EFFECT OF VERAPAMIL ON THE CALCIUM AND MAGNESIUM TRANSPORTS OF RAT KIDNEY CORTEX MITOCHONDRIA

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Accepted May 14, 1979

Abstract—The effect of verapamil on Ca\(^{2+}\) and Mg\(^{2+}\) accumulation was investigated in isolated rat kidney cortex mitochondria. For the 50% inhibition of Ca\(^{2+}\) accumulation, 2 x 10\(^{-4}\) M verapamil concentration was required in the presence of ATP (2 mM) and phosphate (5 mM). Omission of phosphate from the medium increased the inhibitory effect of verapamil on Ca\(^{2+}\) accumulation. Verapamil had no effect on Ca\(^{2+}\) accumulation in the presence of both ATP and succinate (7.8 mM), but further addition of phosphate resulted in a significant inhibition of Ca\(^{2+}\) accumulation by verapamil. Mg\(^{2+}\) accumulation of mitochondria was similarly depressed by verapamil. The same tendency was found as for the modification of verapamil effect by acetate in mitochondrial Ca\(^{2+}\) and Mg\(^{2+}\) accumulation. Succinate oxidation of mitochondria was not affected by verapamil in the absence of phosphate, but was inhibited by verapamil in the presence of phosphate. Therefore, it seemed reasonable to assume that the depression of Ca\(^{2+}\) and Mg\(^{2+}\) transport of mitochondria by verapamil is modulated by permeant anions.

It is well documented that verapamil acts as an inhibitor of Ca\(^{2+}\) translocation across the cell membrane in different tissues (1–4). A stimulative action of verapamil on urine flow and urinary Na\(^{+}\) and Ca\(^{2+}\) excretion upon infusion into the renal artery has also been demonstrated (5). On the other hand, calcium transport of mitochondria has been proposed to play an important role in the regulation of intra cellular Ca\(^{2+}\) environment in the kidney cell (6). It has also been shown that Mg\(^{2+}\) is accumulated by kidney cortex mitochondria (7). However, the precise mode of action of verapamil on transport of bivalent cations into mitochondria remains to be elucidated. In the present paper the effect of verapamil on Ca\(^{2+}\) and Mg\(^{2+}\) accumulation by kidney cortex mitochondria is described.

MATERIALS AND METHODS

Mitochondria were prepared from the kidneys of 200 g male Wistar rats by a modification of method used originally by Schnaitman and Greenawalt (8). The animals were decapitated and the tissues homogenized at 0°C in 0.25 M sucrose containing 0.38 mM tris (hydroxymethyl) aminomethane (Tris) and 0.1 mM ethylene glycol bis(\(\beta\)-aminoether)-N,N'-tetraacetic acid (EGTA). The homogenate was centrifuged at 850 \(\times\) g for 10 min at 3°C. The resultant supernatant fluid was recentrifuged at 850 \(\times\) g for 10 min at 3°C, yielding a second supernatant fraction which was centrifuged at 10,000 \(\times\) g for 10 min at 3°C. The resultant precipitate was resuspended in 0.25 M sucrose containing 0.38 mM Tris and
0.1 mM EGTA, and washed twice at 10,000 x g for 10 min at 3°C. The final pellets were resuspended in 0.25 M sucrose to yield a uniform suspension containing about 2.5 mg protein/ml. About 20 min were required to resuspend the final pellets before use. Protein concentration was determined by the method of Lowry et al. (9) using bovine serum albumin as a standard.

The basic medium consisted of 140 mM KCl, 10 mM Tris-HCl buffer (pH 7.4), 50 mM sucrose, 2 mM MgCl₂, 0.5 mM CaCl₂, 2 mM ATP (Tris salt). Other additions depending on experimental conditions are indicated in the legend. The reaction was started by the addition of 0.4 to 0.5 mg of mitochondrial protein in a total volume of 2 ml. The reaction was carried out at 30°C for 10 min and was stopped by removal of mitochondria with a set of membrane filters of three pieces (Sartorius 11305, pore size 0.6 μm). Separated mitochondria on the filter were washed with cold aqueous solution containing 140 mM KCl and 10 mM Tris-HCl buffer (pH 7.4). Ca²⁺ and Mg²⁺ in mitochondria were extracted with 1 ml of 61% nitric acid at room temperature overnight. Both cations were determined by atomic absorption spectrophotometer (Hitachi 170-10). Incorporated amounts of Ca²⁺ and Mg²⁺ were calculated by subtracting the cations content before incubation from that after incubation. Succinoxidase activity of mitochondria was measured by the conventional Warburg apparatus at 30°C for 40 min with air as gas phase. Tris-ATP was purchased from Sigma Chemical Company, verapamil was from the Eisai Co., Ltd., and all other chemicals were of analytical grade. Redistilled water was used throughout.

RESULTS

The effect of altering concentration of verapamil is shown in Fig. 1. By increasing the concentration of the drug, Ca²⁺ accumulation by rat kidney cortex mitochondria was markedly suppressed in the presence of ATP and phosphate as an energy source and a permeant anion, respectively. Ca²⁺ accumulation by kidney cortex mitochondria was depressed about 50% by 2 x 10⁻⁴ M verapamil. Accumulation of Mg²⁺ by kidney cortex mitochondria was also inhibited 60% by 4 x 10⁻⁴ M verapamil (not shown in the figure).

As shown in Fig. 2, Ca²⁺ accumulation by mitochondria in the absence of phosphate was less than that in its presence (5 mM). Inhibitory effect of verapamil (2 x 10⁻⁴ M) increased significantly from 42.3% in the presence of potassium phosphate (5 mM) to 65.0% in phosphate-free (basic) medium. The ad-
FIG. 2. Effect of potassium phosphate and succinate on Ca\(^{2+}\) accumulation of mitochondria in presence and absence of verapamil. Mitochondria were incubated in a basic medium supplemented with 5 mM potassium phosphate and 7.8 mM succinate. Open columns show control accumulation of Ca\(^{2+}\) by mitochondria in the absence of verapamil. Shaded columns show Ca\(^{2+}\) accumulated by mitochondria in the presence of verapamil (2 \(\times\) 10\(^{-4}\) M). Each column represents the mean value of four experiments. Vertical lines indicate the standard error. Significance of difference of verapamil effect from respective control, *P<0.01, **P<0.005, by the t-test.

FIG. 3. Effect of potassium phosphate and succinate on Mg\(^{2+}\) accumulation of mitochondria in the presence and absence of verapamil. Mitochondria were incubated, as described in Methods, in a basic medium supplemented with 5 mM potassium phosphate and 7.8 mM succinate as indicated. Open columns show control accumulation of Mg\(^{2+}\) by mitochondria. Shaded columns show Mg\(^{2+}\) accumulated by mitochondria in the presence of verapamil (2 \(\times\) 10\(^{-4}\) M). Each column represents the mean value of four experiments. Vertical lines show the standard error. Significance of difference of verapamil effect from respective control, *P<0.05, **P<0.025, ***P<0.005, by the t-test.

FIG. 4. Effect of potassium acetate and succinate on Ca\(^{2+}\) accumulation of mitochondria in the presence and absence of verapamil. Mitochondria were incubated in a basic medium supplemented with 5 mM potassium acetate and 7.8 mM succinate. Open columns show control accumulation of Ca\(^{2+}\) by mitochondria. Shaded columns show Ca\(^{2+}\) accumulated by mitochondria in the presence of verapamil (2 \(\times\) 10\(^{-4}\) M). Each column represents the mean value of three experiments. Vertical lines show the standard error. Significance of difference of verapamil effect from respective control, *P<0.005, by the t-test.
dition of succinate (7.8 mM) to the basic medium increased Ca\(^{2+}\) accumulation by mitochondria from 158.2±26.1 nmoles/10 min/mg protein to 550.2±30.6 nmoles/10 min/mg protein. No effect of verapamil (2 × 10\(^{-4}\) M) was seen on mitochondrial Ca\(^{2+}\) accumulation in the presence of succinate (7.8 mM) in the basic medium. However, further addition of potassium phosphate (5 mM) to the basic medium containing succinate (7.8 mM) resulted in 32.7% inhibition of mitochondrial Ca\(^{2+}\) accumulation by verapamil (2 × 10\(^{-4}\) M).

Figure 3 shows accumulation of Mg\(^{2+}\) by mitochondria and the effect of verapamil. The amount of Mg\(^{2+}\) accumulation, however, was relatively less than that of Ca\(^{2+}\). In general, Mg\(^{2+}\) accumulation of mitochondria was suppressed by verapamil (2 × 10\(^{-4}\) M) in a manner similar to the effect of the drug on Ca\(^{2+}\) accumulation. Verapamil at 2 × 10\(^{-4}\) M had no significant effect on Mg\(^{2+}\) accumulation by kidney cortex mitochondria in a basic medium containing succinate (7.8 mM), but the drug at the same concentration inhibited the accumulation of Mg\(^{2+}\) by 48.0% when potassium phosphate (5 mM) was added to the basic medium containing succinate.

We then attempted to determine why the verapamil action on Ca\(^{2+}\) and Mg\(^{2+}\) accumulation by mitochondria was affected by the presence of potassium phosphate in the medium, and the interaction of verapamil with acetate, another permeant anion, was studied. As shown in Fig. 4, the presence of 5 mM potassium acetate in the basic medium decreased Ca\(^{2+}\) accumulation by kidney cortex mitochondria from 299.2±29.2 nmoles/10 min/mg protein to 116.4±15.2 nmoles/10 min/mg protein (61.1% reduction) and Ca\(^{2+}\) accumulation in the basic medium containing succinate was also decreased by further addition of potassium acetate. The inhibition of Ca\(^{2+}\) accumulation by verapamil in a basic medium was abolished by the addition of potassium acetate. Verapamil significantly inhibited Ca\(^{2+}\) accumulation by mitochondria in much the same manner as that described for the presence of phosphate when acetate was added to the basic medium containing succinate.

The effect of verapamil on succinate oxidation of rat kidney cortex mitochondria was studied (Table 1). When succinate (15.6 mM) was present as a substrate in the basic medium, oxygen consumption was not influenced by verapamil (2 × 10\(^{-4}\) M). However, further addition of potassium phosphate resulted in the significant inhibition of succinate oxidation by verapamil.

**Table 1. Effect of verapamil on succinate oxidation of kidney cortex mitochondria**

| Addition                   | Control     | Verapamil (2 × 10\(^{-4}\) M) | % change |
|----------------------------|-------------|-------------------------------|----------|
| None                       | 1.02±0.05   | 1.07±0.32                     | -4.9%    |
| 5 mM Potassium phosphate   | 0.87±0.08   | 0.63±0.12*                    | -27.5%   |

Succinoxidase activity of mitochondria was measured at 30°C with a conventional Warburg apparatus. Indicated concentrations of potassium and verapamil were added to the basic medium containing succinate (15.6 mM). Succinoxidase activity was expressed as µl of O\(_2\) consumption/min per mg protein. Each value is the mean ±S.E. from three experiments. *Significantly different from control (p<0.05).
DISCUSSION

Kidney cortex mitochondria in the presence of potassium phosphate were able to accumulate a large amount of Ca\(^{2+}\), as reported by several groups of workers (10-13), from studies on rat liver mitochondria. Mg\(^{2+}\) was also accumulated from the reaction medium by kidney cortex mitochondria but Mg\(^{2+}\) accumulation was much less with respect to the magnitude of cation flux than accumulation of Ca\(^{2+}\), as described previously by Carafoli et al. (14), on rat liver mitochondria. The results given in Fig. 2 show that the inhibitory ability of verapamil on Ca\(^{2+}\) accumulation may relate to the presence of phosphate in the reaction medium. We determined preliminarily the concentration of inorganic phosphate in the medium after incubation (two experiments). When no phosphate was added to the basic medium, the concentration of phosphate was 1.06 mM and such was probably released by ATP hydrolysis in mitochondria. While, when no phosphate was added to the basic medium containing succinate, the concentration of phosphate was 0.54 mM, this being lower than that in the basic medium (succinate-free). When potassium phosphate (5 mM) was added to the medium at the onset of incubation, the phosphate level after incubation was 5.37 mM in the basic medium and 3.90 mM in that containing succinate. It is difficult to discuss the relationship between medium phosphate and verapamil effect from the standpoint of our present data, however, the concentration of phosphate in the medium may affect the inhibitory effect of verapamil on kidney cortex mitochondrial Ca\(^{2+}\) accumulation.

Frey and Janke (15) reported that 1 mM verapamil inhibited 50% of Ca\(^{2+}\) uptake by rat cardiac muscle mitochondria. It is difficult to actually compare their results with our data regarding the inhibitory ability of verapamil on Ca\(^{2+}\) accumulation by mitochondria because they did not describe the experimental conditions in their abstract. However, a much lower concentration of verapamil, 2 x 10\(^{-4}\) M, seemed to cause 50% inhibition of Ca\(^{2+}\) accumulation by kidney cortex mitochondria in the basic medium containing potassium phosphate (Fig. 1).

Inhibition of Mg\(^{2+}\) accumulation by verapamil was similar to that of Ca\(^{2+}\) accumulation by the drug in kidney cortex mitochondria (Fig. 3). Figure 3 shows that the presence of phosphate may be required for the inhibitory effect of verapamil on Mg\(^{2+}\) accumulation in a similar manner to that of the drug on Ca\(^{2+}\) accumulation.

Rasmussen et al. (11) suggested that acetate could replace phosphate and enters into mitochondria passively with Ca\(^{2+}\). Thus, acetate was chosen as a permeant anion of mitochondrial membrane and the effect of acetate on verapamil action was examined (Fig. 4). In contrast to the increase in Ca\(^{2+}\) accumulation after addition of potassium phosphate, the presence of potassium acetate (5 mM) in the basic medium decreased Ca\(^{2+}\) accumulation of kidney cortex mitochondria by 61.1% and further addition of potassium acetate to the basic medium containing succinate decreased that by 42.6% (Fig. 4). Verapamil had no effect on Ca\(^{2+}\) accumulation by mitochondria in the presence of potassium acetate in the basic medium, but the drug inhibited Ca\(^{3+}\) accumulation by 55.2% by the addition of potassium acetate in the basic medium containing succinate. This together with the observations that the inhibitory effect of verapamil on Ca\(^{2+}\) accumulation was decreased by the addition of phosphate to the basic medium and observed in the presence of phosphate in the medium
containing succinate seems to indicate that the verapamil effect on Ca\(^{2+}\) accumulation of kidney cortex mitochondria is influenced by phosphate and also by acetate, in an analogous fashion. Namely, if phosphate or acetate is added to the basic medium, the process of Ca\(^{2+}\) and Mg\(^{2+}\) accumulation by kidney cortex mitochondria is less sensitive to the effects of verapamil, while if phosphate or acetate is added to the basic medium containing succinate, that of Ca\(^{2+}\) and Mg\(^{2+}\) accumulation becomes sensitive to verapamil effect.

It was observed by Sakurada et al. (16) that verapamil enhanced the succinate oxidation of heart mitochondria, but their reaction medium lacked the presence of phosphate. It was also reported that verapamil caused a reduction in the myocardial oxygen demand (2). The present results show that succinate oxidation by kidney cortex mitochondria is depressed by \(2 \times 10^{-4}\) M verapamil in the presence of phosphate in the medium under the condition which Ca\(^{2+}\) and Mg\(^{2+}\) accumulation is inhibited by the same concentration of verapamil.

We are particularly concerned with the possibility that the inhibitory effect of the drug may account for the altered permeability of mitochondrial membrane to permeant anions, phosphate and acetate. Further studies to determine whether or not verapamil acts to diminish permeability of phosphate and acetate into mitochondria are in progress in our laboratory.

Acknowledgements: We thank Professor K. Yamamoto for pertinent advice and Miss F. Matsuda, (a student at our college), for technical assistance. Verapamil was kindly provided by Eisai Co., Ltd.

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