EFFECT OF DIURETICS ON ION TRANSPORT OF KIDNEY CORTEX MITOCHONDRIA

II. MODE OF ACTION OF ETHACRYNIC ACID ON MITOCHONDRIAL CALCIUM PUMP

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Abstract—Effects of ethacrynic acid on mitochondrial Ca\(^{2+}\)-accumulation were examined in the rat kidney. The inhibitory effect of the drug on Ca\(^{2+}\)-accumulation was reversed by cysteine or glutathione. The addition of phosphate to the medium abolished the inhibitory effect of ethacrynic acid on Ca\(^{2+}\)-accumulation of kidney cortex mitochondria. On the other hand, acetate enhanced that of the drug. The inhibition of Ca\(^{2+}\)-accumulation by mersalyl was reversed by cysteine, but was not by phosphate, differing from ethacrynic acid. Ethacrynic acid inhibited the activity of mitochondrial ATPase observed in the presence of CaCl\(_2\), but not the enzyme activity in the presence of MgCl\(_2\). The main inhibitory effect of ethacrynic acid on mitochondrial Ca\(^{2+}\)-accumulation may be due to an action on mitochondrial ATPase.

Cellular effects of ethacrynic acid are currently under active investigation. Most examinations in vitro show that membrane (Na\(^+\)-K\(^+\))-ATPase (ATP phosphohydrolase, EC, 3.6.1.3) (1-4) and mitochondrial respiration (5-7) are inhibited by ethacrynic acid, and also that ethacrynic acid depresses the glycolytic pathway (8, 9) and modifies permeability of the cell membrane (10). There is currently no substantially documented evidence that in vitro effects of ethacrynic acid on metabolism appear related to the diuretic mechanisms. Emphasis was thus placed on the study of an other cellular effect of the compound in vitro.

In a previous paper, the present author reported that when ethacrynic acid was incubated with rat kidney cortex mitochondria, the diuretic inhibited the calcium and magnesium accumulation which was dependent on mitochondrial metabolism (11).

The purpose of the present paper was to study further the mode of action of ethacrynic acid on mitochondrial Ca\(^{2+}\)-accumulation.

MATERIALS AND METHODS

Male Wistar rats, weighing 250 to 350 g, were employed. Rat kidney cortex mitochondria were isolated by the method of Hogeboom (12), as described in a previous paper (13).

Ca\(^{2+}\)-accumulation

The conditions of incubation in media containing respiratory substrate, ATP and various ions were in general those described by Gemba (11). At the end of the incubation...
tion period, the reaction medium was filtered through a membrane filter (25 mm Milliporefilter, type DA, size 0.65 μ). Calcium accumulation was estimated by determining its appearance in extract (99.0% of formic acid) of the mitochondria as described in a previous paper (11). Calcium was determined by an atomic absorption spectrophotometer (Hitachi 207).

**Assay of ATPase**

The medium contained the following: Tris-HCl, pH 7.4 (12.5 mM at a final concentration), ATP (tris salt, 2 mM), sucrose (250 mM), and other additions depending upon experimental conditions (total volume of 1.0 ml). Incubation was carried out in a test tube at 30°C. The enzyme reaction was initiated by addition of the mitochondrial suspension (ca. 0.360 mg of protein) and after 10 min, it was terminated by addition of 0.5 ml of 30% trichloroacetic acid. Inorganic phosphate was determined by a modified method (14) of Fiske and Subbarow (15), i.e., perchloric acid was used instead of sulfuric acid.

Mitochondrial protein was determined by the method of Lowry et al. (16). Crystaline bovine serum albumin was used as a protein standard. ATP (di-tris salt) was obtained from Sigma Chemical Co. and employed after the pH had been adjusted to 7.4 with tris. Ethacrynic acid (2, 3-dichloro-4-(2 methylbutylryl)-phenoxyacet acid) and mersalyl were kindly provided by the Japan Merck Banyu Co. and by Professor Saburo Muraoka of the University of Tokushima, respectively.

**RESULTS**

It has been shown in a previous paper (11) that Ca²⁺-accumulation of rat kidney cortex mitochondria was remarkably inhibited by 1×10⁻⁴ M of ethacrynic acid. Subsequently, the effect of sulfhydryl reagents on ethacrynic acid action in kidney cortex mitochondria was examined (Fig. 1). Following the addition of ethacrynic acid (1×10⁻⁴ M) to the medium, Ca²⁺-accumulation of mitochondria decreased from 712.3±68.5 μequiv./mg protein/10 min of the control experiment to 152.9±56.5 μequiv./mg protein/10 min. Cysteine or gluthathione (4×10⁻⁴ M, respectively) greatly increased the Ca²⁺-accumulation by 60.5% and 41.9%, respectively, and the inhibitory effect of ethacrynic acid was reversed by both sulfhydryl reagents. The reversible effect of cysteine was greater than that of gluthathione. The former abolished almost completely the inhibitory effect of ethacrynic acid.

Inhibitory effects of ethacrynic acid (1×10⁻⁴ M) on Ca²⁺-accumulation of mitochondria were completely abolished by phosphate (4×10⁻³ M) (Fig. 2). Inhibition of Ca²⁺-accumulation by ethacrynic acid was partially abolished by the low concentration of phosphate. The percent inhibition of Ca²⁺-accumulation by ethacrynic acid was decreased from 90.8% to 39.3% by the addition of phosphate (4×10⁻⁴ M) (Values are not shown in the figure).

On the other hand, acetate (4×10⁻³ M), which is similar to phosphate in permeability property of the mitochondrial membrane (17), enhanced the inhibitory effect of ethacrynic acid on Ca²⁺-accumulation, and rather extruded calcium from mitochondria, differing...
**FIG. 1.** Effect of sulfhydryl reagents on inhibition by ethacrynic acid upon Ca^{2+}-accumulation in kidney cortex mitochondria. The incubation medium contained 10 mM tris-HCl (pH 7.4), 2 mM MgCl₂, 0.5 mM CaCl₂, 6 mM NaCl, 4 mM KCl, 1 mM tris-ATP, 7.8 mM succinate, 250 mM sucrose, and mitochondria (ca. 2.5 mg of protein) in a final volume of 5 ml. The indicated final concentration of ethacrynic acid and sulfhydryl reagents was added to the medium. The incubation time was 10 min at 30°C. The vertical lines indicate the standard errors. n = 4.

**FIG. 2.** Effect of phosphate and acetate on the inhibition of Ca^{2+}-accumulation by ethacrynic acid. Final concentration of phosphate and acetate was 4 x 10⁻³ M. Other conditions are the same as those given in Fig. 1. The vertical lines indicate the standard errors (n=4), but the values in the presence of acetate are the mean of analysis obtained with two preparations.
Tyler (18) has shown that the phosphate exchange-diffusion carrier of mitochondrial membrane, which permits the entry of phosphate into mitochondria, is inactivated by organic mercurial diuretic, mersalyl. The effects of ethacrynic acid on Ca\(^{2+}\)-accumulation were compared with those of mersalyl in kidney cortex mitochondria. Mersalyl (1 x 10\(^{-4}\) M) inhibited completely Ca\(^{2+}\)-accumulation of kidney cortex mitochondria and produced an efflux of calcium from mitochondria (Fig. 3). This effect of mersalyl (1 x 10\(^{-4}\) M) was reversed by cysteine (4 x 10\(^{-4}\) M), which was similar to ethacrynic acid, but was not by phosphate (4 x 10\(^{-3}\) M). Percent inhibition of Ca\(^{2+}\)-accumulation by mersalyl was only some 24.0% in the presence of cysteine. There was significant difference between the effects of phosphate on inhibitory action of ethacrynic acid and mersalyl on Ca\(^{2+}\)-accumulation of kidney cortex mitochondria.

Ca\(^{2+}\)-accumulation of kidney cortex mitochondria was reduced to 42.1% with phosphate. The indicated concentration of mersalyl was added to the medium. Final concentration of cysteine and phosphate was 4 x 10\(^{-4}\) M and 4 x 10\(^{-3}\) M, respectively. Other conditions were the same as those given in Fig. 1. The vertical lines indicate the standard errors, n=4.

**Table 1. Effect of ethacrynic acid on the mitochondrial ATPase of kidney cortex.**

| Additions          | Mitochondrial ATPase activity* |
|--------------------|--------------------------------|
|                    | None                           | Ethacrynic acid 1 x 10\(^{-4}\) M | Percent inhibition |
| Without MgCl\(_2\) | Control                        | 47.6±1.6                        | 44.1±1.4           | 25.8%       |
|                    | CaCl\(_2\) 5 x 10\(^{-4}\) M    | 95.1±1.9                        | 79.3±3.7           |             |
| \(\Delta\)Ca\(^{2+}\)-ATPase | 47.5±1.1                       | 35.2±3.3                       | 25.8%       |
| MgCl\(_2\) 2 x 10\(^{-2}\) M Control | 52.0±2.9                       | 55.2±3.3                       |             |
|                    | CaCl\(_2\) 5 x 10\(^{-4}\) M    | 140.4±4.3                       | 61.5±2.7           | 25.8%       |
| \(\Delta\)Ca\(^{2+}\)-ATPase | 88.4±3.9                       | 6.3±3.8                        | 92.9%       |

Kidney cortex mitochondria (ca. 0.360 mg protein) were incubated for 10 min at 30°C. The incubation medium contained 12.5 mM tris-HCl (pH 7.4), 2 mM ATP (tris salt), 250 mM sucrose and other additions as indicated. Total volume was 1.0 ml.

Results are expressed as mean±SE, n=6.

*ATPase: mmoles of Pi liberated/mg protein/min.
oligomycin (10 μg/ml), the inhibitor of mitochondrial ATPase (Values are not shown in the figure). The effect of ethacrynic acid was examined on the mitochondrial ATPase of the kidney cortex (Table 1). The enzyme activity was stimulated slightly by 9.3% with MgCl2 (2×10^{-4} M) alone, but, by 100.0% with CaCl2 (5×10^{-4} M), and largely by 195.0% with both CaCl2 and MgCl2. Ethacrynic acid hardly affected the enzyme activity either in the absence of both MgCl2 and CaCl2, or in the presence of MgCl2 alone. However, the enzyme activity stimulated by CaCl2 was repressed by 25.8% with ethacrynic acid in the absence of MgCl2. The repressive effect of ethacrynic acid on the enzyme activity stimulated by CaCl2 was greatly enhanced from 25.8% to 92.9% in the presence of MgCl2.

The effect of cysteine and phosphate was examined on the inhibition of Ca^{2+}-stimulated ATPase activity by ethacrynic acid (Table 2). Following addition of cysteine and phosphate (4×10^{-4} M, respectively), the percent inhibition of Ca^{2+}-stimulated ATPase activity by ethacrynic acid decreased from 89.3% to 6.1 and 65.3%, respectively.

### Table 2. Effect of cysteine and phosphate on the inhibition of Ca^{2+}-stimulated ATPase activity by ethacrynic acid.

| Additions        | Ca^{2+}-stimulated ATPase of mitochondria* |
|------------------|-------------------------------------------|
|                  | None | Ethacrynic acid 1×10^{-4} M | Percent inhibition |
| Cysteine         | 103.9±4.8 | 11.1±2.8 | 89.3% |
| Phosphate        | 4×10^{-4} M | 105.1±4.5 | 98.7±6.3 | 6.1% |
|                  | 128.4±5.9 | 44.5±8.2 | 65.3% |

ATPase activity was measured at pH 7.4 (Tris-HCl, 12.5 mM) with 2 mM Tris-ATP, 2 mM MgCl2, 250 mM sucrose, kidney cortex mitochondria (ca. 0.33 mg protein), and other additions as indicated in the presence and absence of CaCl2 (0.5 mM). Ca^{2+}-stimulated ATPase is the increase in activity in the presence of added CaCl2. Results are expressed as mean±SE, n=6.

*ATPase: μmoles of Pi liberated/mg protein/min.

### DISCUSSION

It has been demonstrated that the diuresis produced by ethacrynic acid may be related to its capacity for binding sulfhydryl groups of renal cellular proteins (19). It was also reported that the inhibitory effect of ethacrynic acid on (Na^+ | K^+)-ATPase of the kidney and red cell membrane was reversed by cysteine (20, 21). The present in vitro results of reversible effect of cysteine and glutathione on inhibitory action of ethacrynic acid also showed that ethacrynic acid reacted with functionally important sulfhydryl groups in Ca^{2+}-accumulation of kidney cortex mitochondria (Fig. 1). Rat kidney cortex mitochondria remarkably accumulated calcium from surrounding medium in the presence of ATP without the addition of phosphate (Fig. 2).

Effect of ethacrynic acid on Ca^{2+}-accumulation of kidney cortex mitochondria resembles that of mersalyl, with respect to the inhibitory effect on Ca^{2+}-accumulation and the reverse of that by sulfhydryl reagent. However, inhibitory mechanism of ethacrynic acid on Ca^{2+}-accumulation seems to differ from that of mersalyl, known as inhibitor of
the phosphate exchange-diffusion carrier in mitochondria (18), in view of the present observation of the effect of phosphate on the inhibition of Ca\(^{2+}\)-accumulation by two diuretics (Fig. 2, 3).

In addition, there was a difference between the effects of the two diuretics on mitochondrial ATPase activity. ATPase activity observed in the presence of MgCl\(_2\) was not affected by ethacrynic acid (Table 1), but the enzyme activity increased 70.6\% with mersalyl (Values are not shown in the figure).

The question as to how phosphate reverses the inhibitory effect of ethacrynic acid cannot be definitively substantiated at this time, but the fact that acetate does not affect the action of ethacrynic acid suggests that the mode of action of phosphate derives from other reasons than the property as permeable anion at mitochondrial membrane.

Ethacrynic acid inhibited not only Ca\(^{2+}\)-accumulation of kidney cortex mitochondria, but also mitochondrial ATPase as observed in the presence of CaCl\(_2\). Ethacrynic acid (1 x 10^{-4} M) repressed the respiration of kidney cortex mitochondria some 44.0\% in the same reaction medium in the experiment of Ca\(^{2+}\)-accumulation as reported previously (11), however, the diuretic inhibited Ca\(^{2+}\)-accumulation of mitochondria about 80 to 90\% (Fig. 1, 2) (11) and the Ca\(^{2+}\)-stimulated ATPase activity about 91\% (Table 1, 2). Cysteine (4 x 10^{-4} M) abolished almost completely not only the inhibitory effect of ethacrynic acid on Ca\(^{2+}\)-accumulation of kidney cortex mitochondria but also that on Ca\(^{2+}\)-stimulated ATPase, and the low concentration of phosphate (4 x 10^{-4} M) partially abolished both inhibitory effects of the drug (Fig. 1, Table 2). It is therefore reasonable that the inhibitory effect of ethacrynic acid on Ca\(^{2+}\)-accumulation of kidney cortex mitochondria closely parallels that of the diuretic on the ATPase activity stimulated by CaCl\(_2\) in the presence of MgCl\(_2\).

Brierley et al. (22) reported Ca\(^{2+}\)-stimulated ATPase activity and its relationship to calcium accumulation in heart mitochondria. On the basis of the discussions presented above, it seemed reasonable to assume that the main inhibitory effect of ethacrynic acid on mitochondrial Ca\(^{2+}\)-accumulation depends on the action of mitochondrial ATPase.

Lehninger suggested that ion-transport function of mitochondria may play an important role in physiological regulation of calcium and active transcellular transport of reabsorbive substances in renal tubule cells (23). It has been reported that ethacrynic acid reaches the cytoplasm of kidney cells (10, 24). Further work is in progress on the effect of ethacrynic acid on mitochondrial calcium pump in vivo with respect to intracellular calcium homeostasis in the kidney.

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