety of complementary approaches with both model and biological membrane preparations (10,11).

MATERIALS AND METHODS

Newcastle Disease Virus. Newcastle disease virus (NDV) strain HP16, was propagated in ova and isolated as previously described (2). A vertical super speed rotor (Sorvall SS-90) was used to facilitate rapid isopycnic banding of the virus particles. The sucrose and renograffin gradients were spun at 20,000 g for 1 h.

Photoreactive Probe. The synthesis and purification of the photoreactive glycolipid probe 12-(4-azido-2-nitrophenoxy)stearoylglucosamine-[1-^{14}C] has been described (12). The specific activity of the radiolabeled probe was 50 mCi/mmole. It was stored in absolute ethanol (12.5 μCi/ml) at 5°.

Cholera Toxin. Cholera toxin, the generous gift of Dr. John Mekalanos, was purified according to the method of Mekalanos, Romig and Collier (13). It was diluted to 6 mg/ml with 0.1 M sodium phosphate buffer, pH 7.

Labeling Protocol. Experiments were carried out under red safety lights until the completion of electrophoresis to avoid activation of probe in unirradiated controls. Typically, 400 μg of NDV as protein was suspended in 6.2 ml of 5 mM sodium phosphate buffered saline, 1 mM in EDTA, pH 7.4 (PBSE). This sample received 50 μl of photoreactive probe in ethanol and was incubated for 15 min at 37°. After 15 min, one 2.4 ml aliquot of the sample NDV was mixed with cholera toxin (144 μg) for 15 min at 37° and another 2.4 ml aliquot was brought to 0° and then mixed with cholera toxin (144 μg) for 15 min at 0°. A third aliquot of virus (1.2 ml) was brought to 0° and incubated without toxin for 15 min at 0°.

After the designated incubation with toxin at 37° or 0°, two 0.6 ml

aliquots were removed from each 2.4 ml sample, placed in 6 X 50 mm Kimble disposable borosilicate glass culture tubes, irradiated for 1 min at 366 nm on a mineral lamp (UV-Products, San Gabriel, CA). Two 0.6 ml aliquots at each temperature served as unirradiated controls. One-half of the sample of NDV without toxin was similarly irradiated and half served as an unirradiated control. Next, cholera toxin was diluted to 72 μg/1.2 ml with PBSE; 10 μl of probe was added and after two 15-min incubations at 37°, one 0.6 ml aliquot was irradiated and one 0.6 ml aliquot served as an unirradiated control. The latter two samples received 60 μl of an ice cold 50% trichloroacetic acid solution. All 12 samples were spun on a Beckman SW50.1 rotor (with 0.7 ml adaptors) at 149,000 g for 30 min. Supernatants were removed and 50 μl of SDS-solubilizing buffer with tracking dye (14) was added to each pellet. The two cholera toxin samples received reducing buffer. The two NDV pellets without toxin received nonreducing buffer; one set of NDV with toxin pellets received reducing buffer and the duplicate set received nonreducing buffer. Electrophoresis and Fluorography. Solubilized samples were applied to SDS-polyacrylamide gels (10-15% acrylamide gradient with a 2.5% stack). After electrophoresis (15), the gels were stained for protein with Coomassie blue, photographed, and then prepared for fluorography (16). Pre-flashed film (17) was exposed to the gels for 30 days before development.

RESULTS

Having demonstrated that unlabeled cholera toxin competes for ^{125}I-labeled cholera toxin binding sites on NDV (unpublished data), we initiated an investigation into the transmembrane dynamics of the associated toxin. Figure 1 shows the photoreactive probe used in these studies. This photoreactive glycolipid spontaneously inserts into membranes (∿ 85-90% of the total counts added). When virus were incubated with this probe for 15 min at 37° and then mixed with cholera toxin at either 0° or 37° for 15 min and irradiated, the viral proteins were identically labeled; however, incubation at 37° was the only condition which led to radiolabeling of cholera toxin.

The gels shown in Figure 2 show the proteins present in each sample described in the Materials and Methods section. Cholera toxin is in lanes 3 and 12, NDV in lanes 1 and 2, NDV with cholera toxin (0°, 15 min) in lanes 4, 5, 8, 9 and NDV with cholera toxin (37°, 15 min) in lanes 6, 7, 10, 11. Lanes 3-7, and 12 contain reduced samples; lanes 1, 2, 8-11 contain unreduced samples. β-mercaptoethanol which diffused into sample lanes 8 and 11 allows us to visualize the reduction of the cholera toxin $A_1S\text{-}SA_2$ peptide to A_1SH (and A_2SH not stained). The unreduced $A_1S\text{-}SA_2$ subunit seen in lanes 3-7 and 12 represents the linear $\underline{A_1S\text{-}SA_2}$ peptide before peptidase cleavage. After enzymic cleavage, A_1 and A_2 are held together only at the

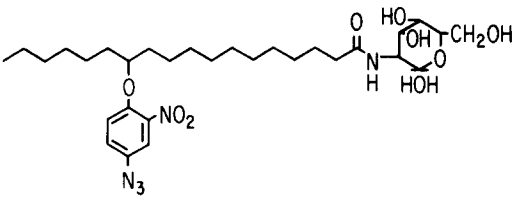

Figure 1. The photoreactive probe 12-(4-azido-2-nitrophenoxy)-stearoylglucosamine-[1-^{14}C].

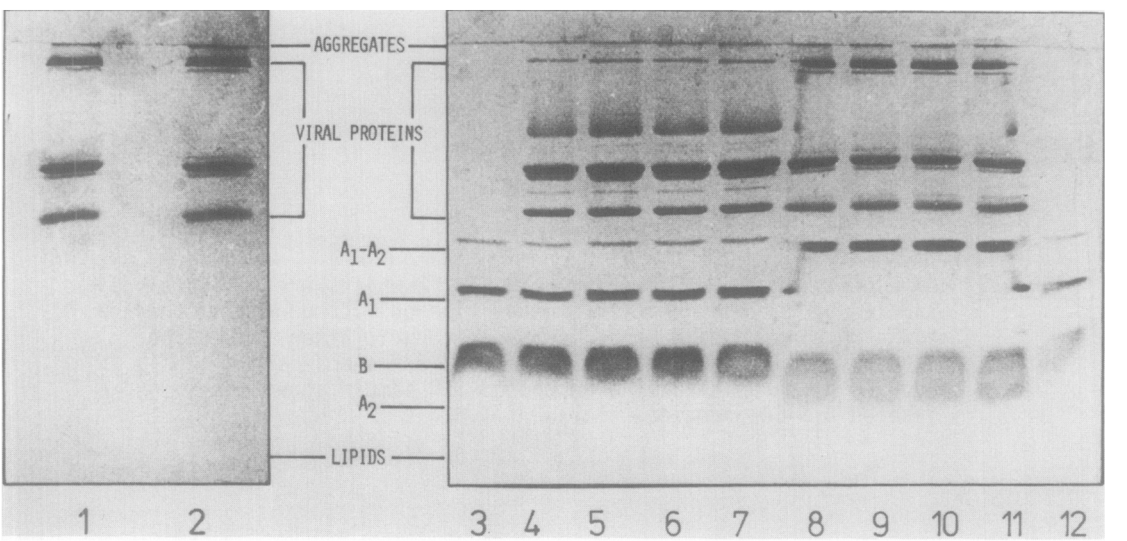

Figure 2. SDS-gel electrophoresis pattern of Newcastle disease virus and cholera toxin proteins. Lanes 1 and 2 contain irradiated and unirradiated NDV samples (unreduced). Lanes 3 and 12 contain irradiated and unirradiated cholera toxin samples (reduced). Lanes 4 and 5 contain reduced unirradiated and irradiated samples of NDV plus cholera toxin (15 min at 0°). Lanes 8 and 9 represent the unreduced counterparts of 4 and 5. Lanes 6 and 7 contain reduced unirradiated and irradiated samples of NDV plus cholera toxin (15 min at 37°). Lanes 10 and 11 represent the unreduced counterparts of 6 and 7.

disulfide bond. Fluorography of the gels shown in Figure 2 reveals the labeling of viral proteins in all irradiated samples, no labeling of cholera toxin in solution, and radiolabeling of the active A_1 subunit of cholera toxin only after a 37° incubation with virus for 15 min.

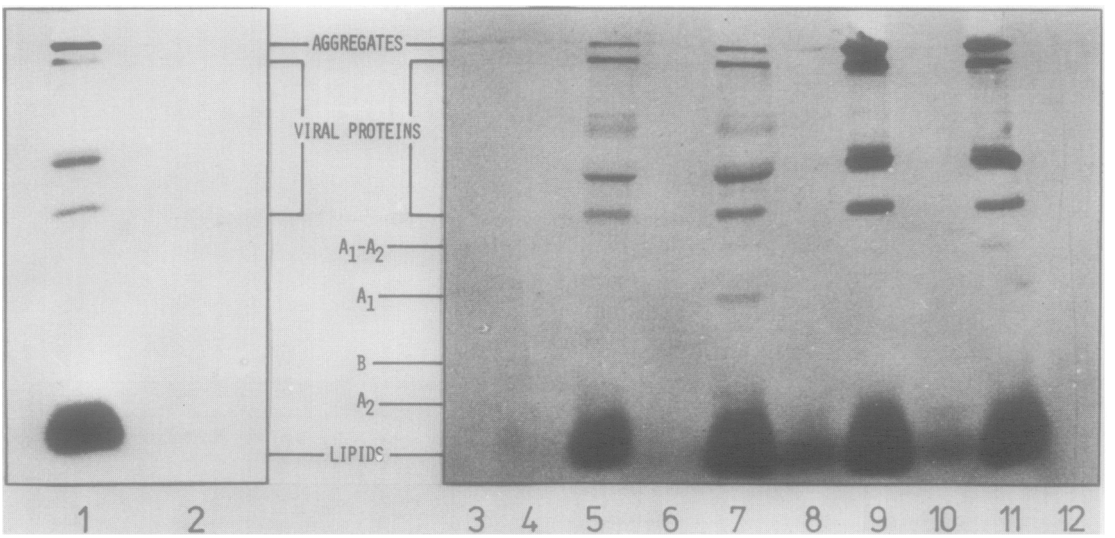

Figure 3. Components of the samples shown in Fig. 2 which were radioactively labeled by the photoreactive probe. The only component of cholera toxin which becomes labeled is the A_1 subunit after incubation with NDV at 37° for 15 min. The reduced 37° sample shows the labeled A_1 subunit and the unreduced 37° sample shows the labeled A_1S-SA_2 peptide.

DISCUSSION

These results demonstrate that there is no radioactivity associated with cholera toxin under conditions where toxin is allowed to bind but not express activity (i.e., with NDV at 0° for 15 min). When the cholera toxin experiments were carried out with a solution of cholera toxin and ^{14}C-probe in concentrations idential to those used in all other treatments but without NDV there was no indication of radiolabeling of any toxin subunits to the extent seen in lanes 7 and 11 where incubation was with virus for 15 min at 37°.

Unirradiated controls contain no radiolabeled proteins. These results confirm our ability to label viral membrane proteins, provide further evidence that the photoreactive probe employed in these studies effects very few contacts with soluble proteins and suggest that the active A_1 subunit of

cholera toxin extends into or crosses the membrane bilayer after the B pentamer binds to receptor gangliosides. By photoactivating azido probes residing within the membrane envelope of NDV at various times after the addition of cholera toxin or specific subunit combinations, we hope to elucidate the transmembrane dynamics of toxin subunits. Other membrane systems can readily be substituted for NDV, e.g., human or pigeon erythrocytes and artificial lipid vesicles. In this way, more complete information can be obtained concerning events which precede expression of toxin activity, i.e., activation of adenylate cyclase on the cytoplasmic face of the membrane.

ACKNOWLEDGEMENTS

Research was supported by USPHS Grant GM22240, the University of California Cancer Research Co-ordinating Committee and the Academic Senate. B.J.W. is the recipient of USPHS Research Career Development Award GM00228 and J.S.B. is a Fulbright-Hayes Postdoctoral Scholar.

REFERENCES

1. Bramhall, J.S., Shiflett, M.A., and Wisnieski, B.J. (1978) Biochem. J., in press.
2. Wisnieski, B.J., and Iwata, K.K. (1977) Biochemistry 16,1321-1326.
3. Chakrabarti, P., and Khorana, H.G. (1975) Biochemistry 14,5021-5032.
4. Greenberg, G.R., Chakrabarti, P., and Khorana, H.G. (1976) Proc. Natl. Acad. Sci. USA 73,86-90.
5. Stoffel, W., Salm, K.-P., and Körkemeier, U. (1976) Hoppe-Sezler's Z. Physiol. Chem. 357,917-924.
6. Stoffel, W., Därr, W., and Salm, K.-P. (1977) Hoppe-Sezler's Z. Physiol. Chem. 358,453-462.
7. Blough, H.A., and Choppin, D.E.M. (1968) Virology 36,286-292.
8. Rott, R., and Klenk, H.-D. (1977) Virus Infection and the Cell Surface, eds. Poste, G. and Nicolson, G., North-Holland, N.Y., pp 47-67 .
9. Cuatrecasas, P. (1973) Biochemistry 12,3547-3558.
10. Moss, J., Fishman, P.H., Manganiello, V.A., Vaughan, M., and Brady, R.O. (1976) Proc. Natl. Acad. Sci. USA 73,3480-3483.
11. Moss, J., Fishman, P.H., Richards, R.L., Alving, C.R., Vaughan, M., and Brady, R.O. (1976) Proc. Natl. Acad. Sci. USA 73,1034-1037.
12. Bramhall, J., Ishida, B., and Wisnieski, B. (1978) J. Supramol. Struct. 9, in press.
13. Mekalanos, J., Romig, W.R., and Collier, R.J. (1978) Infect. Immun. 20,552-558.
14. O'Farrell, P.H. (1975) J. Biol. Chem. 250,4007-4021.
15. Laemmli, U.K. (1970) Nature 227,680-685.
16. Bonner, W.N., and Laskey, R.A. (1974) Eur. J. Biochem. 46,83-88.
17. Laskey, R.A., and Mills, A.D. (1975) Eur. J. Biochem. 56,335-341.