EFFECT OF DIURETICS ON ION TRANSPORT OF KIDNEY CORTEX MITOCHONDRIA

III. SPECIES DIFFERENCE IN CALCIUM ACCUMULATION AND IN ETHACRYNIC ACID EFFECT

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Abstract—Effect of inorganic phosphate (4×10^{-3} M) on Ca^{++} -accumulation was examined in kidney cortex mitochondria. Ca^{++} -accumulation of rat kidney cortex mitochondria was slightly influenced by inorganic phosphate. On the other hand, dog kidney cortex mitochondria did not accumulate calcium from the incubation medium until the inorganic phosphate had been added. Ca^{++} -accumulation of rabbit kidney cortex mitochondria was markedly stimulated by inorganic phosphate. When ethacrynic acid was added to the reaction medium in the absence of inorganic phosphate, Ca^{++} -accumulation of rat kidney cortex mitochondria was depressed and the decrease in calcium content of rabbit and dog kidney cortex mitochondria was enhanced. In the presence of inorganic phosphate, the inhibition of Ca^{++} -accumulation by ethacrynic acid was observed only on dog kidney cortex mitochondria. Subsequently, the effect of inorganic phosphate (4×10^{-1} M) and ethacrynic acid (1×10^{-4} M) on Ca^{++} -ATPase was examined in kidney cortex mitochondria. The low concentration of inorganic phosphate (4×10^{-1} M) activated Ca^{++} -ATPase of kidney cortex mitochondria in all animal species. The greatest activation of Ca^{++} -ATPase occurred in rabbits, but the activity of the enzyme was lower than that in rats and dogs. Inhibition of Ca^{++} -ATPase by ethacrynic acid was depressed by the addition of inorganic phosphate in kidney cortex mitochondria of experimental animals. Ca^{++} -accumulation may be regulated through the stimulating effect of inorganic phosphate and the inhibitory effect of ethacrynic acid on Ca^{++} -ATPase in kidney cortex mitochondria. Species difference in ethacrynic acid effect on Ca^{++} -accumulation in kidney cortex mitochondria of rats, rabbits and dogs is discussed.

It is well known that renal tubule cells, especially proximal tubule cells, contain a great number of large sized mitochondria. It is assumed that ion accumulation in mitochondria plays an important role in cell membrane transport mechanism through a regulation of intracellular calcium concentration. In a previous paper, Gemba reported that calcium was actively accumulated in isolated kidney cortex mitochondria of the rat, and this accumulation (Ca^{++} -accumulation) was inhibited by a potent diuretic, ethacrynic acid (1). ATP phosphohydrolase [Ca^{++} -ATPase, EC, 3.6.1.4] stimulated by the addition of CaCl_2 of kidney cortex mitochondria was also inhibited by the diuretic. In that experiment, the activity of Ca^{++} -accumulation paralleled that of Ca^{++} -ATPase. It was concluded that calcium transport and Ca^{++} -ATPase were possibly two aspects of the same system (2).

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Ethacrynic acid has different diuretic effects on different species of experimental animals, i.e., potent diuretic effect is observed in the dog, a mild effect in the rabbit, while that in the rat is slight (3, 4). Whether or not ethacrynic acid reveals differences among the species regarding inhibitory effects on subcellular organella of experimental animals is a subject which holds great interest.

The present investigation was undertaken to compare these effects in rats, rabbits and dogs.

MATERIALS AND METHODS

Animals were anesthetized with sodium pentobarbital, and the kidneys were removed from male Wistar rats, weighing 250 to 350 g, male rabbits, weighing 2 to 3 kg, and mongrel dogs of both sexes, weighing 10 to 20 kg. Kidneys were cooled immediately in ice cold sucrose (0.25 M) and the cortex was separated from medulla. Kidney cortex mitochondria were prepared with cold sucrose (0.25 M) by the method of Hogeboom (5).

Accumulation of calcium in mitochondria. The mitochondria (ca. 2.5 mg protein) were incubated in a reaction medium containing 10 mM Tris-HCl, pH 7.4, 2 mM MgCl_2 , 0.5 mM CaCl_2 , 6 mM NaCl, 4 mM KCl, 1 mM ATP (tris salt), 7.8 mM succinate, 250 mM sucrose and other additions depending upon experimental conditions in a total volume of 5.0 ml at 30°C, while shaking gently. After a 10 min incubation, the reaction medium was filtered through a millipore filter (type DAWP, size 0.65 μ) to rapidly separate the mitochondria. Ca^{++} -accumulation was estimated by determining its appearance in the extract (99% of formic acid) of mitochondria as described previously (1). Calcium was determined by an atomic absorption spectrophotometer (Hitachi 207).

Assay of ATPase. ATP hydrolysis by kidney cortex mitochondria was measured by estimating inorganic phosphate released in the medium containing 12.5 mM Tris-HCl, pH 7.4, 2 mM ATP (tris salt), 2 mM MgCl_2 , 250 mM sucrose and other additions depending upon experimental conditions in a total volume of 1.0 ml at 30°C for 10 min as described in a previous paper (2). Ca^{++} -ATPase activity was calculated as follows; ATPase activity observed in the presence of MgCl_2 (2 mM) was subtracted from total ATPase activity obtained in the reaction medium which contained both MgCl_2 (2 mM) and CaCl_2 (0.5 mM). ATPase activity was expressed as m μ moles of liberated inorganic phosphate/mg protein/min.

Protein concentration of mitochondria was estimated according to the procedure of Lowry et al. (6). Crystalline bovine serum albumin was used as a protein standard. ATP (ditris salt) was obtained from Sigma Chemical Co.. ATP and succinic acid were employed after the pH had been adjusted to 7.4 with tris (hydroxymethyl) aminomethane. Ethacrynic acid, 2,3-dichloro-4-(2 methylenbutyryl)-phenoxyacetic acid, was kindly provided by Japan Merck Banyu Co. and prepared in Tris-HCl, pH 7.4.

RESULTS

After incubation of kidney cortex mitochondria in a standard medium, a similar Ca^{++} -accumulation was observed in rats and rabbits; 741.3 ± 76.6 (SE) m μ equiv/mg protein/

FIG. 1. Effect of inorganic phosphate on Ca^{++} -accumulation in kidney cortex mitochondria.

The medium was incubated for 10 min at 30°C . The indicated final concentration of phosphate was added to the medium as $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer, pH 7.4. Each bar denotes the mean $\pm$ standard error (SE). $n=5$.

10 min for rats and 685.9 ± 122.5 (SE) $\mu\text{equiv./mg protein/10 min}$ for rabbits. On the other hand, dog mitochondria did not accumulate calcium from the standard medium but rather extruded it as shown in Fig. 1. Chapell et al. reported that calcium uptake by rat liver mitochondria was stimulated by the addition of inorganic phosphate (7). We, therefore, examined the effect of inorganic phosphate on Ca^{++} -accumulation to make a comparison among the three species. Generally, inorganic phosphate (4×10^{-3} M) stimulated Ca^{++} -accumulation in the mitochondria of all these species, but the extent was different. The greatest stimulation was observed in rabbits and the least in rats. In the dog, calcium movement turned from efflux to influx by the addition of inorganic phosphate (Fig. 1).

In our previous work, we reported that ethacrynic acid inhibited Ca^{++} -accumulation of rat kidney cortex mitochondria (1). In the present paper, the inhibitory effect of the diuretic was examined in three different animal species and compared. Ethacrynic acid (1×10^{-4} M) decreased Ca^{++} -accumulation of mitochondria in all species. In rats, Ca^{++} -accumulation was depressed by 82.1%, and the value was almost the same as that reported previously. In rabbits, Ca^{++} -accumulation turned to extrusion by the addition of ethacrynic acid, and in dogs, calcium extrusion was enhanced by ethacrynic acid (Fig. 2).

Inhibitory effects of ethacrynic acid on Ca^{++} -accumulation of mitochondria were examined in the presence of inorganic phosphate (4×10^{-3} M) (Fig. 3). Inorganic phosphate abolished the inhibitory effect of ethacrynic acid in rats and rabbits. However, in dog mitochondria, Ca^{++} -accumulation was still inhibited by approximately 75.0% by ethacrynic acid even in the presence of inorganic phosphate. There was a marked difference in Ca^{++} -accumulation of kidney cortex mitochondria in the presence of inorganic

FIG. 2. Effect of ethacrynic acid on Ca^{++} -accumulation in kidney cortex mitochondria in the absence of inorganic phosphate.

Final concentration of ethacrynic acid was 1×10^{-4} M. Other conditions were the same as in Fig. 1. $n=5$.

FIG. 3. Effect of ethacrynic acid on Ca^{++} -accumulation in kidney cortex mitochondria in the presence of inorganic phosphate.

The incubation medium described contained inorganic phosphate (4×10^{-3} M, pH 7.4). Addition was made at the final concentration of 1×10^{-4} M ethacrynic acid. Other conditions were as in Fig. 1. $n=5$.

phosphate in the control experiment using all three species. The highest Ca^{++} -accumulation occurred in rabbits, and next in rats. Dog kidney cortex mitochondria had the lowest Ca^{++} -accumulation rate among the three species.

In a second series of experiments, mitochondrial ATPase of each animal was determined with and without CaCl_2 . With the absence of CaCl_2 in the reaction medium, the order of ATPase activity was rats, rabbits and dogs (Fig. 4). Although the extent of Ca^{++} -ATPase in rats and dogs was almost the same, the activity was less in the rabbit. Ethacrynic acid

FIG. 4. Effect of ethacrynic acid on mitochondrial ATPase of kidney cortex.

The medium containing about 0.35 mg mitochondrial protein/ml was incubated for 10 min at 30°C in the absence or presence of CaCl_2 (5×10^{-4} M). Black columns show the enzyme activities in the presence of MgCl_2 and white columns show Ca^{++} -ATPase activities. Each bar denotes the mean $\pm$ SE. $n=6$.

EA: Ethacrynic Acid (1×10^{-4} M)

FIG. 5. Effect of inorganic phosphate on Ca^{++} -ATPase of kidney cortex mitochondria.

The indicated final concentration of inorganic phosphate, pH 7.4, was added to the medium. Other conditions were as in Fig. 4. $n=6$.

showed a similar effect on both ATPase activities in rats and rabbits, but a different effect was observed in the dog. In rat and rabbit mitochondria, ATPase activity was not influenced by ethacrynic acid in the absence of CaCl_2 , but Ca^{++} -ATPase was strongly inhibited by ethacrynic acid. On the contrary, in dog mitochondria, ethacrynic acid inhibited Ca^{++} -ATPase slightly, while the diuretic rather stimulated ATPase in the absence of CaCl_2 .

The effect of a low concentration of inorganic phosphate on Ca^{++} -ATPase of the mitochondria was examined. The concentration (4×10^{-4} M) of inorganic phosphate was one tenth of that (4×10^{-3} M) in Ca^{++} -accumulation experiment, because of the measure-

FIG. 6. Effect of inorganic phosphate on the percent inhibition of Ca^{++} -ATPase by ethacrynic acid.

Values are the mean of analysis obtained from six preparations.

ment of inorganic phosphate liberating from ATP for the ATPase assay. As shown in Fig. 5, the addition of inorganic phosphate activated Ca^{++} -ATPase of the kidney cortex mitochondria in all the species. The greatest degree of activation was seen in the rabbit, but the specific activity of the enzyme was lower than that in the other animal species. ATPase activity observed in the absence of $CaCl_2$ was not affected by inorganic phosphate ($4 \times 10^{-4} M$) in kidney cortex mitochondria of these experimental animals (Values are not shown in the figure). Subsequently, the effect of inorganic phosphate on the inhibitory effect of ethacrynic acid was examined in Ca^{++} -ATPase of kidney cortex mitochondria. Fig. 6 shows that the addition of inorganic phosphate resulted in a decrease in the percent inhibition of Ca^{++} -ATPase by ethacrynic acid in all species. In rat kidney cortex mitochondria, the percent inhibition of Ca^{++} -ATPase by ethacrynic acid was decreased from 91.0% in the control experiment to 64.6% following the addition of inorganic phosphate.

DISCUSSION

It has been reported that Ca^{++} -ATPase activity is related to Ca^{++} -accumulation in heart mitochondria (8). ATP and calcium appear to have an influence on each other in their transport across the mitochondrial inner membrane. Spencer and Bygrave have reported that calcium stimulated adenine nucleotide translocase activity in rat liver mitochondria (9). The increased ATP translocated in the presence of calcium ion would be available for hydrolysis by ATPase. The energy produced by ATP breakdown would then serve for Ca^{++} -accumulation. Previous work in this series has shown that the main inhibitory effect of ethacrynic acid on Ca^{++} -accumulation depends on the action on Ca^{++} -ATPase in rat kidney cortex mitochondria (2). The present experiment showed that ethacrynic acid inhibited Ca^{++} -ATPase of kidney cortex mitochondria not only in rats but also in rabbits and dogs (Fig. 4). In rabbit kidney cortex mitochondria, Ca^{++} -ATPase was inhibited completely by ethacrynic acid, and the decrease of calcium content was observed

by the addition of the diuretic (Fig. 2). The decrease of calcium content was also enhanced by the diuretic in dog kidney cortex mitochondria. On the basis of these data, it is reasonable to assume that inhibition of Ca^{++} -ATPase by ethacrynic acid may relate not to the inhibition of calcium efflux but to the inhibition of calcium influx in kidney cortex mitochondria.

Inorganic phosphate did not affect ATPase activity observed in the presence of MgCl_2 , but rather stimulated Ca^{++} -ATPase in kidney cortex mitochondria (Fig. 5). It is more likely that inorganic phosphate may function to regulate the activity of Ca^{++} -ATPase of kidney cortex mitochondria in the intact cells of these experimental animals. Stimulative action of phosphate and inhibitory action of ethacrynic acid on mitochondrial Ca^{++} -accumulation may appear through the influence on Ca^{++} -ATPase activity. Whether or not inorganic phosphate competes with ethacrynic acid concerning the action on mitochondrial Ca^{++} -ATPase is a subject of further investigation.

Our present results show that dog kidney cortex mitochondria releases calcium in the absence of inorganic phosphate and that the ability to accumulate calcium was lower than in rabbits and rats even in the presence of inorganic phosphate. It would thus appear that the rate of calcium efflux is highest in dogs. In addition, the differences in the response of mitochondrial Ca^{++} -accumulation among rats, rabbits and dogs may be related to differences in the activity of Ca^{++} -ATPase and the extent of sensitivity of inorganic phosphate or ethacrynic acid to the enzyme. It is of further interest that ethacrynic acid increased ATPase activity in the presence of MgCl_2 in dog kidney cortex mitochondria unlike the results in rats and rabbits (Fig. 4), thus resembling the action of uncoupling agents on the ATPase of mitochondria. We also have data which show that 2,4-dinitrophenol (5×10^{-5} M), well known as an uncoupler of mitochondria, stimulates 92.0% of ATPase observed in the presence of MgCl_2 and depresses 53.8% of Ca^{++} -accumulation in rat kidney cortex mitochondria (Values are not shown in the figure). The action of ethacrynic acid resembling the action of uncoupler on the ATPase was not depressed by inorganic phosphate (unpublished), and therefore, may relate in part to the inhibition of Ca^{++} -accumulation observed in the presence of inorganic phosphate in dog kidney cortex mitochondria (Fig. 3).

Lehninger has suggested that the ion transport function of mitochondria might play an important role in physiological regulation of calcium and active transcellular transport of reabsorptive substances in renal tubule cells (10). From the present results, it may be concluded that ethacrynic acid affects the intracellular calcium homeostasis of the kidney, to a different extent in each of the three species. Further investigation of the intracellular concentration of inorganic phosphate in the kidney cells of animal species employed in the present study is warranted.

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