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Inhibition of synaptosome membrane-bound integral enzymes by organic solvents

by Marja Korpela, MD¹

KORPELA M. Inhibition of synaptosome membrane-bound integral enzymes by organic solvents. *Scand J Work Environ Health* 1989;15:64–68. The possible mechanism of the depressive effect of organic solvents on the central nervous system (CNS) was studied with synaptosome membranes as a model. The changes in the activities of the membrane-bound integral enzymes acetylcholinesterase, total adenosinetriphosphatase, and magnesium-activated adenosinetriphosphatase were determined after treatment with different concentrations of organic solvents in vitro. Aromatic hydrocarbons and chlorinated aliphatic hydrocarbons inhibited all the enzyme activities concentration dependently. Alcohols had no significant effect at the same dose levels. The results of the present study suggest that the CNS depressive effect of organic solvents may be based on their interaction with membrane integral proteins.

Key terms: alcohols, aliphatic chlorinated hydrocarbons, aromatic hydrocarbons, membrane effects.

Organic solvents are widely used in industry (eg, in spray painting, glueing, degreasing) and in households. Their most important toxic effect is the depression of the activity of the central nervous system (CNS) by a mechanism which is still unclear. The action of a wide variety of compounds capable of inducing anesthesia is thought to be either on lipid or on protein components of the membrane. One of the theories on anesthesia suggests that the potency of anesthetics is directly proportional to the lipid solubility of the anesthetic in question and that changes in membrane lipids are the critical factors leading to anesthesia (1). However, the action of several anesthetics does not fit this theory. Another theory, which is supported by the studies of Franks & Lieb (2–4), suggests that lipid-soluble anesthetics can interact nonspecifically with large hydrophobic regions of membrane proteins.

In previous studies by my co-workers and I, the interaction of organic solvents with integral proteins of erythrocyte membranes has been demonstrated in humans and rats at anesthetic and lower concentrations (5–7). In the present study I have investigated the changes in the activities of acetylcholinesterase (AChE), total adenosinetriphosphatase (total ATPase), and magnesium-activated adenosinetriphosphatase (Mg^{2+} -ATPase) in rat synaptosome membranes exposed to organic solvents under in vitro conditions to evaluate further the mechanism of the solvent-induced CNS depression on the cell membrane.

Materials and methods

Organic solvents

The organic solvents studied were benzene, toluene, styrene, and *o*-xylene (analytical grade, E Merck, Darmstadt, Federal Republic of Germany), 1,1,1-trichloroethane (analytical grade, Fluka AG, Buchs, Switzerland), trichloroethylene, 1,1,2,2-tetrachloroethane, and tetrachloroethylene (analytical grade, E Merck), methanol (analytical grade, E Merck), ethanol (minimum 99.5 % grade of purity, Oy Alko Ab, Finland), 1-propanol, and 1-butanol (analytical grade, E Merck).

Preparation of synaptosomes

Male Sprague-Dawley rats (weight 200 g) were decapitated. The cerebrum was gently separated from the cerebellum, brain stem, and meninges and immediately placed in ice.

The isolation of the synaptosomes was performed by the Percoll gradient method (8). Briefly, the samples were homogenized at 800 revolutions/min with a Teflon-glass homogenizer in isolation medium containing 0.32 M sucrose, 5 mM N-2-hydroxyethyl-piperazine-N'-2-ethane-ethylenediaminetetraacetic acid (HEPES), pH 7.5 (Sigma Chemical Co, St Louis, Missouri, United States) and 0.1 mM ethylenediaminetetraacetate (EDTA) (Sigma Chemical Co), and centrifuged at 1 000 g for 10 min to give a pellet containing nuclear and cell debris and a low-speed supernatant, which was further centrifuged at 12 000 g for 20 min to produce the crude mitochondrial pellet, which was resuspended in 3 ml of isolation medium per gram of original wet tissue and homogenized gently.

The stock solution of isosmotic Percoll (SIP) (Sigma Chemical Co) was made by the addition of nine parts (volume/volume) of the original Percoll solution

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to one part (volume/volume) of 2.5 M sucrose, and the lower density media by the dilution of SIP with a solution containing 0.25 M sucrose, 5 mM HEPES (pH 7.2), and 0.1 mM EDTA. The pH of the gradient solutions was adjusted to 7.5. Percoll step gradients were made by the overlaying of 4 ml of 16 % Percoll solution with 4 ml of 10 % Percoll solution. The resuspended mitochondrial pellet was diluted with eight volumes of 8.5 % Percoll solution to give a final concentration of Percoll 7.5 %, and 4.5 ml of this suspension was layered onto the 10/16 % Percoll gradients. After centrifugation at 15 000 g for 20 min, the synaptosomes were collected and stored in ice until used. All the procedures used for preparing the synaptosomes were carried out at 0–4°C.

Determination of acetylcholinesterase activity

In determining the AChE activity in synaptosome membranes, the method of Ellman et al (9) was used.

In the reaction mixture [10.4 ml of synaptosome suspension and 2.6 ml of 0.1 M phosphate buffer (pH 8.0)], different concentrations of organic solvents were added with a Hamilton microsyringe. After 30 min of incubation, in 3-ml, glass-stoppered tubes at 37°C with continuous shaking, the samples were transferred into a thermostated cuvette, and 100 µl of 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Co) was added. The absorbance at 412 nm was set at zero, and the reaction was started by the addition of 20 µl of 0.075 M acetylthiocholine iodide. The change in the absorbance was measured for 5 min. The AChE activity was expressed as moles of substrate hydrolyzed per minute per gram of protein, and the activities of the solvent-treated samples were given as the percentage of the activities of the controls.

Determination of adenosinetriphosphatase activities

In the determination of the ATPase activities, a modification of the discontinuous method based on the determination of inorganic phosphate (10) was used.

The organic solvents were added to the 2-ml reaction mixture [50 mM tris(hydroxymethyl)aminomethanehydrochloride acid (Tris-HCl) buffer (pH 7.6), 1.5 mM adenosine-5'-triphosphate (ATP) (Sigma Chemical Co), 6.0 mM magnesium chloride, 100 mM sodium chloride, 20 mM potassium chloride, and 0.1 ml of synaptosome suspension] with a Hamilton microsyringe, and the tubes were glass-stoppered and mixed. The total ATPase activity was measured with sodium (Na⁺), potassium (K⁺), and magnesium (Mg²⁺) ions present in the reaction mixture, and Mg²⁺-ATPase activity with only the magnesium ion present in the mixture. After 1 h of incubation at 37°C with continuous shaking, 2 ml of ice-cold 10 % trichloroacetic acid (TCA) was added. After centrifugation at 3 000 g for 5 min, 1 ml of the supernatant was removed for the assay, and 3 ml of 0.1 N sodium acetate, 0.4 ml of 1 % molybdate/1 N sulfuric acid solu-

tion, and 2 ml of ascorbic acid were added. The absorbances of the samples were read at 770 nm after 25 min. The enzyme activities were expressed as moles of inorganic phosphate formed per hour per milligram of protein, and the activities of the solvent-treated samples were given as the percentage of the activities of the controls.

Protein determination

The method described by Lowry et al (11) was used for the protein determinations.

Statistical methods

All the solvent concentrations were measured in triplicate, and three independent measurement series were carried out for each solvent studied. In the statistical calculations, Student's t-test (4 degrees of freedom) was used.

Results

Acetylcholinesterase activity

The AChE activity of the control samples was 0.10 (SE 0.05) mmol of substrate hydrolyzed per minute per gram of protein.

The aromatic hydrocarbons and chlorinated aliphatic hydrocarbons inhibited the AChE activity concentration dependently. AChE was significantly ($P < 0.001$) inhibited by toluene, styrene, and *o*-xylene at concentrations of 3 mM or higher and by benzene at concentrations of 9 mM ($P < 0.01$) or higher (figure 1). Of the chlorinated aliphatic hydrocarbons, 1,1,2,2-tetrachloroethane was strongly AChE-inhibiting, and it showed a clear concentration dependency. The other solvents of this group had a slighter but still significant inhibitory effect, ie, trichloroethylene and tetrachloroethylene from the concentration of 3 mM up ($P < 0.01$) and 1,1,1-trichloroethane from the concentration of 6 mM ($P < 0.05$) up (figure 1). Of the alcohols studied, 1-butanol was slightly AChE-activating at the highest concentrations (20 and 30 mM). Methanol, ethanol, and 1-propanol had no effect on the enzyme activities (figure 1).

Adenosinetriphosphatase activities

The control samples had a total ATPase activity of 0.54 (SE 0.01) µmol of inorganic phosphate per hour per milligram of protein and a Mg²⁺-ATPase activity of 0.21 (SE 0.01) µmol of inorganic phosphate per hour per milligram of protein.

The total ATPase was significantly inhibited by all the aromatic hydrocarbons at concentrations of 3 mM or higher. [The significance at 3 mM was $P < 0.05$ for benzene and styrene, $P < 0.01$ for *o*-xylene, and $P < 0.001$ for toluene (figure 2).] At the two highest concentrations, styrene and *o*-xylene were the most effective compounds of this group. 1,1,2,2-Tetrachloro-

Figure 1. Effect of different concentrations of aromatic hydrocarbons, chlorinated aliphatic hydrocarbons, and alcohols on acetylcholinesterase (AChE) activity in rat synaptosome membrane. The means and standard error of the means of nine independent measurements are shown.

Figure 2. Effect of different concentrations of aromatic hydrocarbons, chlorinated aliphatic hydrocarbons, and alcohols on total adenosinetriphosphatase (ATPase) activity in rat synaptosome membrane. The means and standard error of the means of nine independent measurements are shown.

ethane inhibited total ATPase even more than AChE at all the concentrations. The other chlorinated aliphatic hydrocarbons were inhibitory, too, but to a less extent (figure 2). Alcohols had no effect on enzyme activity.

Mg^{2+} -ATPase was inhibited by all the aromatic hydrocarbons and, of the chlorinated aliphatic hydrocarbons, by 1,1,2,2-tetrachloroethane, tetrachloroethylene, and trichloroethylene. Alcohols had no significant effect on the Mg^{2+} -ATPase activity (figure 3).

Discussion

The results of the present study show that the studied aromatic hydrocarbons and chlorinated aliphatic hydrocarbons inhibit AChE and ATPase activities in rat synaptosome membrane concentration dependently. These results agree with previous findings with erythrocyte membrane (5–7). Organic solvents affect both erythrocyte membrane AChE, which is situated on the outer half of the membrane, and ATPase, the location of which is mainly on the cytoplasmic side of

Figure 3. Effect of different concentrations of aromatic hydrocarbons, chlorinated aliphatic hydrocarbons, and alcohols on magnesium-activated adenosinetriphosphatase (Mg^{2+} -ATPase) activity in rat synaptosome membrane. The means and standard error of the means of nine independent measurements are shown.

the erythrocyte membrane (12). Many other compounds have also been shown to inhibit both erythrocyte and synaptosome membrane enzymes, eg, local anesthetics (13–16), fatty acids (17, 18), and alcohols (19–23). The similarity of the effects of organic solvents on synaptosome membrane and erythrocyte membrane suggests that even the erythrocyte membrane is a suitable model in studies of the mechanism of the anesthetic-like CNS effects of organic solvents.

The enzyme inhibition induced by organic solvents in the present study was mainly related to the lipid solubilities of the solvents. However, 1,1,2,2-tetrachloroethane, which is the most toxic of the chlorinated hydrocarbons, was a very potent enzyme inhibitor, although its lipid solubility is low in comparison to, eg, that of the aromatic hydrocarbons.

The molecular structure of a compound may be one of the factors determining its action on biological membranes. Usually, the CNS-depressive properties of solvents are enhanced by halogenation and by an increase in the length of the carbon chain. It has also been shown that the inhibition of AChE by local anesthetics is dependent on the structure of these anesthetics (24).

In the present study, the ATPase activities were inhibited more than the AChE activity, although the location of these enzymes would indicate that AChE is more easily affected.

No significant changes were found between the total ATPase and the Mg^{2+} -ATPase inhibitions. Only 1,1,2,2-tetrachloroethane inhibited the total ATPase significantly more than Mg^{2+} -ATPase. Synaptosomal Na^+ - K^+ -ATPase, and Mg^{2+} -ATPase also have identical sensitivities to local anesthetics (24). This phenomenon may indicate a structural relationship,

similarity, or identity between these two proteins. The alcohols tested had no significant effects on the enzyme activities at the concentrations used. Higher concentrations of alcohols have been shown to inhibit synaptosomal enzymes (19–22).

The present results suggest that organic solvents interact with membrane-bound integral proteins even at concentrations too low to cause any changes in the lipid components of the membrane. Not even clinical concentrations of anesthetics have been found to cause any changes in the bilayer structure in short exposures (2). The studies of Greenberg & Tsong (25, 26) indicate that the local anesthetic site in the membrane is an integral protein. Thus the changes in the integral enzymes AChE and ATPase may be the critical factor which determines the anesthetic CNS effect of solvents.

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