Laboratory of Molecular Pharmacology
Developmental Therapeutics Program
Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20205, U.S.A.

YVES POMMIER*
LEONARD A. ZWELLING
RONALD E. SCHWARTZ
MICHAEL R. MATTERN
KURT W. KOHN

REFERENCES

1. L. A. Zwelling, S. Michaels, L. C. Erickson, R. S. Ungerleider, M. Nichols and K. W. Kohn, *Biochemistry* **20**, 6553 (1981).
2. Y. Pommier, D. Kerrigan, R. Schwartz and L. A. Zwelling *Biochim. biophys. Res. Commun.* **107**, 576 (1982).
3. W. E. Ross, D. L. Glaubiger and K. W. Kohn, *Biochim. biophys. Acta* **519**, 23 (1978).
4. M. Gellert, *A. Rev. Biochem.* **50**, 879 (1981).
5. P. R. Cook and I. A. Brazell, *J. Cell Sci.* **22**, 287 (1976).
6. J. Filipski and K. W. Kohn, *Biochim. biophys. Acta* **698**, 280 (1982).
7. K. W. Kohn, R. A. G. Ewig, L. C. Erickson and L. A. Zwelling, in *DNA Repair: A Laboratory Manual of Research Techniques* (Eds. E. C. Friedberg and P. C. Hanawalt), pp. 379–401. Marcel Dekker, New York (1981).
8. M. O. Bradley and K. W. Kohn, *Nucleic Acids Res.* **7**, 793 (1979).
9. K. W. Kohn, L. C. Erickson, R. A. G. Ewig and C. Friedman, *Biochemistry* **15**, 4629 (1976).
10. M. S. Center, *Biochem. biophys. Res. Commun.* **89**, 1231 (1979).
11. H. M. Wu, N. Dattagupta, M. Hogan and D. M. Crothers, *Biochemistry* **19**, 626 (1980).
12. Y. Pommier, M. Mattern, R. Schwartz, K. Kohn and L. Zwelling, *Biochemistry* **23**, 2922 (1984).

* To whom correspondence should be addressed at: Building 37, Room 5A19, 9000 Rockville Pike, Bethesda, MD 20205.

Biochemical Pharmacology, Vol. 33, No. 23, pp. 3912–3914, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Cepharanthine protection of Na^+,K^+-activated adenosinetriphosphatase of plasma membranes from rat cerebral synaptosomes against inhibition by ascorbate

(*Received* 25 *January* 1984; *accepted* 9 *June* 1984)

Cepharanthine, a biscoclaurine-type alkaloid obtained mainly from *Stephania cephalantha*, is known to protect a variety of tissues by stabilization of biological membranes that are under attack from bilirubin, phospholipase, X-ray irradiation, oncogenic agents, etc. There are two proposed mechanisms of membrane stabilization by the alkaloid: cepharanthine represses a fluidity change of the membrane lipid bilayer [1–3], and it prevents peroxidation of the membrane lipids [4, 5]. However, there could be another stabilization mechanism that involves membrane enzymes or proteins. For instance, the drug prevents K^+ loss of erythrocytes caused by noxious agents which inhibit Na^+, K^+-activated adenosinetriphosphatase (EC 3.6.1.3) (Na^+, K^+-ATPase) of the plasma membrane [1, 2]. One of the underlying mechanisms may possibly be cepharanthine protection of the Na^+, K^+ pump from the agents.

Plasmalemmal Na^+, K^+-ATPase has been shown to be inhibited by ascorbate, which is particularly abundant in the brain and adrenal medulla. Using synaptosomal plasma membranes of rat cerebrums, we reported that the inhibition mechanism may not involve lipid peroxidation but may be the direct attack of ascorbate radicals on the enzyme molecules [6], unlike the conventional belief that the inhibition is via lipid peroxidation. Our conclusion was based mainly on the facts that Na^+, K^+-ATPase was strongly inhibited, in spite of little peroxidation of the membrane lipids, in the presence of ascorbate even after the metal contamination had been very carefully removed from all the chemicals used, and that dimethylfuran (an active oxygen scavenger) did not prevent Na^+, K^+-ATPase inhibition but *p*-nitrosodimethylaniline (an eliminator of organic free radicals) did.

Thus, there is a possibility that cepharanthine prevents the free radical attack on Na^+, K^+-ATPase of the plasma membrane. The present work examined this possibility by the use of our determination of Na^+, K^+-ATPase activity of the plasmalemmal preparation from rat cerebral synaptosomes [6].

Materials and methods

Most of the materials and methods were as reported elsewhere [6]. Briefly, synaptosomal plasma membranes were prepared from the forebrains of male rats of the Wistar strain by a slight modification of the method of Lee and Phillis [7]. An ATP preparation (Sigma Chemical Co., Grade 1) was purified by a batch method with Chelex 100 to eliminate the contaminating heavy metals, and other chemicals used were also purified by column chromatography of the same chelating resin. The water preparation had a specific resistance over $18 \times 10^6\ \Omega$/cm. The standard assay medium for ATPase determination contained 120 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 50 mM imidazol–HCl buffer (pH 7.4), plasmalemmal fraction, and the additions (specified in Table 1) in a final volume of 1 ml. Incubation was at 37° for 10 min. Na^+, K^+-ATPase activity was expressed as the difference between the amount of P_i liberated in the presence of Mg^{2+}, Na^+, and K^+ and that liberated in the presence of Mg^{2+} alone. The degree of lipid peroxidation was determined by the method of Ottolenghi [8]. For peroxidation determination the membrane fraction was incubated at 37° for 10 min in a medium (final volume, 1 ml) containing 50 mM Tris buffer (pH 7.4) and the additions as specified in Table 2. Cepharanthine (6′, 12′-dimethoxy-2, 2′-dimethyl-6, 7-[methylenebis(oxy)]oxyacanthan) was a gift of the Kaken Pharmaceutical Co., Japan. The drug was dissolved in a small amount of ethanol, and the ethanol solution was added to the incubation mixtures. The final ethanol concentration was 17.4 μM, which affected neither ATPase activity nor lipid peroxidation.

Results and discussion

Na^+-K^+-ATPase of synaptosomal plasma membrane was inhibited by over 50% in the presence of 0.1 mM ascorbate alone (Table 1a) as we have reported [6]. When cepharanthine (0.1 mM) was added together with ascorbate, the inhibition was about 10%. The presence of ascorbate and

Table 1. Effects of cepharanthine on Na^+, K^+-ATPase inhibition by ascorbate, Fe^{3+}, ethanol, ouabain, Pb^{2+}, and Hg^{2+}*

Addition	Na^+, K^+-ATPase activity (%)
(a) None	100 (control)
Ascorbate (0.1 mM)	45.0
Fe^{3+} (12 μM)	87.9
Cepharanthine (0.1 mM)	95.4
Ascorbate + Fe^{3+}	26.4
Ascorbate + cepharanthine	88.5
Ascorbate + Fe^{3+} + cepharanthine	87.3
(b) Ethanol (1.74 mM)	16.0
Ouabain (0.1 mM)	16.2
Pb^{2+} (0.05 mM)	70.1
Hg^{2+} (0.01 mM)	8.1
Ethanol + cepharanthine	12.6
Ouabain + cepharanthine	20.2
Pb^{2+} + cepharanthine	80.9
Hg^{2+} + cepharanthine	6.2

* In the absence of the added chemicals, Na^+, K^+-ATPase activity was 20 μmoles P_i/mg protein/hr. Chloride salts were used for Fe^{3+} and Hg^{2+}, and acetate salt for Pb^{2+}. Values are the averages of six experiments, and S.E.M.s were smaller than 10% of the means. See the text for more detailed incubation conditions.

Fe^{3+}, a catalyst of active oxygen formation, decreased the enzyme activity to one-fourth of the control. This strong inhibition was also eliminated by the alkaloid, and the inhibition remained merely about 10% as well. To examine the effects of cepharanthine on other types of inhibition, ethanol (hydrophobic interaction), ouabain (specific binding), Pb^{2+}, and Hg^{2+} (SH attack) were tested (Table 1b). All of them decreased the activity drastically at the concentrations used, and cepharanthine did not restore it. Thus, although cepharanthine did not prevent other types of Na^+, K^+-ATPase inhibition, the alkaloid might prevent at least two types of inhibition: (1) that by the peroxidation of membrane lipids, in which the enzyme is integrated, and (2) that by organic radical attack.

This tentative conclusion was examined by a comparison of lipid peroxidation of the plasmalemmal preparation (Table 2) with inhibition of its Na^+, K^+-ATPase activity. Ascorbate alone produced no effects on lipid peroxidation, although ascorbate strongly inhibited Na^+, K^+-ATPase. Fe^{3+} increased peroxidation by about 30%, and there was no change in peroxidation in the presence of ascorbate plus ATP. When ascorbate was added along with Fe^{3+}, peroxidation increased more than two times over the control. It was enhanced almost 3-fold by the combination of ascorbate, ATP, and Fe^{3+}. Although cepharanthine alone did not affect peroxidation, the drug stopped the peroxidation increase induced by ascorbate plus Fe^{3+}, and it reduced to about one-fourth the peroxidation increase caused by ascorbate, ATP, plus Fe^{3+}.

The results indicate two types of cepharanthine action on Na^+, K^+-ATPase of the synaptosomal plasmalemmas prepared from rat cerebrums. (1) The alkaloid inhibited lipid peroxidation induced by active oxygen and prevented, in turn, ATPase inactivation caused by peroxidation. The action observed in the present work may be expected from the reports that biscoclaurine-type alkaloids, to which cepharanthine belongs, prevent lipid peroxidation of various biological membranes induced by Fe^{2+} [4] or X-ray

Table 2. Effects of cepharanthine on lipid peroxidation induced by ascorbate, ATP, and Fe^{3+}*

Addition	Peroxidation (%)
None	100
Ascorbate (0.1 mM)	101
ATP (4 mM)	79†
Fe^{3+} (0.012 mM)	129
Cepharanthine (0.1 mM)	102
Ascorbate + ATP	88.9
Ascorbate + Fe^{3+}	230
Ascorbate + Fe^{3+} + ATP	280
Ascorbate + Fe^{3+} + cepharanthine	115
Ascorbate + Fe^{3+} + ATP + cepharanthine	148

* Lipid peroxidation was about 20 nmoles malonaldehyde/mg protein/hr when there was no addition. Values are the averages of five experiments, and S.E.M.s were smaller than 15% of the means. See the text for the determination conditions.

† Taken from Ref. 6.

irradiation [5]. One type of stabilizing action by cepharanthine on the biological membrane and the artificial lipid bilayer is by this mechanism. (2) Cepharanthine prevented Na^+, K^+-ATPase inhibition caused by ascorbate alone in the absence of Fe^{3+}. Since ascorbate radicals are responsible for the Na^+, K^+-ATPase inhibition under these conditions [6], it is tempting to conclude that the alkaloid may protect the membrane enzyme from ascorbate radicals although the exact mechanism is unclear. The alkaloid is reported to have no ability to scavenge free radicals [5], and hence the mechanism is unlikely to involve the elimination of ascorbate radicals by cepharanthine. Whether the drug inhibits radical formation itself is unknown; no data are available in the literature. The cepharanthine protection may also not be through the mechanisms that are relevant to the inhibition by alcohol, ouabain, and SH reagents, inasmuch as the inhibition by these agents was unaffected by the presence of the alkaloid (Table 2). Thus, cepharanthine may exert its action of sheltering biologically active membranes by protection not only of the lipid component but also of the enzyme component.

In summary, cepharanthine, a biscoclaurine-type alkaloid obtained from *S. cephalantha*, was investigated for its protective action of Na^+, K^+-activated ATPase of the plasma membranes from rat cerebral synaptosomes. The results indicate that there might be two types of mechanisms of cepharanthine action: the alkaloid prevented both membrane lipid peroxidation, which in turn inhibited the ATPase integrated in the lipid bilayer, and the deleterious effects of ascorbate, whose effective form was its radical, although the exact mechanism remains to be clarified.

Acknowledgement—The present work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education.

Department of Biochemistry
Nagoya City University Medical School
Mizuho-ku, Nagoya 467, Japan

KASHIKO GOTO
RYO TANAKA*

* To whom all correspondence should be addressed.

REFERENCES

1. K. Utsumi, M. Miyahara, M. Inoue, M. Mori, K. Sugiyama and J. Sasaki, *Cell Struct. Funct.* **1**, 133 (1975).
2. K. Utsumi, M. Miyahara, K. Sugiyama and J. Sasaki, *Acta histochem. cytochem.* **9**, 59 (1976).
3. M. Miyahara, K. Aono, J. S. Queseda, K. Shimono, Y. Baba and S. Yamashita, *Cell Struct. Funct.* **3**, 61 (1978).
4. N. Shiraishi, T. Arima, K. Aono, B. Inouye, Y. Morimoto and K. Utsumi, *Physiol. Chem. Physics* **12**, 299 (1980).
5. S. Nagatsuka and T. Nakazawa, *Biochim. biophys. Acta* **691**, 171 (1982).
6. K. Goto and R. Tanaka, *Brain Res.* **207**, 239 (1981).
7. S. L. Lee and J. W. Phillis, *Can. J. Physiol. Pharmac.* **55**, 961 (1977).
8. A. Ottolenghi, *Archs. Biochem. Biophys.* **79**, 355 (1959).

Biochemical Pharmacology, Vol. 33, No. 23, pp. 3914–3917, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Binding of phospholipase A_2 to isolated heart muscle

(*Received* 20 *January* 1984; *accepted* 8 *May* 1984)

Phospholipases A_2 (EC 3.1.1.4) isolated from snake venoms vary in lethal potency and enzymatic activity [1, 2]. They are used extensively as enzymatic probes with which to study phospholipid function and organization in membranes [1, 3]. Many basic snake venom phospholipases A_2 have potent pharmacological effects while other venom phospholipases A_2, usually acidic or neutral, are much weaker in their pharmacological properties [1, 2, 4–6]. However, the acidic or neutral phospholipases have enzymatic activities, as measured in various *in vitro* systems, that are often much greater than those of the basic enzymes [1, 2, 4–6]. Even though there is a lack of correlation between pharmacological and enzymatic activities, it has been suggested that the pharmacological properties of phospholipases A_2 are due to phospholipid hydrolysis [1]. Our previous studies with native and chemically modified derivatives of the basic *Naja nigricollis* phospholipase A_2 and the acidic *Naja naja atra* phospholipase A_2 have shown that the extent of phospholipid hydrolysis, as measured directly in the tissue being studied, does not correlate with their pharmacological properties [4–12]. These results led us to conclude that some pharmacological effects of snake venom phospholipases A_2 are due to a non-enzymatic action.

The phospholipase A_2 from *N. nigricollis* venom has potent cardiotoxic effects which do not correlate with the very low levels of tissue phospholipid hydrolysis or production of phospholipid hydrolytic products [4–6,*]. The *N. n. atra* phospholipase A_2, in contrast, shows little cardiotoxicity even though it causes the same low level of phospholipid hydrolysis [4–6,*] as the *N. nigricollis* enzyme. Since cardiotoxicity appears to be due to a non-enzymatic action of this phospholipase A_2, it was of interest to investigate whether differences in potency correlated with the extent of binding to heart tissue.

* Unpublished results.

Materials and methods

Lyophilized *N. nigricollis* snake venom was obtained from the Miami Serpentarium Laboratories (Miami, FL), and lyophilized *N. n. atra* snake venom was collected in Kaohsiung, Taiwan, by one of the authors (C. C. Y.). The methods for purification and homogeneity determination of the most basic (pI 10.6) phospholipase A_2 from *N. nigricollis* snake venom and the major acidic (pI 5.2) phospholipase A_2 from *N. n. atra* snake venom are identical to those previously described [13, 14].

Radiolabeled *N. nigricollis* and *N. n. atra* enzymes were prepared by the iodine monochloride (Sigma) method of MacFarlane [15] as modified by Vogel *et al.* [16]. The specific activity of the labeled *N. n. atra* enzyme was 44.2 Ci/mmole protein. To obtain adequate amounts of material and to perform all experiments within 3 weeks following iodination, it was necessary to prepare two iodinated batches of *N. nigricollis* phospholipase A_2 (sp. act. of 14.1 and 0.87 Ci/mmole protein).

Following iodination (up to 4 weeks), these phospholipases retained 50–100% of their enzymatic activity (measured on lecithin-Triton mixed micelles) and 50–100% of their original lethal potency (intraventricular injection into rats).

Hearts from male Sprague–Dawley rats (150–300 g), obtained from the Charles River Breeding Laboratories (Wilmington, MA), were rapidly removed under ether anesthesia. The hearts were rinsed twice in Tyrode's solu-