Photoaffinity Labeling of the Electroplax Sodium Channel with a Photoreactive μ-Conotoxin Carrying a Radioactive and Chromogenic Diazirine

Yasumaru HATANAKA,*,[a] Hitoshi NAKAYAMA,[a] and Yuichi KANAOKA[b]

Faculty of Pharmaceutical Sciences, Hokkaido University,[a] Sapporo 060, Japan and Toyama Women's College,[b] 444 Gankaiji, Toyama 930–01, Japan.
Received March 16, 1992

Photoreactive derivatives of μ-conotoxin GIIIA carrying a radioactive and chromogenic diazirine have been prepared as a photoaffinity labeling reagent for muscle-type sodium channels. When the reagent was photolyzed in the presence of the eel sodium channel, radioactivity was specifically incorporated into the channel protein.

Keywords photoaffinity labeling; μ-conotoxin; peptide toxin; sodium channel; radioisotope labeling; diazirine; carbene

In the past few years, the primary structures of ion channels have been deduced from cyclic complementary deoxyribonucleic acid (cDNA) clones, and it has been concluded that the principal subunits of voltage-sensitive Na^+, Ca^{2+}, and K^+ channels are homologous members of a gene family.[1,2] Models relating the primary structures of these principal subunits to their functional properties have been proposed, and structural studies have been started to define a functional map of these proteins.[2] Chemical approaches are dependent upon the use of a number of specific neurotoxins that act at the receptor sites as molecular probes of channel structure and function. Since μ-conotoxins, polypeptide neurotoxins from *Conus geographus*, specifically block ion flow through muscle channels,[3] determination of the location of their receptor region within the primary structure of the sodium channel α-subunit will help to define the molecular mechanisms of ion flux. The analysis of the tertiary structure of μ-conotoxin GIIIA, a member of the conotoxin family, by means of two-dimensional proton nuclear magnetic resonance methods is of current interest in connection with the structure–function relationship of the toxin with the channel protein.[4] Photoreactive derivatives of this toxin will be potentially useful tools for structural analyses of the binding sites at the molecular level. Because of several deficiencies of nitrene-yielding aryl azide, the diazirine, a three-membered nitrogen-containing heterocycle, has become an important candidate that generates carbene as a highly reactive intermediate.[5] Several aryl azide derivatives of conotoxins have recently been reported.[6] However, during the course of our chemical studies of ion channel structure, we obtained better results in photoaffinity labeling with diazirine derivatives than with azides.[7] We have already reported the first example of photoreactive μ-conotoxin GIIIA carrying a diazirine,[8] [[2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]]phenoxy]-acetic acid (NDPA), which was recently developed by us as a chromogenic photoreactive carbene precursor for photoaffinity labeling.[9] The present paper deals with synthesis and application of radioactive analogs of these photoreactive conotoxins.

Results and Discussion

Synthesis of the photolabeling reagent was carried out with a synthetic μ-conotoxin GIIIA[10] (Chart 1). Reaction of the μ-conotoxin with the *N*-hydroxysuccinimide ester of carbon-14-labeled NDPA resulted in a mixture of [[2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]]phenoxy]-acetyl (NDP) derivatives which showed a quite similar profile on high-performance liquid chromatography (HPLC) to that described previously.[8] The first three peaks correspond to the 9-, 8-, or 11-substituted mono-NDP conotoxins, respectively, and binding constants and modification sites on the toxin have already been described.[8] Isolation yields of these three products from starting NDPA were 4%, 22%, and 7%, respectively, based on the amounts of radioactivity incorporated.

Solubilized and partially purified sodium channel was incubated with the major NDP derivative obtained above. Under the equilibrated incubation conditions described in

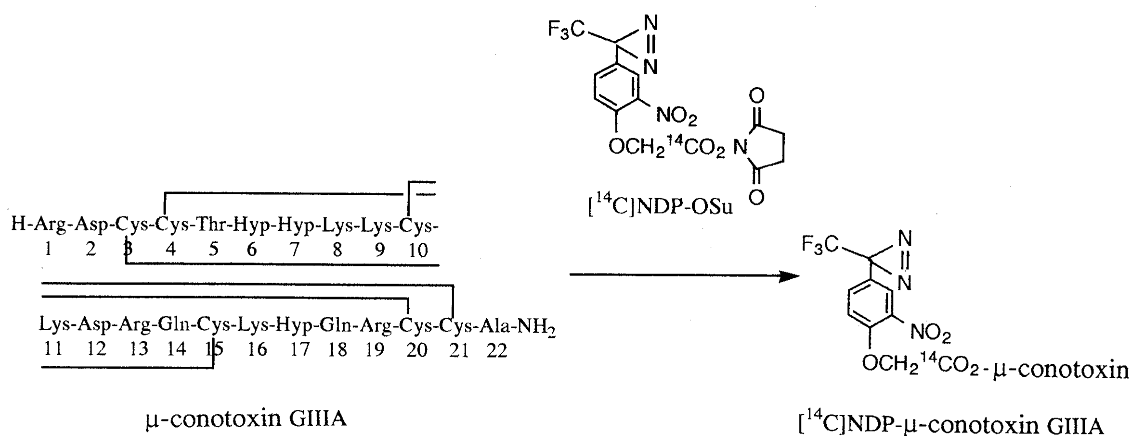

Chart 1

© 1992 Pharmaceutical Society of Japan

NII-Electronic Library Service

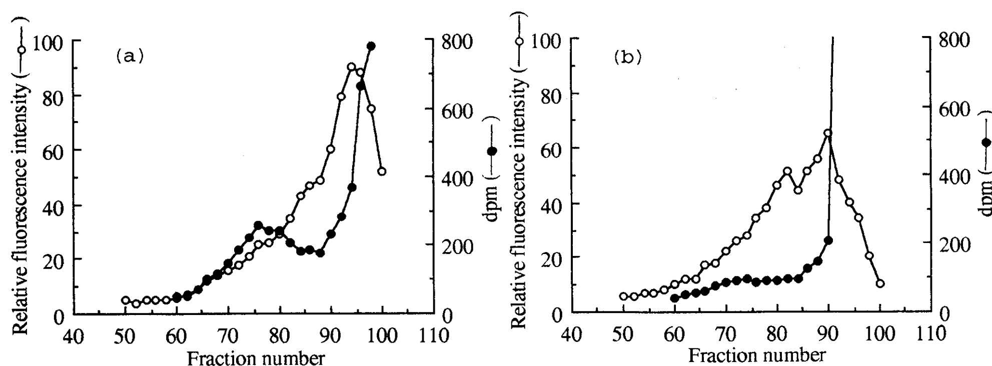

Fig. 1. Sepharose CL-4B Chromatography of the Sodium Channel Photolabeled with Radioactive NDP Derivative of μ-Conotoxin GIIIA

(a) The photolabeled sample was developed on a column (3.2×85 cm) with 50 mM sodium phosphate buffer (pH 7.5) containing 0.1% SDS at a flow rate of 30 ml/h. Fractions of 6 ml were collected. Protein (○; 20 μl of each fraction) and radioactivity (●; 200 μl of each fraction) were monitored by fluorescamine assay and liquid scintillation counting, respectively. (b) A sample photolabeled in the presence of tetrodotoxin was developed similarly, and fractions were monitored as described for (a).

the experimental section, 97% of the channel was observed to form a complex with the reagent. After the photolysis, the sample was heat-denatured, subjected to reductive carboxymethylation, and then purified by gel-filtration chromatography on Sepharose 4B.

Most of the channel protein was eluted in fractions 66—84. The specific labeling of the sodium channel protein is clearly demonstrated by a comparison of the profile of incorporated radioactivity (Fig. 1a) with that of the control experiment (Fig. 1b). Eluted sodium channel fractions were pooled and the specific labeling yield was determined to be 2% of the total channel protein. Similarly low efficiency was also obtained in photoaffinity labeling with derivatives of tetrodotoxin[7a)] or scorpion toxin.[11)] Although the incorporation yield of photolabeling was low, labeled regions are expected to be identifiable by the special method of probing labeled proteolytic fragments with a battery of sequence-specific antibodies recognizing different segments of the channel protein.[7c)] We have successfully identified the labeled region with a photoreactive tetrodotoxin derivative using this approach,[12)] and attempts at mapping the binding site of μ-conotoxin are under way.

Experimental

Synthesis μ-Conotoxin GIIIA was synthesized as described before.[10)] The carbon-14 labeled NDPA was obtained from the reaction of radioactive methyl bromoacetate (American Radiolabeled Chemicals, St. Louis, U.S.A.; specific activity 56 mCi/mmol) and a nitrophenol derivative of diazirine according to the procedure described previously.[9)] The hot NDPA (0.5 μmol) was mixed with a 0.1 M acetonitrile solution of *N*-hydroxy succinimide (10 μl, 1 μmol) and a 0.1 M acetonitrile solution of dicyclohexyl carbodiimide (10 μl, 1 μmol) at room temperature. The mixture was allowed to react at room temperature for 1 h, then diluted with 80 μl of dry acetonitrile. Ten microliter aliquots of the active ester solution obtained were added to a solution of μ-conotoxin GIIIA (13.0 mg, 5 μmol) in 1 ml of borate buffer (50 mM, pH 8.5) at room temperature. The same addition procedure was repeated until all of the solution of active ester had been added to the reaction mixture. After the addition, the reaction was allowed to proceed for 1 h. The reaction mixture was directly subjected to HPLC. Reversed-phase HPLC was performed with a Waters chromatography unit equipped with a 250×4.6 mm Chemcosorb C_{18} column (Chemco Scientific Co., Japan; 7 μm particles, 80 Å pores) as described before.[8)] The first three peaks were purified to homogeneity and were chromatographically identified by co-injection into the HPLC column with the corresponding cold samples. The yields of purified products were determined from the incorporated radioactivity.

Photolabeling A partially purified sodium channel protein, obtained by solubilization and diethylaminoethyl (DEAE) ion-exchange chromatography,[13)] was used for labeling experiments. Solubilized sodium channel (8 nmol of [^{3}H]saxitoxin binding sites) in 13 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 0.3% Lubrol PX/phosphatidylcholine (7 : 1) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mM iodoacetamide, 1 μM pepstatin A, and 5 mM ethylenediaminetetraacetic acid) was incubated with the major product of photoreactive conotoxin (1.2 μM) at 0 °C in the dark. After 30 min, a 200 μl aliquot of the incubation mixture was taken out to measure the complex formed by the rapid gel filtration method.[7a,14)] The irradiation was performed with a 20 W black-light lamp (National FL 20S-BL-B) at a distance of 5 cm for 1 h on an ice bed. As a control experiment, the sample in the presence of 1.8 μM tetrodotoxin was similarly incubated and photolyzed. All the irradiated samples were heat-denatured at 90 °C for 5 min in the presence of 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol, followed by reductive carboxymethylation as described previously.[1)] The samples were then chromatographed on a column of Sepharose CL-4B (Pharmacia) as described by Miller *et al.*[15)] Fractions were monitored by measuring radioactivity and by fluorescamine assay for proteins. Eluted sodium channel fractions of high molecular weight (*ca.* 250 kDa),[7a)] monitored by SDS–polyacrylamide gel electrophoresis (PAGE) on 6% polyacrylamide gel, were pooled and the photoincorporated amounts of radioactivity per mole of sodium channel protein isolated were determined.

Acknowledgement This work was supported by a Grant-in-Aid (No. 02670938, Y. H.) and a Grant-in-Aid for Scientific Research on Priority Areas (No. 03215103, Y. K.) from the Ministry of Education, Science and Culture, Japan.

References

1) M. Noda, S. Shimizu, T. Tanabe, T. Takai, T. Kayano, T. Ikeda, H. Takahashi, H. Nakayama, Y. Kanaoka, N. Minamino, K. Kanagawa, H. Matsuo, M. A. Raftery, T. Hirose, S. Inayama, H. Hayashida, T. Miyata, and S. Numa, *Nature* (London), **312**, 121 (1984).
2) *a*) W. A. Catterall, *Science*, **242**, 50 (1988); *b*) *Idem*, *Curr. Opinion Neurobiol.*, **1**, 5 (1991).
3) W. R. Gray, B. M. Olivera, and L. J. Cruz, *Ann. Rev. Biochem.*, **57**, 665 (1988).
4) *a*) J. Lancelin, D. Kohda, S. Tate, Y. Yanagawa, T. Abe, M. Satake, and F. Inagaki, *Biochemistry*, **30**, 6908 (1991); *b*) K. Ott, S. Becker, R. D. Gordon, and H. Rüterjans, *FEBS Lett.*, **278**, 160 (1991); *c*) K. Sato, Y. Ishida, K. Wakamatsu, R. Kato, H. Honda, Y. Ohizumi, H. Nakamura, M. Ohya, J. Lancelin, D. Kohda, and F. Inagaki, *J. Biol. Chem.*, **266**, 16989 (1991).
5) H. Bayley, "Laboratory Techniques in Biochemistry and Molecular Biology: Photogenerated Reagents in Biochemistry and Molecular Biology," ed. by T. S. Work and R. H. Burdon, Elsevier, Amsterdam, Chapter 3, 1983.
6) *a*) S. Becker, R. Liebe, and R. D. Gordon, *FEBS Lett.*, **272**, 152 (1990); *b*) R. A. Myers, G. C. Zafaralla, W. R. Gray, J. Abbott, L. J. Curz, and B. M. Olivera, *Biochemistry*, **30**, 9370 (1991).
7) *a*) E. Yoshida, H. Nakayama, Y. Hatanaka, and Y. Kanaoka,

NII-Electronic Library Service

Chem. Pharm. Bull., **38**, 982 (1990); *b*) M. Taki, H. Nakayama, and Y. Kanaoka, *FEBS Lett.*, **283**, 259 (1991); *c*) H. Nakayama, M. Taki, J. Striessnig, H. Glossmann, W. A. Catterall, and Y. Kanaoka, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9203 (1991).

8) Y. Hatanaka, E. Yoshida, H. Nakayama, and Y. Kanaoka, *FEBS Lett.*, **260**, 27 (1990).

9) Y. Hatanaka, E. Yoshida, H. Nakayama, and Y. Kanaoka, *Bioorganic Chem.*, **17**, 482 (1989).

10) Y. Hatanaka, E. Yoshida, H. Nakayama, and Y. Kanaoka, *Chem. Pharm. Bull.*, **38**, 236 (1990).

11) F. J. Tejedor and W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8742 (1988).

12) H. Nakayama, Y. Hatanaka, E. Yoshida, K. Oka, M. Takanohashi, Y. Amano, and Y. Kanaoka, *Biochem. Biophys. Res. Commun.*, **184**, 900 (1992).

13) *a*) W. G. Agnew, S. R. Levinson, J. S. Brabson, and M. A. Raftery, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 2606 (1978); *b*) H. Nakayama, R. M. Withy, and M. A. Raftery, *ibid.*, **79**, 7575 (1982).

14) S. R. Levinson, C. J. Curatalo, J. Reed, and M. A. Raftery, *Anal. Biochem.*, **99**, 72 (1979).

15) J. A. Miller, W. S. Agnew, and S. R. Levinson, *Biochemistry*, **22**, 462 (1983).

NII-Electronic Library Service