Inhibition by Chlorpromazine, Metals and L-Ascorbic Acid of Calcium-ATPase and Magnesium-ATPase in Bovine Adrenal Medullary Microsomes

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Abstract—Effects of chlorpromazine, metals and L-ascorbic acid (AA) on Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase in microsomal and granular fractions obtained from the bovine adrenal medulla were studied. Marker enzyme analysis on microsomal subfractions in a discontinuous sucrose density gradient showed a correlation of distribution between ATPase activities and plasma membrane. The two ATPase activities in such plasma membrane-rich microsomes were reduced by chlorpromazine, Hg\(^{2+}\) and Cu\(^{2+}\) (0.3 mM of each), and their effects were greater on the Mg\(^{2+}\)-ATPase activity. Zn\(^{2+}\) (0.3 mM) also reduced only the Mg\(^{2+}\)-ATPase activity. AA (3 mM) reduced the two ATPase activities to an equal extent. Nevertheless, the inhibitions of ATPases by Hg\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) were decreased, unaltered and additively enhanced in combination with AA, respectively. We also observed high Mg\(^{2+}\)-ATPase activity in the granule-rich fraction, but this ATPase activity was unaffected by all of the above agents. These results indicate that Mg\(^{2+}\)-ATPase in the plasma membrane-rich microsome of adrenal medulla is inhibited by chlorpromazine, Hg\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) more significantly than Ca\(^{2+}\)-ATPase, but Mg\(^{2+}\)ATPase in the granular fraction is unaffected, and that AA changes the potency of inhibition by some metals of ATPases diversely.

The universal role of calcium in stimulus-secretion coupling has been well established (1). Catecholamine (CA) secretion from adrenal chromaffin cells is stimulated by an influx of calcium across the plasma membrane (2-4) which produces a rise in free intracellular calcium, and this is terminated by restoration of a low resting level of intracellular calcium (5).

Such reduction of internal calcium concentration is achieved mainly through extrusion of calcium by a specific calcium transporter (or calcium pump) in the cell membrane (6), as well as through calcium uptake by subcellular organelles (5, 7). According to biochemical (6, 8) and histochemical (9) studies, calcium-stimulated adenosine triphosphatase (Ca\(^{2+}\)-ATPase) was considered the enzyme involved in the plasma membrane calcium transport of adrenal chromaffin cells.

Heavy metals (10, 11) and chlorpromazine (12, 13) produce a prolonged CA secretion from the adrenal medulla. Such stimulatory effects of metals seemed to be due to a blockade of the calcium extrusion mechanism in the chromaffin cell membrane (14) which leads to a sustained increase in cytosolic free calcium. However, metals produced only a small reduction of Ca\(^{2+}\)-ATPase activity in the plasma membrane-rich microsome from the adrenal medulla, and this effect was not correlated with marked inhibition of calcium uptake by the plasma membrane vesicles (14).
Chlorpromazine is an inhibitor of calmodulin (15) that is required for activation of the ATPase representing calcium pump (16, 17), whereas this agent had no effect on the Ca\(^{2+}\)-ATPase in the adrenal medulla (18). These evidence indicate no relation between Ca\(^{2+}\)-ATPase activity and prolonged CA secretion induced by these agents, and they also seem incompatible with the earlier presumption that Ca\(^{2+}\)-ATPase may represent the calcium pump in the chromaffin cell membranes.

Though it has been shown that magnesium-stimulated adenosine triphosphatase (Mg\(^{2+}\)-ATPase) is also present on the chromaffin cell membrane with a prominent enzyme activity (6, 8, 9, 19), little is known about the significance of this enzyme in the plasma membrane. Thus, we examined the effects of chlorpromazine and metals on Mg\(^{2+}\)-ATPase in plasma membrane-rich microsomes from the adrenal medulla in comparison with those on Ca\(^{2+}\)-ATPase and granular Mg\(^{2+}\)-ATPase (20, 21). In this paper, the effects of l-ascorbic acid (AA) on ATPases are also described since the adrenal medulla contains a large amount of AA (22, 23), an inhibitor of ATPase (24, 25).

Materials and Methods

Preparation of microsomal and granular fractions: The microsomal and granular fractions of the adrenal medulla were prepared according to the method of Leslie and Borowitz (6), with minor modification. Bovine adrenal glands obtained at a local slaughter house were kept on ice during transport and used within 1 hr post mortem. The medullary tissue (20 g) was dissected free from cortex and homogenized in 10 vol. of 0.3 M sucrose using a Potter-Elvehjem glass homogenizer with a loose-fitting Teflon pestle. The homogenate was centrifuged at 800xg for 10 min to sediment nuclei and cell debris. The supernatant was centrifuged at 27,000xg for 20 min to separate mitochondria and chromaffin granules. The resultant supernatant was further centrifuged at 105,000xg for 60 min. The final pellet which contained purified microsomes was resuspended in 8 ml of 0.3 M sucrose containing 0.1% sodium deoxycholate and layered on a 0.6 to 1.2 M discontinuous sucrose density gradient containing 5 mM Tris-HCl and 0.1% sodium deoxycholate (pH 7.8). This gradient was then centrifuged at 105,000xg for 120 min. Eight subfractions were obtained from the density gradient with a pasteur pipette. The volume of each subfraction was about 1.1–1.2 ml. The pellet which contains mitochondria and chromaffin granules was resuspended in 0.3 M sucrose and layered on a 1.4 to 2.25 M discontinuous sucrose density gradient. Thereafter, the gradient was centrifuged at 105,000xg for 120 min, and six subfractions were obtained. These operations were carried out in a cold room (0–4°C).

Enzyme assay: Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase activities were assayed by the presence of either MgCl\(_2\) or CaCl\(_2\) in the incubation medium, respectively. For Mg\(^{2+}\)-ATPase activity, a 0.1 ml aliquot (200–400 \(\mu\)g protein) of each subfraction of microsome was preincubated in the reaction medium containing 4 mM MgCl\(_2\) and 33 mM Tris-HCl (pH 7.0) for 15 min at 37°C, and the reaction was started by an addition of ATP (disodium salt; final concentration of 4 mM) in a final volume of 3 ml. The reaction was stopped 20 min later with 5% trichloroacetic acid, and free inorganic phosphate was measured by the method of Fiske and Subbarow (26). Ca\(^{2+}\)-ATPase assay was carried out in the incubation medium for Mg\(^{2+}\)-ATPase except with a replacement of MgCl\(_2\) by 2 mM CaCl\(_2\). In blank experiments, MgCl\(_2\) and CaCl\(_2\) were removed from the medium. (Na\(^{+}\)+K\(^{+}\))-ATPase assay was done in the incubation medium containing 4 mM MgCl\(_2\) and 33 mM Tris-HCl, with and without 110 mM NaCl and 10 mM KCl. These ATPase activities were linear with incubation time to 60 min and protein in a range of 100 to 800 \(\mu\)g. Drugs and divalent metals were dissolved in the incubation medium.

Thiamine pyrophosphatase was assayed in the incubation medium for Mg\(^{2+}\)-ATPase, except ATP was replaced by thiamine pyrophosphate with and without MgCl\(_2\). Acetylcholinesterase (AChE) activity was determined by the method of Ellman et al. (27) using 20 \(\mu\)l samples (40–80 \(\mu\)g protein), and succinate dehydrogenase (SDH) activity
was measured by the method of Williams and Kamin (28) using a 0.5 ml aliquot (1–2 mg protein) of each sample. The AChE and SDH activities were expressed as absorbances at 412 nm and 500 nm, respectively. CA and protein were estimated by the methods of Weil-Malherbe (29) and Lowry et al. (30), respectively.

Experiments with catecholamine secretion from the granules: The experiment with CA secretion from the isolated granules was performed after the granule-rich fraction on a sucrose density gradient was returned to an isotonic density. The experiment was started by the addition of the granule fraction (1–1.5 mg CA/400–500 μg protein) into the incubation medium of 40 mM Tris-HCl buffer containing 150 mM KCl (pH 7.0) at 37°C (final volume of 3 ml). The reaction was stopped 5 min later by adding 4 ml of cold incubation buffer. This reaction medium was then centrifuged at 36,000×g for 20 min, and CA in the supernatant was estimated.

Materials: The agents used were as follows: l-ascorbic acid (Wako), aspirin (Hoei), caffeine (Shizuoka Caffeine), chlorpromazine hydrochloride (Takeda), pentobarbitone sodium (Tokyo Kasei), picrotoxin (Wako), barium chloride (Wako), cupric sulfate (Wako), ferrous sulfate (Wako), manganese chloride (Koso), mercuric chloride (Wako) and Zinc sulfate (Wako).

Statistics: The data obtained in this study was statistically analyzed by Student’s t-test. The values were considered significant when the P value was less than 0.05 as compared with the control value.

Results

Subcellular localization of ATPase activities in the adrenal medulla: Ca²⁺-ATPase, Mg²⁺-ATPase and (Na⁺+K⁺)-ATPase activities were observed in the microsomal fraction obtained from the bovine adrenal medulla. The optimum ratio of ATP and divalent cations for activation of Ca²⁺-ATPase and Mg²⁺-ATPase was 2 : 1 (data not shown), in agreement with an observation by Wilson and Kirshner (8).

Figure 1 shows the distribution of ATPase and other enzyme activities in eight subfractions of the microsomes on a sucrose density gradient. Ca²⁺-ATPase activity was observed in all subfractions, and its activity was high in fractions 3 through 5. Mg²⁺-ATPase activity was also high in fractions 3 through 6, but (Na⁺+K⁺)-ATPase activity was observed only in fraction 3. Among these enzymes, Ca²⁺-ATPase showed the highest activity.

Acetylcholinesterase activity (marker for plasma membrane) was present in a high amount in all the subfractions from 3 through 6, but thiamine pyrophosphatase activity (marker for Golgi apparatus) was observed in subfractions 3, 4 and 6. The activity of succinate dehydrogenase (marker for mitochondria) was not found in any of the subfractions, and only a small amount of CA (2–4 μg/mg protein) was detected in subfractions 1 through 3. These results indicate that subfractions 3 through 5 (corresponding to 0.6–0.8 M sucrose layer) are rich in the plasma membrane. Thus, these subfractions were used as the plasma membrane-rich microsomes.

Figure 2 shows the localization of ATPase activities on a sucrose density gradient of mitochondria and granule fraction. Subfractions 2 and 3 represent the mitochondrial fraction as shown by the high activity of succinate dehydrogenase. On the other hand, subfraction 5 represents the granule-rich fraction as shown by the specific localization of CA. The mitochondrial fractions showed relatively similar activities of Ca²⁺-ATPase and Mg²⁺-ATPase, but the granular fraction showed higher activity of Mg²⁺-ATPase than Ca²⁺-ATPase. Thus, subfraction 5 (corresponding to the 1.8–2.0 M sucrose layer) was used as the granule-rich fraction.

Effects of chlorpromazine on Ca²⁺-ATPase and Mg²⁺-ATPase in the plasma membrane-rich microsomes: As shown in Table 1, chlorpromazine (0.3 mM) reduced Ca²⁺-ATPase activity to 54%, and also, but more significantly, reduced Mg²⁺-ATPase activity to 31%. Although we tested the effects of aspirin (0.3 mM), caffeine (1 mM), pentobarbital (0.3 mM) and picrotoxin (0.3 mM) on the ATPase activities, none of the agents showed any effect on the ATPases.

Effects of metals and l-ascorbic acid on Ca²⁺-ATPase and Mg²⁺-ATPase in the
Fig. 1. Distribution of ATPases, acetylcholinesterase, thiamine pyrophosphatase, succinate dehydrogenase, catecholamines and protein in sucrose density gradient subfractions of the bovine adrenal medullary microsomes. Each point represents the mean value of three separate experiments and vertical bars show S.E.

Table 1. Effects of several drugs on Ca$^{2+}$-ATPase and Mg$^{2+}$-ATPase activities in the plasma membrane-rich microsomes

| Drug               | Ca$^{2+}$-ATPase activity (μmoles Pi/hr/mg protein) | Mg$^{2+}$-ATPase activity (μmoles Pi/hr/mg protein) |
|--------------------|--------------------------------------------------|---------------------------------------------------|
| Control            | 21.4±2.3 (n=5)                                   | 8.9±0.6 (n=4)                                     |
| Aspirin (0.3 mM)   | 16.6±3.1 (n=5)                                   | 7.7±0.3 (n=4)                                     |
| Caffeine (1 mM)    | 19.1±2.3 (n=5)                                   | 8.7±0.6 (n=4)                                     |
| Chlorpromazine (0.3 mM) | 11.5±0.5* (n=5)                               | 2.8±0.6* (n=4)                                   |
| Pentobarbital (0.3 mM) | 19.2±2.3 (n=5)                                  | 8.8±0.7 (n=4)                                     |
| PicROTOXIN (0.3 mM) | 17.4±2.6 (n=5)                                   | 9.1±0.3 (n=4)                                     |

*: Significantly different from the control value at P<0.01. n: number of separate experiments.
plasma membrane-rich microsomes: Figures 3 and 4 show the effects of metals and AA on ATPase activities in the microsomal subfractions. Ca²⁺-ATPase activity was reduced by Hg²⁺ (80.6±4.1%) or Cu²⁺ (69.8±1.7%) at a concentration of 0.3 mM, and Mg²⁺-ATPase activity was also, but more significantly, reduced by both metals (Hg²⁺: 88.7±2.4%, Cu²⁺: 96.2±2.1%). Zn²⁺ (0.3 mM) markedly reduced Mg²⁺-ATPase activity (46.6±7.5%) without affecting Ca²⁺-ATPase activity. Both ATPase activities were equally (about 40%) depressed by AA (3 mM). Fe²⁺, Mn²⁺ and Ba²⁺ (0.3 mM of each) had little effect on the activities of these enzymes.

AA has been suggested to inhibit (Na⁺+K⁺)-ATPase by interacting with an endogenous heavy metal ion (24). Thus, we further examined the effect of AA on the inhibition by heavy metals of ATPases. The inhibition by Hg²⁺ of Ca²⁺-ATPase and Mg²⁺-ATPase was decreased in the presence of AA by 39.6±2.4% and 33.7±0.9%, respectively. Cu²⁺-inhibition was unaffected by a combination with AA, whereas Zn²⁺-inhibition was additively enhanced by AA, resulting in a marked reduction of Mg²⁺-ATPase activity (89.6±2.9%). Ba²⁺ and Fe²⁺ did not produce significant alteration of AA inhibition, but Ca²⁺-ATPase inhibition by Mn²⁺ was enhanced by AA.

Effects of chlorpromazine, metals and L-ascorbic acid on Mg²⁺-ATPase and catecholamine secretion in the granule-rich fraction: ATP and Mg²⁺ (constituents for the Mg²⁺-ATPase assay) stimulate CA secretion from the isolated granules (21). Therefore, Mg²⁺-ATPase activity was measured from the amount of phosphate released for 5 min in the presence of ATP (4 mM) and MgCl₂ (2 mM), that is, the same conditions, as the CA release experiments. The Mg²⁺-ATPase activity (8.2±0.6 µmoles Pi/hr/mg protein, n=4) was unaffected by chlorpromazine, AA and metals such as Hg²⁺, Cu²⁺ and Zn²⁺.

Fig. 2. Distribution of Ca²⁺-ATPase, Mg²⁺-ATPase, succinate dehydrogenase, catecholamines and protein in sucrose density gradient subfractions of the adrenal medullary mitochondria and granule fraction. Each point represents the mean value of four separate experiments.
This was accompanied by no alteration of the (ATP+Mg2+)-stimulated CA secretion (113.9 ±12.1 μg/5 min, n=3) from the granules, except an enhancement by Cu2+ (0.3 mM) to 9 fold (1039±20 μg/5 min, n=3; P<0.01).

Fig. 3. Effects of metals and L-ascorbic acid on Ca2+-ATPase activity in bovine adrenal medullary microsomes. AA (3 mM) and metals (0.3 mM) were dissolved in the incubation medium. ATPase activity was expressed as μmoles Pi/hr/mg protein or percent of the control (Cont) activity, and vertical bars show S.E. of three or four separate experiments. Open column: AA or metals alone. Filled column: combination of metals with AA. *P<0.05, **P<0.01: Significant difference from control value.

Fig. 4. Effects of metals and L-ascorbic acid on Mg2+-ATPase activity in bovine adrenal medullary microsomes. Representations are the same as those described in Fig. 3.
Discussion

In several subfractions from the sucrose density gradient of adrenal medullary microsomes, the distribution of Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase correlated with that of the marker enzyme (acetylcholinesterase) for plasma membrane and partially with that for the Golgi apparatus (thiamine pyrophosphatase). These subfractions had little succinate dehydrogenase activity and only a small amount of CA, suggesting that the contamination of mitochondria and chromaffin granules was negligible. Thus, our marker enzyme analysis supports earlier reports (6, 8, 19) indicating that the microsomal fraction is rich in the plasma membrane of chromaffin cells and that two ATPase activities are localized in the plasma membrane as well as in the Golgi apparatus.

In such plasma membrane-enriched subfractions of microsomes, chlorpromazine reduced Ca\(^{2+}\)-ATPase activity. This observation was different from a report by Leslie et al. (18) who showed no reduction of the ATPase activity by chlorpromazine up to 1 mM. This difference may be due to their use of whole microsomes in the ATPase assay. Nevertheless, our interesting finding is that the inhibitory effect of chlorpromazine was greater on the Mg\(^{2+}\)-ATPase activity. Similarly, the two enzyme activities were significantly reduced by Hg\(^{2+}\) and Cu\(^{2+}\), but their effects were greater on the Mg\(^{2+}\)-ATPase. Zn\(^{2+}\) reduced the Mg\(^{2+}\)-ATPase activity without notable change in the Ca\(^{2+}\)-ATPase activity. These inhibitions by metals are unlikely due to a nonspecific denaturation of the ATPases since other metals (Ba\(^{2+}\), Fe\(^{2+}\) and Mn\(^{2+}\)) produced no significant reduction of the ATPase activities. These evidence indicates that Mg\(^{2+}\)-ATPase in the plasma membrane-rich microsome may be more susceptible to the inhibitory actions by these agents, as compared with the Ca\(^{2+}\)-ATPase.

Chlorpromazine, Hg\(^{2+}\) and Zn\(^{2+}\) have been shown to evoke a prolonged adrenal CA secretion (10–13). The dose for these agents used in this study is less than that required for the stimulation of adrenal CA secretion. Thus, a significant reduction of Mg\(^{2+}\)-ATPase activity might be more closely associated with the secretory response induced by these agents, as compared with Ca\(^{2+}\)-ATPase.

Recently, the enzymatic representation of the calcium pump has been established as Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-dependent ATPase in erythrocyte (31) and islet plasma membranes (16). In the earlier and present studies, Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase were separately assayed only by the addition of either CaCl\(_2\) or MgCl\(_2\) into the incubation medium, respectively. At present, it is unclear which ATPase represents the calcium pump in chromaffin cell membranes.

Interestingly, the ATPase representing the calcium pump in erythrocyte and islet cell membranes required calmodulin, a calcium binding protein (32), for maximal enzyme activity (16, 31). On the other hand, Ca\(^{2+}\)-ATPase in skeletal muscle sarcoplasmic reticulum (32) and in the islet cell endoplasmic reticulum (16), each of which is a single enzyme, is not known to be regulated by calmodulin. Calmodulin is well known to be inhibited by chlorpromazine (15). Thus, ATPase that is activated by endogenous calmodulin may be inhibited by chlorpromazine, but not an ATPase like Ca\(^{2+}\)-ATPase in the endoplasmic reticulum. A large amount of calmodulin has been detected in chromaffin cells (33), so that some relation between ATPases and calmodulin would be expected in the adrenal medulla. Accordingly, our finding of a potent inhibition of Mg\(^{2+}\)-ATPase by chlorpromazine may imply the possibility that this ATPase might be the enzyme related to the endogenous calmodulin. Little or no inhibition of Ca\(^{2+}\)-ATPase by chlorpromazine seems indicative of minor or no interaction with calmodulin.

Furthermore, Mg\(^{2+}\)-ATPase was significantly inhibited by Hg\(^{2+}\) and Zn\(^{2+}\), metals which have been proposed to decrease the function of calcium extrusion in chromaffin cell membranes (14). Based on these evidence, the enzymatic representation of the calcium pump in chromaffin cells seems to be Mg\(^{2+}\)-ATPase rather than Ca\(^{2+}\)-ATPase. This possibility may be supported by a report of Wilson and Kirshner (8) suggesting that Ca\(^{2+}\)-ATPase in the plasma membrane-rich microsome is a single enzyme, but Mg\(^{2+}\)-
ATPase has two enzymatic components, one of which corresponds to Ca\(^{2+}\)-ATPase. However, further studies are needed to clarify the significance of Mg\(^{2+}\)-ATPase in the calcium transfer mechanism of the chromaffin cell membranes and whether there is also an involvement of calmodulin.

AA has been identified as an endogenous modulator of (Na\(^{+}\)+K\(^{+}\))-ATPase in the brain, interacting with an endogenous heavy metal ion, an inhibitor of the ATPase (24). Thus, we examined the effect of AA on the ATPase activities. As the result, AA depressed both ATPase activities in the plasma membrane-rich microsome to an equal extent. Nevertheless, the inhibitions by Hg\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) of ATPase activities were decreased, unaffected and additively enhanced in combination with AA, respectively. The coexistence of Mn\(^{2+}\) and AA resulted in a marked reduction of Ca\(^{2+}\)-ATPase activity. These results indicate that the potency of inhibition by some metals of the ATPases can be changed by a combination with AA, although underlying mechanisms are still unclear. Since AA is abundantly present in the adrenal medulla (22, 23), some AA-related regulation of ATPase activities may be expected.

In addition to the Mg\(^{2+}\)-ATPase in the microsomal fraction, we confirmed the presence of high Mg\(^{2+}\)-ATPase activity in the granule-rich fraction. However, none of the agents tested showed any effects on the ATPase activity, although Cu\(^{2+}\) enhanced granular CA release.

Conclusively, our study indicated that Mg\(^{2+}\)-ATPase in the plasma membrane-rich microsome is inhibited by chlorpromazine, Hg\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) more significantly than Ca\(^{2+}\)-ATPase, whereas Mg\(^{2+}\)-ATPase in the granular fraction is unaffected, and that the potency of inhibition by some metals of the ATPases can be changed by the presence of AA.

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