Contribution of Lipid Dynamics on the Inhibition of Bovine Brain Synaptosomal Na^+-K^+-ATPase Activity Induced by 4-Hydroxy-2-nonenal

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The effects of lipid hydroperoxide degradation products, such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), on bovine brain synaptosomal ATPase activities and their membrane lipid organization were examined. When the synaptosomes were treated with HNE, this resulted in the decrease of Na^+-K^+-ATPase activity with the loss of sulfhydryl (SH) groups in the membrane proteins. In contrast, MDA treatment of the synaptosomes did not induce an appreciable decrease in the ATPase activity or a loss of SH groups. The decreases in ATPase activity and SH content by treatment with HNE were also observed, as a Na^+-K^+-ATPase preparation was used in place of the synaptosomes. On the other hand, HNE had very little effect on synaptosomal Ca^{2+}- and Mg^{2+}-ATPase activities. The results of the kinetic analysis of the Na^+-K^+-ATPase activity indicated that the decrease in the activity by HNE-modification is due to a decreased affinity for the substrate. ATP completely protected the ATPase from the HNE attack. Modification of the synaptosomes with HNE caused a decrease in the membrane lipid fluidity near the lipid/water interface, not the lipid layer interior. In addition, it was found that there is a good relationship between the lipid fluidity and the Na^+-K^+-ATPase activity under the presence of various concentrations of HNE, suggesting that the lipid dynamics are closely related to HNE-induced inhibition of the ATPase activity. On the other hand, MDA did not induce change in the membrane lipid fluidity. HNE and MDA are mainly incorporated into the lipid and protein fractions in the synaptosomal membranes, respectively. Based on these results, we proposed a possible mechanism of HNE-induced inhibition of synaptosomal Na^+-K^+-ATPase activity associated with alterations in the membrane lipid organization.

Key words 4-hydroxy-2-nonenal; malondialdehyde; lipid peroxidation product; Na^+-K^+-ATPase; lipid fluidity; brain synaptosome

Lipid peroxidation in cell membrane phospholipids induced by reactive oxygen species and/or free radicals leads to alterations in the membrane structure and functions of cells, and has been proposed as a major mechanism for the onset of several pathological events *in vivo*, including postischemic-reperfusion injury, cancer, senile dementia and aging.[1] A number of studies have shown that peroxidation of membrane lipids in cells gives rise to highly reactive α,β-unsaturated aldehydes from the lipid hydroperoxides as secondary oxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA).[2—4] Pryor and Porter have reported that HNE can be formed from omega-6-polyunsaturated fatty acids.[5] Among the aldehydes generated as the result of membrane lipid peroxidation, HNE can be produced in relatively large concentrations up to 5 mM in cells, and is the most damaging to cells.[3,6] Koster *et al.* also reported that the concentration of HNE in the lipid bilayer of peroxidized microsomes is about 4.5 mM.[7]

HNE directly interacts with several amino acid residues of protein, *i.e.*, the sulfhydryl (SH) group of cysteine, the imidazole moiety of histidine and the ε-amino group of lysine through a Michael-type nucleophilic addition.[2] Several investigators have reported that this reaction leads to the structural modification and functional impairment of proteins.[3,8,9]

Previous studies have shown that free and/or protein-bound HNE accumulate in a variety of non-neural cells exposed to oxidative insult,[3] in pyramidal neuron cytoplasm and on neurofibrillary tangles in the brain of Alzheimer's disease patients.[10—12] Mark *et al.* have also recently reported that amyloid β-peptide-mediated neuronal cell death is related to a large increase in the levels of free and protein-bound HNE.[13] In addition, several investigators have also reported that HNE disrupts neuronal ion homeostasis by impairing the function of membrane-bound iron-motive ATPases such as Na^+-K^+-ATPase and Ca^{2+}-ATPase.[14—16] These findings strongly suggest that the aldehydes generated by lipid peroxidation of the membrane are serious toxic products for the onset of oxidative injury. However, the exact mechanism of the onset of cellular injury induced by HNE is still unclear at present.

In the present study, we examined the effects of HNE on membrane-bound ATPases of bovine brain synaptosomes in the context of their membrane lipid dynamics in order to obtain further information concerning mechanisms of the onset of HNE toxicity. This paper suggests the possibility that changes in the lipid dynamics are related to HNE-induced inactivation of the synaptosomal Na^+-K^+-ATPase.

MATERIALS AND METHODS

Materials Pepstatin A (microbial source), aprotinin (bovine lung, 5.2 TIU/mg solid), leupeptin hydrochloride (microbial source), soybean trypsin inhibitor (type 1-S), 1,6-diphenyl-1,3,5-hexatriene (DPH), phenylmethylsulfonyl fluoride (PMSF), 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl), *p*-hydroxy-mercuribenzoic acid (PHMB) and Na^+-K^+-ATPase (EC 3.6.1.3; porcine cerebral cortex, 0.42 U/mg protein) were purchased from Sigma (St. Louis, MO, U.S.A.). 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulfate (SDS), *N*-ethylmaleimide (NEM) and dithiothreitol were obtained from Wako Pure Chemical Co. (Osaka, Japan). *n*-Heptyl-β-D-thioglucoside and 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were from Dojin Chemical Co. (Kumamoto,

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Japan). ATP (disodium salt) was purchased from Oriental Yeast Co. (Tokyo, Japan). (−)-Ouabain octahydrate was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). HNE and malonaldehyde bis(dimethylacetal) were obtained from Cayman Chemical Co. (Ann arbor, MI, U.S.A.) and Tokyo Kasei Co. (Tokyo, Japan), respectively. A MDA stock solution (100 mM) was prepared by dissolving malonaldehyde bis(dimethylacetal) in 1 N HCl and adjusted by 1 N NaOH to pH 7.4. All other chemicals were of the highest grade purity.

Synaptosome Preparation Isolation of synaptosomes from bovine brain was performed according to the procedure described by Hensley *et al.*[17] with a slight modification. Bovine brain cortices (5 g) were homogenized in 10 volumes of 20 mM Hepes–NaOH buffer (pH 7.4) containing 0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 0.2 μg/ml PMSF, 20 μg/ml trypsin inhibitor, 2 mM EDTA and 1 mM dithiothreitol (Buffer A) in a Potter-Elvehjem glass homogenizer (six strokes with 1000 rev/min). The homogenate was centrifuged at 1500×g for 10 min at 4 °C, and the supernatant obtained was then centrifuged at 20000×g for 10 min at 4 °C. The pellets were washed twice and suspended in Buffer A (9 ml). Samples were layered onto a discontinuous gradient containing 10 ml of 1.18 M sucrose, 10 ml of 1 M sucrose and 10 ml of 0.85 M sucrose, then centrifuged at 82500×g for 2 h at 4 °C. Synaptosome fraction was obtained at the 1 M sucrose/1.18 M sucrose interface, and washed twice in 25 volumes of 20 mM phosphate buffer (pH 7.4) containing 4.6 mM KCl, 0.6 mM $MgCl_2$ and 117 mM NaCl (Buffer B). After centrifugation at 20000×g for 10 min, the final synaptosome fraction was re-suspended in the same buffer.

Modification of Synaptosomes with Aldehydes The synaptosomes (1 mg protein/ml) were treated with 500 μM aldehyde in Buffer B in the presence of 0.5% *n*-heptyl-β-D-thioglucoside for 30 min at 37 °C, unless otherwise specified. The reaction was terminated by dilution with a large volume of the same buffer and centrifugation at 20000×g for 10 min at 4 °C. The pellets obtained were washed twice in Buffer C containing 20 mM Hepes–NaOH buffer (pH 7.4), 4.6 mM KCl, 0.6 mM $MgCl_2$, 1.1 mM KH_2PO_4 and 137 mM NaCl. After centrifugation at 20000×g for 10 min, the pellets were suspended in the same buffer, and used as aldehyde-modified synaptosomes.

ATPase Activity Assay All assays were carried out in a final volume of 1 ml with the use of 30 μg synaptosomal protein. (1) Na^+-K^+-ATPase activity was measured in the assay medium containing 50 mM Tris–HCl buffer (pH 7.4), 110 mM NaCl, 10 mM KCl, 0.01 mM EDTA, 5 mM $MgCl_2$ and synaptosomes. The reaction was started by the addition of 3 mM ATP (as the final concentration) to the assay medium, and incubation was carried out at 37 °C for 30 min, unless otherwise specified. Liberated inorganic phosphate was measured according to the procedure described by Fiske and Subbarow.[18] The control value (subtracted) was determined in the presence of 1 mM ouabain. In the kinetic studies of Na^+-K^+-ATPase activity, the ATP concentration was varied from 0.3 mM to 3 mM, and the assay was performed within 2 min, because the ATP hydrolysis proceeds linearly within 5 min. (2) Ca^{2+}-ATPase activity was measured in 50 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM EGTA, 2 mM $CaCl_2$, 2 mM sodium azide, 1 mM ouabain and synaptosomes. The control value (subtracted) was determined in the presence of 0.2 mM EGTA. (3) Mg^{2+}-ATPase activity was measured in the same assay medium employed for Ca^{2+}-ATPase activity, except 0.2 mM $MgCl_2$ was used in place of $CaCl_2$. The control value (subtracted) was determined in the presence of 0.2 mM EGTA. In the latter two ATPases, other experimental conditions of the activity assays were the same as those described for the Na^+-K^+-ATPase activity assay.

Measurements of SH Content The SH content of the synaptosomal membrane proteins was determined according to the procedure described by Ellman.[19] The synaptosomes (0.3 mg protein/ml) were treated with 5 mM DTNB for 10 min at 37 °C in 20 mM Tris–HCl buffer (pH 8.0) in the presence of 3% SDS. The SH content was calculated using the molar extinction coefficient of 13600 $M^{-1}\,cm^{-1}$ at 412 nm.

Fluorescence Measurements of DPH- and TMA-DPH-Labeled Synaptosomes The aldehyde-modified synaptosomes (0.1 mg protein/ml) were incubated with 20 μM DPH or TMA-DPH for 10 min at 37 °C in buffer C. The fluorescence measurements were carried out at 25 °C using a Hitachi F-4500 spectrofluorometer. The excitation and emission wavelengths used in the fluorescence measurements of DPH- and TMA-labeled synaptosomes were 330 and 430 nm, respectively. The steady-state fluorescence anisotropy (γ) is defined as the value of $(I_V-I_H)/(I_V+2I_H)$, where I_V and I_H represent the fluorescence intensities of the vertically and horizontally polarized emitted lights with vertically polarized excitation, respectively.[20] The contribution of the products formed by modification of the synaptosomes with HNE or MDA to the fluorescence measurements was negligible.

Separation of Aldehyde-Modified Protein and Lipid Fractions HNE- or MDA-modified synaptosomes (3 mg protein/ml) were extracted with 3 ml of a chloroform/methanol (1 : 2 v/v) mixture and centrifuged at 1600×g for 10 min at room temperature to separate the protein and lipid fractions. The protein fraction obtained at the organic solvent/aqueous phase interface was collected and washed at once with the same chloroform/methanol mixture (3 ml), and then dissolved in 1 ml of 3% SDS. On the other hand, the combined chloroform layer (lipid fraction) was dried under N_2 gas flow and then dissolved in 1 ml of chloroform. The emission spectra of aldehyde-modified protein and lipid fractions were monitored at 25 °C, with an excitation wavelength of 355 nm.

Protein Determination The protein determination was performed according to the procedure of Lowry *et al.*[21] using bovine serum albumin as the standard.

Statistical Analysis Data were represented as the mean±S.D. of three independent determinations. To determine statistical significance between groups, the data were analyzed by an ANOVA Bonferroni's multiple *t*-test.

RESULTS

Changes in Synaptosomal ATPase Activities and SH Content by Aldehyde-Modification As shown in Table 1 and Fig. 1, treatment of the synaptosomes with 500 μM HNE resulted in a marked decrease in Na^+-K^+-ATPase activity in a time-dependent manner. On the other hand, the activities of Ca^{2+}- and Mg^{2+}-ATPases were almost not inhibited (less

Table 1. Effects of HNE Treatment on Synaptosomal ATPase Activities

HNE	Specific activites (μmol Pi/mg protein/min)		
	Na^+-K^+-ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase
−	0.506±0.006	0.213±0.005	0.224±0.003
+	0.137±0.002*	0.180±0.004*	0.175±0.014*

The concentration of HNE was 500 μM. Other experimental conditions are described under Materials and Methods. *$p<0.05$ *vs.* control (no HNE treatment) in each system.

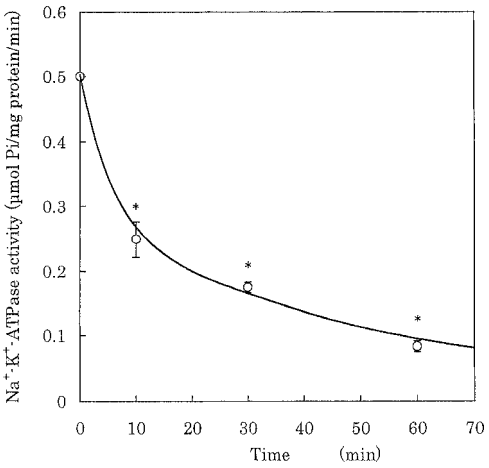

Fig. 1. Time Course of HNE-Induced Inhibition of Na^+-K^+-ATPase Activity

The synaptosomes were treated with 500 μM HNE. *$p<0.05$ *vs.* the activity at 0 time.

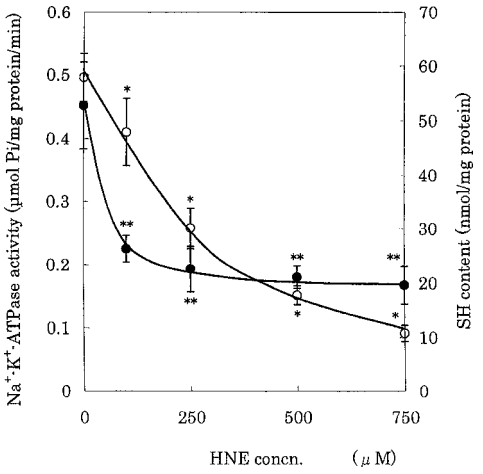

Fig. 2. Concentration Dependence of HNE on Na^+-K^+-ATPase Activity and SH Content in Synaptosomal Membranes

The HNE concentration was varied from 100 to 750 μM. The experimental conditions and procedures are described under Materials and Methods. Symbols: ○, Na^+-K^+-ATPase activity; ●, SH content. *,**$p<0.05$ *vs.* the control (no HNE treatment) in each system.

than 20% inhibition) by the treatment (Table 1).

Figure 2 shows the concentration dependence profiles of HNE on the Na^+-K^+-ATPase activity and SH contents in the membrane proteins. In these experiments, the synaptosomes were treated with various concentrations of HNE (100—750 μM). As can be seen in the figure, binding of HNE to the synaptosomes led to a dose-dependent inactivation of the ATPase activity with the loss of SH content in the membrane proteins. In addition, it was found that the HNE concentration required to induce half-maximal inhibition of the ATPase activity is approximately 250 μM (Fig. 2). In contrast, MDA did not affect the Na^+-K^+-ATPase activity, even at high concentrations (500 μM) of the aldehyde under the same conditions (0.495±0.033 and 0.479±0.021 μmol Pi/mg protein/min for control and MDA-treated synaptosomes, respectively). The SH content also did not change by treatment of the synaptosomes with MDA (51.5±1.6 and 52.7±1.1 nmol/mg protein the systems of control and MDA-treated ones, respectively).

Table 2. Effects of SH Reagents on Na^+-K^+-ATPase Activity and SH Content

SH reagent	Na^+-K^+-ATPase activity (μmol Pi/mg protein/min)	SH content (nmol/mg protein)
Control	0.476±0.003	49.3±1.3
NEM	0.020±0.002*	12.7±2.0*
PHMB	0.011±0.001*	8.3±1.5*

The concentrations of SH reagents were 500 μM. The conditions and procedure of modification of the synaptosomes with NEM or PHMB are the same as those with aldehydes described under Materials and Methods. *$p<0.05$ *vs.* control (no HNE treatment) in each system.

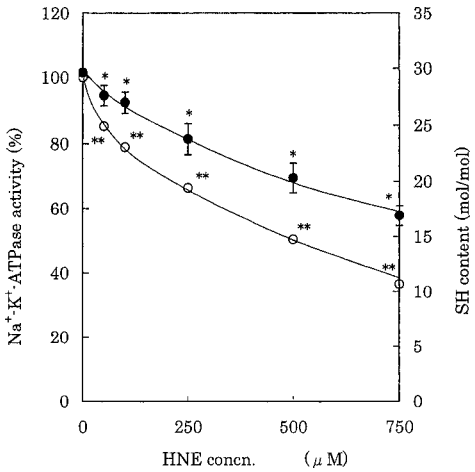

Fig. 3. Effects of Increasing Concentrations of HNE on the Activity and SH Content of Na^+-K^+-ATPase Preparation

Na^+-K^+-ATPase (1 mg/ml) was treated with various concentrations of HNE (50—750 μM) for 30 min at 37 °C. The reaction was terminated by the addition of 2 mM NAC, followed by dialysis against 30 mM Tris–HCl buffer (pH 7.4). The concentrations of the enzyme employed in the ATPase activity and SH content measurements were 10 μg and 1 mg/ml, respectively. The SH content was represented as mol per mol of the enzyme, assuming that the molecular weight of the enzyme is 294000.[36] The control ATPase activity is 0.75±0.01 μmol Pi/mg protein/min. Symbols: ○, Na^+-K^+-ATPase activity; ●, SH content. *,**$p<0.05$ *vs.* the control (no HNE treatment) in each system.

Effects of SH Reagents on Na^+-K^+-ATPase Activity The effects of several SH reagents on the synaptosomal Na^+-K^+-ATPase activity were examined.

As shown in Table 2, the ATPase activity was almost completely inhibited by treatment of the synaptosomes with NEM or PHMB. And, the SH content of the synaptosomal membrane proteins also markedly decreased by the treatment. From these results, it is suggested that modification of the SH groups in the membrane proteins is closely related to HNE-induced inactivation of synaptosomal Na^+-K^+-ATPase.

Effects of HNE-Modification on Enzyme Activity and SH Content in Na^+-K^+-ATPase Preparation To further confirm the involvement of SH-modification in HNE-induced inhibition of Na^+-K^+-ATPase activity, we used a Na^+-

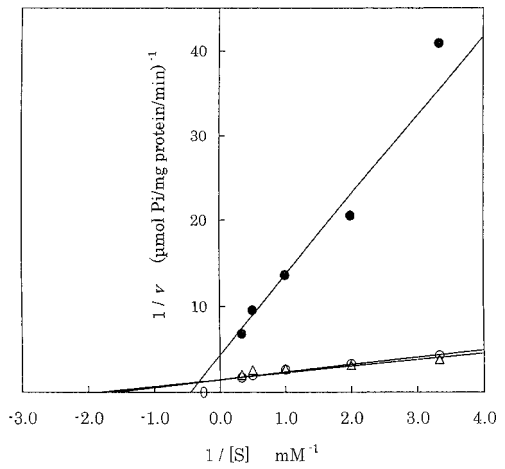

Fig. 4. The Double Reciprocal Plots of Na^+-K^+-ATPase Activity and ATP Concentration

The concentrations of HNE and MDA were 500 μM. Other experimental conditions are the same as those described in the legend to Fig. 2. Symbols: ○, control (no HNE treatment) synaptosomes; ●, HNE-treated synaptosomes; △, MDA-treated synaptosomes.

Table 3. Effects of Aldehyde-Modification on the Kinetic Parameters of Na^+-K^+-ATPase Activity

Addition	K_m (mM)	V_{max} (μmol Pi/mg protein/min)
No	0.60±0.03	0.68±0.01
HNE	2.37±0.51*	0.24±0.04*
HNE+ATP	0.60±0.03	0.65±0.02
MDA	0.52±0.02*	0.66±0.05

* $p<0.05$ *vs.* control (no HNE treatment) in each system.

K^+-ATPase preparation in place of the synaptosomes.

As shown in Fig. 3, the ATPase activity and SH content were decreased by treatment of the enzyme preparation with HNE in concentration-dependent manners, indicating that the loss of SH groups in the enzyme molecule plays an important role in HNE-induced inhibition of Na^+-K^+-ATPase activity.

Kinetic Parameters of Na^+-K^+-ATPase Activity In order to identify the mechanism of HNE-induced inhibition of synaptosomal Na^+-K^+-ATPase activity, the effects of aldehyde-modification on the kinetic parameters of the ATPase activity were examined (Fig. 4), and the results are summarized in Table 3. It is clear that treatment of the synaptosomes with HNE resulted in an increase of the K_m value for ATP and a decrease of the V_{max} value. In contrast, treatment of the synaptosomes with MDA did not affect these enzymatic parameters of Na^+-K^+-ATPase. In addition, treatment of the synaptosomes with 500 μM HNE in the co-presence of 10 mM ATP completely protected the Na^+-K^+-ATPase activity from the HNE toxicity (0.158±0.035 and 0.502±0.019 μmol Pi/mg protein/min for the systems without and with ATP). In this case, the ATPase activity of control synaptosomes was 0.492±0.035. Similar phenomena were also observed in the kinetic parameters of the enzyme activity (Table 3).

Effects of Aldehydes on Lipid Fluidity of Synaptosomal Membrane The effect of HNE- or MDA-modification on the membrane lipid fluidity of the synaptosomes was examined in terms of fluorescence anisotropy measurement.

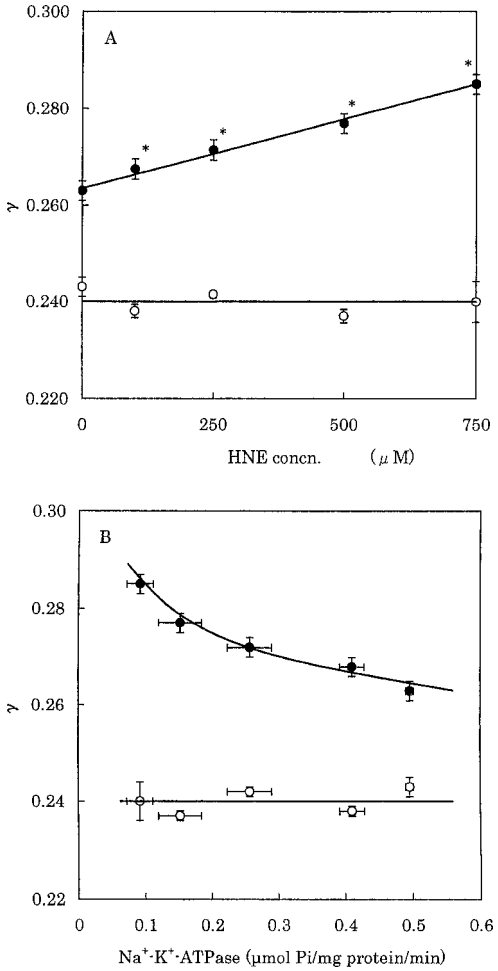

Fig. 5. Effects of HNE-Modification on Fluorescence Anisotropy of DPH- or TMA-DPH-Labeled Synaptosomes

(A) HNE Concentration Dependence. The concentration of HNE was varied from 100 to 750 μM. The procedure and conditions of the fluorescence anisotropy measurement were described under Materials and Methods. * $p<0.05$ *vs.* control synaptosomes (no HNE treatment). (B) Relationship between Na^+-K^+-ATPase Activity and Fluorescence Anisotropy of TMA-DPH-Labeled Synaptosomes. The data of Na^+-K^+-ATPase activity and fluorescence anisotropy were obtained from Figs. 2 and 5A, respectively. Symbols: ○, DPH-labeled synaptosomes; ●, TMA-DPH-labeled synaptosomes.

As shown in Fig. 5A, the fluorescence anisotropy (γ) of DPH-labeled synaptosomes did not change by HNE-modification over the concentration range tested (100—750 μM). In contrast, the γ value of TMA-DPH-labeled synaptosomes increased depending on the concentration of HNE. On the other hand, the extent of the fluorescence anisotropy of DPH-labeled ($\gamma=0.246\pm0.003$) and TMA-DPH-labeled ($\gamma=0.268\pm0.002$) control synaptosomes did not change by treatment with 500 μM MDA ($\gamma=0.242\pm0.002$ and 0.264 ± 0.001 for DPH- and TMA-DPH-labeled ones, respectively). These results suggest that HNE-modification induce decrease in the lipid fluidity at the membrane surface rather than membrane lipid interior. In addition, it was found that there is a good relationship between the Na^+-K^+-ATPase activity and the γ value of TMA-DPH embedded in the synaptosomal membranes (Fig. 5B).

Localization of Bound Aldehydes As shown in Fig. 6A, HNE- and MDA-modified synaptosomes showed characteristic emission spectra exhibiting the maximum at 425 nm and

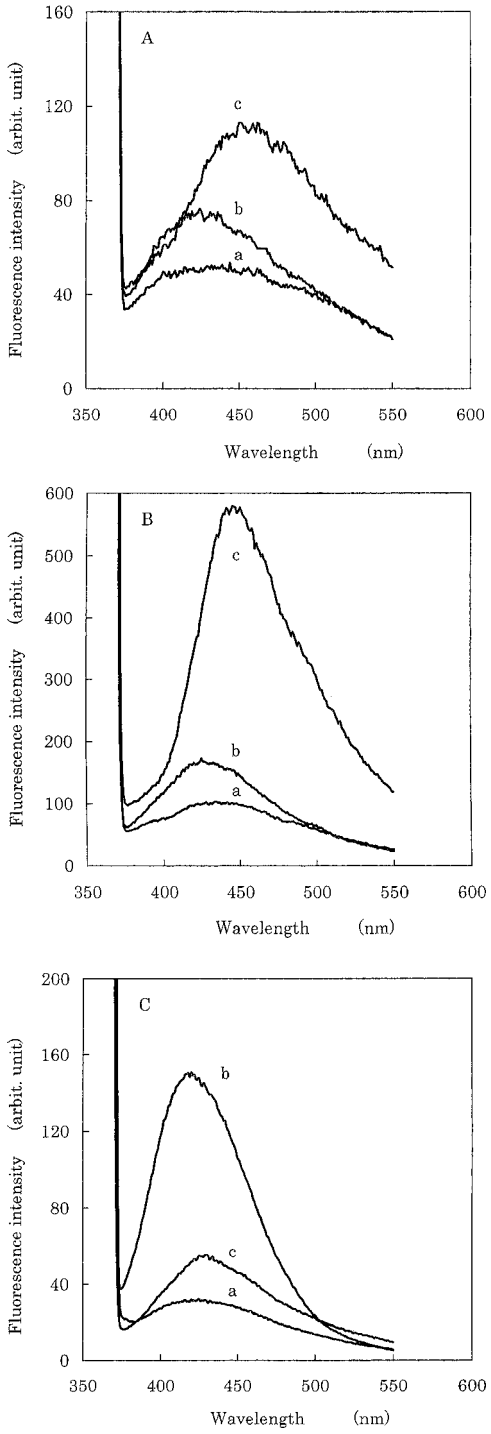

Fig. 6. Emissions Spectra of Fluorescent Products Formed by HNE- or MDA-Modification of Synaptosomes

The concentrations of HNE and MDA were 500 μM. The emissions spectra were monitored with the excitation wavelength at 355 nm. Other experimental conditions and the procedure of separating protein and lipid fractions from aldehyde-modified synaptosomes are described under Materials and Methods. (A) aldehyde-modified synaptosomes; (B) protein fractions; (C) lipid fractions. a, control (no HNE treatment); b, HNE-treated synaptosomes; c, MDA-treated synaptosomes.

457 nm, respectively. In addition, the analysis using the protein and lipid fractions separated from aldehyde-modified synaptosomes showed that HNE and MDA were mainly incorporated into the lipid and protein fractions, respectively (Figs. 6, B and C). In addition, the concentration dependence profile of HNE showed that fluorescent product(s) formation

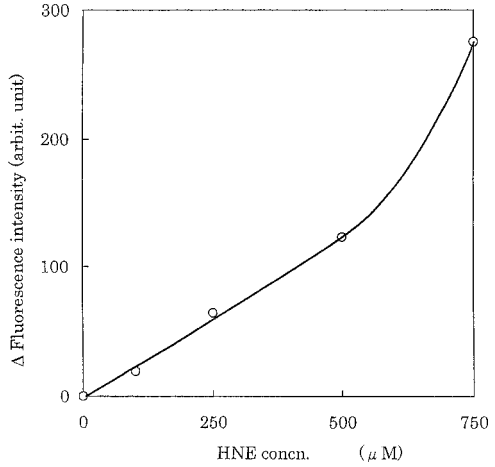

Fig. 7. Effects of Increasing Concentrations of HNE on Fluorescent Product(s) Formation in Lipid Fraction

The concentration of HNE was varied from 100 to 750 μM. The excitation and emission wavelengths were 355 and 430 nm, respectively. Other experimental conditions were the same as those described in the legend for Fig. 6. △ Fluorescence intensity is represented as the difference in the intensities of the systems with and without HNE-modification.

in the lipid fractions was dependent on the concentration of HNE (Fig. 7).

DISCUSSION

Treatment of the synaptosomes with HNE resulted in a time- and concentration-dependent inhibition of Na^+-K^+-ATPase activity (Figs. 1 and 2). The concentration of HNE required to induce half-maximal inhibition of the ATPase activity was determined to be approximately 250 μM (Fig. 2), which is in the range of the values previously reported for other enzymes.[3] On the other hand, HNE did not induce an appreciable inhibition of Ca^{2+}- or Mg^{2+}-ATPase activity (Table 1), suggesting that HNE-modification mainly affects Na^+-K^+-ATPase, rather than Ca^{2+}- and Mg^{2+}-ATPases, in the synaptosomes.

It was also found that HNE-modification causes the loss of SH groups in the membrane proteins (Fig. 2). In addition, modification of the synaptosomes with SH-directed reagents such as NEM and PHMB markedly inhibited the ATPase activity (Table 2). These results suggest that the inhibition of synaptosomal Na^+-K^+-ATPase activity by HNE treatment is mainly due to modification of the SH groups in the ATPase molecule. The results with the Na^+-K^+-ATPase preparation also supported this hypothesis (Fig. 3). As is well known, HNE can also react with lysine at both the double bond and the carbonyl moiety of the aldehyde to form secondary amine or Schiff's bases.[22] Xu has reported that Na^+-K^+-ATPase possesses a number of lysine residues in the active site, and that the ATPase is deactivated by modification of lysine residues.[23] However, a preliminary experiment using DNS-Cl showed that treatment of the synaptosomes with DNS-Cl (500 μM) for 30 min at 37 °C did not induce an appreciable inhibition of the Na^+-K^+-ATPase activity (0.476 ± 0.025 and 0.395 ± 0.036 μmol Pi/mg protein/min for control and DNS-treated ones, respectively). From this finding, we have ruled out the possibility that modification of lysine residues located in the active site of Na^+-K^+-ATPase is directly involved in

HNE-induced inhibition of the ATPase. The kinetic data indicated a decreased binding affinity of the substrate for the enzyme (Fig. 4, Table 3). In addition, the inhibitory effect of HNE against Na^+-K^+-ATPase activity was completely prevented by pre-treatment with the substrate, suggesting that inhibition of the enzyme activity associated with HNE-modification may reflect alterations in or near the active site in the enzyme molecule.

The results of the fluorescence anisotropy measurements showed that the γ value of TMA-DPH molecules embedded in the synaptosomal membrane lipids increased by treatment with HNE in a concentration-dependent manner (Fig. 5A). In contrast, the γ value of DPH-labeled synaptosomes did not change in the concentration range of HNE tested. Because TME-DPH is a positively charged probe, these results suggest that HNE-modification of the synaptosomal membranes cause a change in their lipid dynamics at the lipid/water interface on the membrane surface, not in the lipid hydrocarbon interior. An increased fluorescence anisotropy reflects a restricted motion of the fluorescence probes located in the membrane lipid layers due to increased lipid-lipid interactions.[24] From these findings, therefore, it is suggested that the lipid fluidity at the membrane surface decreased by modification of the synaptosomes with HNE. This finding is not consistent with the reports by Subramanian *et al.*[25] and Buko *et al.*,[26] in which they reported that modification of synaptosomal membranes[25] and liver plasma membranes[26] with HNE caused an increased lipid fluidity of the membranes.

As shown in Fig. 5B, there is good correlation between the Na^+-K^+-ATPase activities and the γ values of TMA-DPH-labeled synaptosomes in the presence of various concentrations of HNE. It has been well known that the dynamic environment around membrane proteins plays an important role in regulation of the activities of membrane-bound enzymes *via* alterations of lipid-lipid and lipid-protein interactions.[27—29] Based on these findings, it is suggested that lipid dynamics are an important factor in the inactivation mechanism(s) of synaptosomal Na^+-K^+-ATPase by HNE treatment, and we speculate that HNE-induced inhibition of the synaptosomal Na^+-K^+-ATPase activity may be related to changes in the reactivity of the SH groups located in or near the active site in the enzyme *via* changes in their membrane lipid dynamics.

It has been reported that lipid peroxidation products easily bind to proteins and phospholipids in membranes to form fluorescent products.[30] As shown in Fig. 6A, both HNE and MDA are bound to synaptosomal membrane components, resulting in the formation of fluorescence products exhibiting emission maxima at 425 and 457 nm, respectively. The results with the lipid and protein fractions separated from HNE- and MDA-modified synaptosomes showed that HNE and MDA were mainly incorporated into the lipid and protein fractions, respectively (Figs. 6, B and C), suggesting that the reactivity of HNE and MDA against the synaptosomal membrane components are different. In addition, it is clear that the extent of fluorescent product(s) formation in the lipid fraction is dependent on the concentration of HNE (Fig. 7). It is of interest that MDA-modification did not affect the fluorescence anisotropy of TMA-DPH- and DPH-labeled synaptosomes, nor Na^+-K^+-ATPase activity (Table 3), although MDA is also an important agent for inducing oxidative damage of cellular membranes.[3,31] And, the emission maximum of MDA-modified synaptosomes was 457 nm, which is significantly different from the emission maximum (425 nm) of HNE-modified ones (Fig. 6A), suggesting that the fluorescence is due to chromophores of another structure.[32] From these findings, it seems that the discrepancy observed between HNE- and MDA-modified synaptosomes may reflect the difference in action mechanisms of these aldehydic compounds on the synaptosomal Na^+-K^+-ATPase described above, although the exact mechanisms are unclear at present.

Recent studies have proposed that lipid peroxidation products of membranes, especially HNE, are involved in the onset of a variety of neuronal disorders.[33,34] In addition, several investigators have reported that HNE impairs the function of proteins relating to the regulation of ion homeostasis in cultured hippocampal neurons[13,16] and in non-neuronal cells such as liver cell microsomes.[35] Recently, Keller *et al.* also reported that HNE inhibits ion-motive ATPase and glutamate transport activities of hippocampal neurons.[14] In the present report, we have proposed the possibility that alterations in membrane lipid dynamics play an important role in the onset of HNE-induced inhibition of synaptosomal Na^+-K^+-ATPase activity. Although further detailed experiments using reconstituted membranes including Na^+-K^+-ATPase are necessary to elucidate the precise mechanisms, it seems that the present findings give us insight into the analysis of the mechanisms of HNE toxicity.

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