INHIBITION OF Mg- AND Mg, Na, K-ACTIVATED ADENOSINE TRIPHOSPHATASE ACTIVITY BY L-ASCORBIC ACID AND L-CYSTEINE

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It is now commonly postulated that the electrical excitability of neural cells depends on the concentration gradients of Na⁺ and K⁺ across the cell membrane and the gradients are maintained by active cation transport system. Further, a membrane-bound, Na⁺ and K⁺-stimulated and ouabain-sensitive adenosine triphosphatase (Na-K-ATPase) has been proved to be involved in the active cation transport system in the brain as well as in many other tissues (1). While cardiac glycosides have been well known to be specific inhibitors of Na-K-ATPase, some biogenic substances were also considered to participate in the transport mechanism. In this respect, two biogenic substances, ascorbic acid and cysteine, came to light. They were described as rather specific inhibitors of Na-K-ATPase in the electric organ of eel (2) and the liver of rat (3). The present report describes how ascorbic acid and cysteine affect Na-K-ATPase and Mg-ATPase of the rabbit brain, histochemically and biochemically. For reference the enzymes of rabbit liver were tested.

METHODS

1. Histochemical demonstration of ATPase

Male albino rabbits weighing 1.8 to 2.2 kg were used. Immediately after sacrifice of the animal by exsanguination, the cerebrum and the liver were removed. Further procedures for staining of the cerebral and liver ATPase were followed to the method of McClurkin (4). Small pieces of the cerebrum and the posterior part of the left lobe from the liver were cut out and fixed in an ice-cold 10% formalin with 0.25 M sucrose for 18 to 20 hours. Then the specimens were washed in ice-cold distilled water, placed into the test tubes and immediately frozen in a mixture of aceton and dry ice at about -70°C. The frozen tissues were sectioned at 10 μ thickness in a cryostat at -17°C. The sections were floated on cold 0.2 M Tris-HCl (pH 7.2) and stained for ATPase within 2 hours. The sections were incubated in a freshly prepared medium containing 3 mM ATP-Ba₂⁺ (dissolved in 0.1 N HCl), 24 mM Tris-HCl (pH 7.8), 3 mM Pb(NO₃)₂, and 3 mM MgSO₄ (the final pH 7.2). Some sections were incubated in a medium added with 100 mM NaCl and 30 mM KCl. Incubation was carried out at 37°C for 10 to 30 minutes, depending on the enzyme activity of tissues. After incubation, the sections were rinsed.

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in distilled water, floated on 1% ammonium sulfide solution for one minute, rinsed again in distilled water and mounted in buffered glycerin for microscopy.

In some experiments, instead of ATP-Ba₄ and MgSO₄ in McClurkin's incubation medium, Tris-ATP and MgCl₂ were used according to the reaction mixture in biochemical assay.

In the first series of experiments, agents to be tested were added to the incubation medium. The pH was adjusted to 7.2 with 0.1 M Tris after adding the agents. In the second series of experiments, tissue slices at 3 to 4 mm thickness were immersed in Ringer-Locke's solution containing the test agents and bubbling with oxygen. Control sections were incubated in a medium without substrate at 37°C for 10 to 30 minutes, or incubated in a complete medium after immersion in hot water at 90°C for 5 minutes to destroy the enzyme activity.

2. Biochemical determinations of ATPase activity

Preparation of a microsomal fraction from rabbit cerebrum: Male albino rabbits weighing 1.8 to 2.2 kg were killed, exsanguinated from the common carotid arteries. The brain was removed and the cerebral hemispheres were taken out in an isolation medium (0.25 M sucrose, 5 mM EDTA-Na₂, 30 mM histidine, pH 6.8) described by Skou (5). After removal of the pia-arachnoid membranes, 2 g of brain pieces were placed in an ice-cold Teflon homogenizer (A.H. Thomas Co.; No. 4228-B), added with 0.1% sodium desoxycholate and ground in 9 volumes of the isolation medium. Grinding involved 6 passages of the pestle, rotating at 2,800 rev/min for 1 minute in an ice bath. Immediately thereafter, the homogenates were centrifuged at 8,000 × g for 10 minutes. The microsomal fraction was obtained as sediments by centrifuging of 8,000 × g supernatants for 30 minutes at 100,000 × g in the isolation medium. The final sediments were suspended in 0.25 M sucrose containing 1 mM EDTA-Na₂ and 30 mM histidine at pH 7.0, as described by Skou (5), then kept frozen at -20°C as stock sample. Low speed centrifugation was carried out in an angle head of the usual centrifuge (Kubota), and for high speed centrifugation up to 100,000 × g the no. 40 head of the Spinco model L centrifuge was used.

ATPase assay: Reaction mixtures of 2.0 ml were prepared in tubes in ice, containing 100 mM Tris-HCl (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂ and 0.1 mg protein of microsomal fraction. Preincubation was carried out at 37°C for 5 minutes, then 3 mM Tris-ATP was added. After shaking for 15 minutes, the tube was placed in ice and 0.5 ml of 40% trichloroacetic acid was added. After centrifugation, a 0.6 ml aliquot of the supernatant was provided for determination of inorganic phosphate as described by Fiske and Subbarow (6). The activity of ATPase was expressed as µmoles Pi liberated/mg protein/hr. Protein was determined by the method of Lowry et al. (7), using crystalline bovine plasma albumin as standard.

Tris-ATP: A free form of ATP was made by a passage of disodium ATP through a Dowex-50 cation exchange resin column (H⁺form). Concentration of ATP in the filtrate from the column was determined by reading the extinction at 260 mμ of a dilution, when a millimolar extinction of ATP at 260 mμ was 14.2 at pH 2.0 (8). The free ATP was made
to pH 7.2 with 0.5 M Tris, and water was added to make a final concentration of the solution 30 mm in ATP. The stock solution was stored at -20°C until use. The yield of ATP with this method was 85 to 90% and the contents of inorganic phosphate and sodium ion in the 30 mm ATP solution were found to be less than 1.3 mm and 4 mm, respectively.

Reagents: ATP-Ba$_2$ (Nutritional Biochemical Co.), ATP-Na$_2$ (Sigma Chemical Co.), L-glutathione (SH) (Daiichi Pharmaceutical Co., Tokyo) and ouabain (Merck) were used. Other agents were purchased from Nakarai Chemical Co., Kyoto. Water was distilled after treatment with ion exchange resin.

RESULTS

1. Histochemical demonstration of ATPase in rabbit cerebrum and liver

Cerebrum: When ATP, Mg$^{++}$, Na$^+$ and K$^+$ were present in the incubation medium, the enzyme activity of ATPase was seen as dark stain in the plasma membranes of nerve cells and in the capillary endothelium. Nuclei of them were also stained darkly. In the absence of Na$^+$ and K$^+$ in the incubation medium, there was no stain in the plasma membranes of nerve cells and faint stain appeared in the cytoplasmic area of nerve cells and the capillary endothelium. Nuclei and nucleoli of nerve cells were also stained faintly.

Liver: In the presence of ATP, Mg$^{++}$, Na$^+$ and K$^+$ in the incubation medium, the activity of ATPase was seen in the plasma membranes of liver cells, the endothelial cells of central veins and other blood vessels and the sinusoids. The bile canaliculi, or the plasma membranes of hepatic cells fronting the bile canaliculi were stained heavily. Stellate cells in the sinusoids had also dark stain. In the nuclei and especially nucleoli of liver cells, a dark stain was seen. Sections incubated similarly but without Na$^+$ and K$^+$ showed a weak staining in the endothelial tissues. No stain occurred in the plasma membranes of hepatic cells and the bile canaliculi. The nuclei and nucleoli were not stained or, if any, extremely faint.

Controls: There was no stain in sections of cerebrum and liver incubated in a medium without substrate. Immersion of sections in hot water at 90°C inactivated the enzyme activity and made unstained.

2. Effects of L-ascorbic acid and other agents on ATPase staining in rabbit cerebrum and liver

L-Ascorbic acid, L-cysteine and, as reference agents, L-glutathione-SH, L-methionine and ouabain were examined. In the presence of ATP, Mg$^{++}$, Na$^+$ and K$^+$, the addition of L-ascorbic acid (10$^{-6}$M) reduced the ATPase activity in all regions of the cerebrum (Table 1) and the liver (Table 2). There was no stain in the plasma membranes of the liver cells and the neural membranes in the cerebrum. The bile canaliculi were also unstained. L-Cysteine (10$^{-6}$M) and L-glutathione-SH (10$^{-6}$M) did not affect or, if any, slightly reduced the Na-K-ATPase staining in the cerebrum and the liver. In the absence of Na$^+$ and K$^+$, L-ascorbic acid, L-cysteine and L-glutathione-SH did not significantly affect ATPase staining. The addition of L-methionine (10$^{-4}$M) did not affect the staining, irrespective of Na$^+$ and K$^+$. Ouabain (10$^{-4}$ M) inhibited the Na-K-ATPase activity in the plasma membranes of the liver cells, the bile canaliculi and the neural membranes in
TABLE 1. Effects of drugs on adenosine triphosphatase staining of rabbit brain cortex.

| Drugs          | ATPase staining                | Neuronal cells | Endothelial cells of capillaries |
|----------------|-------------------------------|----------------|---------------------------------|
|                | Plasma membranes | Cytoplasmic area |                    |
|                | Mg++                    | −              | −                 | +                 |
| Mg++ + Na+ + K+| +                        | +              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |
| Mg++ + Na+ + K+| −                        | −              | +                 |

++ : strong staining, + : weak staining, − : no staining.

TABLE 2. Effects of drugs on adenosine triphosphatase staining of rabbit liver.

| Drugs          | ATPase staining                | Liver cells | Bile canaliculi | Endothelial cells of central veins | Stellated cells |
|----------------|-------------------------------|-------------|-----------------|-----------------------------------|-----------------|
|                | Plasma membranes | Cytoplasmic area |                    |                                   |                 |
|                | Mg++                    | −           | +               | −                                 | +               |
| Mg++ + Na+ + K+| +                        | +           | +               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |
| Mg++ + Na+ + K+| −                        | +           | −               | +                                 | +               |

++ : strong staining, + : weak staining, − : no staining.

The essentially similar results were obtained on tissue slices incubated in Ringer-Locke solution containing above described agents. The reducing action of ATPase staining was most obvious in the regions around blood vessels. The use of the incubation medium containing Tris-ATP for ATP-Ba₂ (see Methods) did not alter the results described above.

3. Activity of ATPase in microsomal fraction of rabbit cerebrum

According to the observations of Schwartz et al. (9), 75% of Na-K-ATPase activity in the guinea pig cerebral cortex is present in a microsomal fraction. Therefore, the microsomal fraction was used in the present study. The conditions for ATPase assay were based on those of Skou (5), as described under Methods. There was a linear relationship between

the cerebrum.

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liberation of inorganic phosphate and incubation time for at least 17 minutes. In further experiments, the reaction was carried out for 15 minutes. In order to know the amount of inorganic phosphate liberated from non-enzymatic hydrolysis of ATP or from sources other than substrate ATP, a reaction mixture without microsomal enzyme or ATP was incubated. In a reaction mixture without ATP, even a trace of inorganic phosphate was undetectable at 0 and 15 minutes of incubation, indicating that inorganic phosphate derived from sources other than ATP preparation was negligible. In a reaction mixture without microsomal protein as enzyme, the amount of inorganic phosphate at the beginning of incubation, which was probably due to a contamination of ATP preparation, did not change after 15-minute incubation. This fact indicates that non-enzymatic hydrolysis of ATP within 15 minutes at 37°C was negligible. Inorganic phosphate liberated by enzymatic hydrolysis was thus estimated by subtracting the amount of the phosphate in a reaction mixture without microsomal protein from that in a complete reaction system. There was no linear relationship between the initial velocity of enzymatic reaction and the amount of enzyme between 0.1 and 1.0 mg protein/tube. In further experiments, microsomal protein 0.1 mg/tube was used. Changes in pH of reaction mixture affected only slightly the activity of Mg-ATPase but very markedly Na-K-ATPase. The optimal pH of Na-K-ATPase was 7.8. The effects of substrate concentration of ATPase activity were examined and a nearly maximal activity was obtained with 3 mm ATP for both Mg- and Na-K-ATPase. The concentration of Mg" required for maximal activity was 1.5 mm for Mg-ATPase and 3 mm for Na-K-ATPase. For maximal activity of Na-K-ATPase, 100 mm of Na+ and 20 mm of K+ were sufficient, and at higher concentrations of these ions Mg-ATPase activity was rather somewhat inhibited. The activity of both enzyme increased proportionally to a rise of incubation temperature from 20°C to 45°C.

Thus, suitable conditions for maximal ATPase activities at a physiological temperature were indicated from data obtained above. In next experiments, 100 mm Tris-HCl (pH 7.8), 3 mm MgCl₂, 100 mm NaCl, 20 mm KCl, Tris-ATP 3 mm and 0.1 mg of microsomal protein were used in the reaction mixtures. Incubation was carried out for 15 minutes at 37°C (Table 3). Under these conditions, it was found that ouabain 2 × 10⁻⁵ M inhibited the activity of Na-K-ATPase almost completely, while it had no effect on the activity of Mg-ATPase.

**Table 3. Conditions for assay of adenosine triphosphatase.**

| Reaction mixture | Tris-HCl (pH 7.8) | 100 mm |
|------------------|-------------------|--------|
| MgCl₂            | 3 mm              |
| NaCl             | 100 mm            |
| KCl              | 20 mm             |
| ATP (Tris salt)  | 3 mm              |
| Microsomal protein | 0.1 mg        |

**Incubation time**

| 15 min |

**Incubation temperature**

| 37°C |
4. Effects of L-ascorbic acid and other agents on the microsomal ATPase

The assay conditions were described above, and the effects of L-ascorbic acid, L-cysteine, L-glutathione (SH) and L-methionine were examined (Table 4). L-Ascorbic acid $10^{-4}$ M inhibited both Mg-ATPase and Na-K-ATPase to 64.7% and 23.2%, respectively. L-Cysteine $10^{-4}$ M also inhibited Mg-ATPase and Na-K-ATPase to 60.1% and 43.8% of control, respectively. The activities of Mg-ATPase and Na-K-ATPase were plotted against the concentration of L-ascorbic acid and L-cysteine in a representative experiment with microsomal preparation (Figs. 1 and 2). L-Ascorbic acid and L-cysteine inhibited almost completely Na-K-ATPase at a concentration of $10^{-4}$ M. Unlike L-cysteine, L-glutathione $10^{-4}$ M showed statistically non-significant effects on Mg-ATPase and Na-K-ATPase. Similar results were obtained with three different preparations of microsomal fractions from rabbit brain cortex.

**Table 4. Effects of drugs on adenosine triphosphatase activities of microsomal fractions from rabbit brain cortex.**

| Drugs         | ATPase activities (μ moles Pi/mg protein/hr) | Mg-ATPase activity (%) | Mg-Na-K-ATPase activity (%) |
|---------------|--------------------------------------------|------------------------|----------------------------|
|               |                                            | Mg-ATPase               | Na-K-ATPase activity       |
| L-Ascorbic acid $10^{-4}$ M | 21.1±1.6 (64.7) | 12.9±10.7 (23.2)      |                           |
| L-Cysteine $10^{-4}$ M          | 19.6±2.9 (60.1) | 24.3±3.1* (43.8)       |                           |
| L-Glutathione (SH) $10^{-4}$ M  | 29.2±5.6 (80.6) | 50.1±16.2 (90.3)       |                           |
| L-Methionine $10^{-4}$ M        | 30.2±5.2 (92.6) | 65.0±18.9 (117.1)      |                           |

Mean in three observations ± standard error.

* statistically significant (P<0.05)

**Fig. 1.** Relationship between the concentration of L-ascorbic acid and the activities of Mg-ATPase and Na-K-ATPase in a rabbit brain microsomal fraction.

**Fig. 2.** Relationship between the concentration of L-cysteine and the activities of Mg-ATPase and Na-K-ATPase in a rabbit brain microsomal fraction.
fraction. \textit{L}-Methionine $10^{-4}$ M inhibited neither Mg-ATPase nor Na-K-ATPase in all microsomal preparations tested.

\textbf{DISCUSSION}

In the histochemical demonstration of ATPase, omission of Na$^+$ and K$^+$ from the medium resulted in a loss of stain in the plasma membranes of neural cells in the cerebrum, the plasma membranes of liver cells and the bile canaliculi of the liver. In a medium containing Na$^+$ and K$^+$, ouabain inhibited the staining in these structures. The observations were in agreement with those of McClurkin (4), indicating the presence of ouabain-sensitive Na-K-ATPase in these structures. In the endothelial tissues of the cerebrum and liver, the ATPase staining was made stronger by the addition of Na$^+$ and K$^+$. But the staining was unaffected by the addition of ouabain. These observations may suggest the possible presence of ouabain-insensitive Na-K-ATPase in the endothelial tissues.

On the phosphatase staining of nuclei, Gomori (10) considered the stain in the nuclei to be a result of diffusion of reaction product and selective affinity of nuclear substance to lead complexes. In experiments with the nuclei isolated in non-aqueous media for preventing the diffusion of reaction product, Stern and Mirsky (11) showed the absence of acid phosphatase, alkaline phosphatase and ATPase, while Fisher et al. (12) and Siebert (13) demonstrated the presence of ATPase. According to Gomori, the staining of nucleus with a lead salt method for the demonstration of phosphatase does not always show the enzymatic activity, and the effects of drugs on stain in the nuclei were not necessarily due to the effects on the nuclear enzyme. The significance of the staining of nucleus and the effects of drugs on it must be studied by chemical and biochemical approach.

The distribution of ATPase in the subcellular fractions of brain has been studied by Skou (5), Schwartz (9), Jännefelt (14), Deul and McIlwain (15) and Aldridge (16). The Na-K-ATPase was mostly present in the microsomal fraction. In the rabbit brain microsomal fraction of this study, there were 25 to 35 $\mu$moles Pi/mg protein/hr of specific activity of Mg-ATPase and 35 to 80 $\mu$moles Pi/mg protein/hr of Na-K-ATPase activity. The addition of Na$^+$ and K$^+$ to the reaction system approximately tripled the activity of Mg-ATPase, giving ratio of 2.4 to 4.2. The activation of enzyme by alkali metals averaging threefold was described by Deul and McIlwain (15) and the activation up to eightfold by Skou (5). The latter author used a reaction mixture containing 6 mM Mg$^{2+}$ for Na-K-ATPase assay, but in this study the activation ratio did not increase with 6 mM Mg$^{2+}$.

Histochemical and biochemical studies were performed for testing the effects of L-ascorbic acid and L-cysteine on ATPase activity. In the histochemical studies, both agents $10^{-4}$ M seemed to inhibit only a Na-K-ATPase staining and had likely no effect on the Mg-ATPase staining in the brain and liver. Biochemically, these substances inhibited both Mg-ATPase and Na-K-ATPase in the brain microsomal fraction with a predominat effect on the latter enzyme. Since the ouabain-insensitive Na-K-ATPase, described in a histochemical study, was not detectable in microsomal fraction, the effects of L-ascorbic acid and L-cysteine on this enzyme were not examined biochemically. Regarding the inhibitory
effect of L-ascorbic acid and L-cysteine on Mg-ATPase, the histochemical results were inconsistent with those obtained biochemically. It was possible that the histochemically demonstrable Mg-ATPase was inhibited by formaldehyde fixation and stained weakly, thus further inhibition of the enzyme activity by L-ascorbic acid or L-cysteine was made obscure. L-Glutathione-SH 10^{-4} M showed a slight inhibition on Na-K-ATPase, and L-methionine 10^{-4} M had no effect on ATPase either histochemically or biochemically.

Glynn (2) showed that L-ascorbic acid 10 mm with p-phenylenediamine 1 mm inhibited Na-K-ATPase by 18% without showing any significant effect on Mg-ATPase in electric organ of eel. Emmelot and Bos (3) described the inhibitory effect of L-cysteine on Na-K-ATPase by 30 to 60% and a slight inhibition on Mg-ATPase by 0 to 10%, using rat liver cytoplasmic membranes. Thus, L-ascorbic acid and L-cysteine were recognized as specific inhibitors of Na-K-ATPase. A slight or no effect of ascorbic acid and cysteine on Mg-ATPase may perhaps be due to species or organ difference of nature of Mg-ATPase itself.

Rajalakshmi and Patel (17) reported that a tranquilizer increased the synthesis of ascorbic acid by liver homogenate and raised the concentration of ascorbic acid in the liver, adrenals and brain. It is interesting to investigate whether L-ascorbic acid in higher concentration in the brain would produce a tranquilizing effect and, if so, it might be related to the inhibition of the brain Na-K-ATPase by the acid. Thus, further question remains whether the physiological level of L-ascorbic acid in the brain would play an important role in normal function of the brain.

**SUMMARY**

1. In the histochemical demonstration of Mg-ATPase and Na-K-ATPase, stain location was described in cerebral and liver sections. With cerebral sections, in the presence of Mg^{2+} and ATP in the incubating medium, faint stain of Mg-ATPase was observed in cytoplasmic area of neuronal cells and capillary endothelium. When Na^+ and K^+ were added to the incubating medium, dark stain occurred in plasma membranes of neuronal cells and capillary endothelium. With liver sections, stain and faint stain of Mg-ATPase were also observed in cytoplasmic area and endothelial tissues. When Na^+ and K^+ were added, dark stain was seen in plasma membranes of liver cells, bile canaliculi and endothelial tissues.

2. L-Ascorbic acid (10^{-4} M) reduced the staining of Na-K-ATPase in all regions in cerebral and liver sections, especially in plasma membranes of neuronal cells, and bile canaliculi, but the acid did not affect staining of Mg-ATPase. The effects of L-cysteine and L-glutathione (SH) were similar in nature, but less in intensity on the staining of ATPase. L-Methionine had no effect on the staining of Mg-ATPase and Na-K-ATPase. When tissue slices were incubated in a Ringer-Locke's solution containing test agents, the similar results were obtained especially in regions around blood vessels.

3. In the biochemical assay of ATPase in microsomal fractions of rabbit brain, assay conditions were selected as suitable for maximal activities. The optimal pH was 7.8,
maximal activity of Mg-ATPase was obtained at Mg\(^{2+}\) concentration of 3 mm and maximal stimulation of Mg-ATPase by Na\(^+\) and K\(^+\) occurred at Na\(^+\) and K\(^+\) concentrations of 100 mm and 20 mm, respectively. Ouabain \((2 \times 10^{-4} \text{ M})\) inhibited completely the Na-K-ATPase, but did not the Mg-ATPase.

4. L-Ascorbic acid \((10^{-4} \text{ M})\) and L-cysteine \((10^{-4} \text{ M})\) inhibited both Mg-ATPase and Na-K-ATPase, but the latter was more greatly inhibited by these agents. L-Glutathione and L-methionine had not statistically significant effects on Mg-ATPase and Na-K-ATPase.

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