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EFFECTS OF DIURETICS ON CALCIUM UPTAKE AND RELEASE IN RENAL MICROSOMES

TAKASHI DAN and MUNEKAZU GEMBA*
Department of Pharmacology, Osaka College of Pharmacy, Kawai, Matsubara, Osaka 580, Japan

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Abstract—A variety of diuretics were tested for their effects on Ca^{2+} uptake and release by microsomes isolated from rat kidneys. Mersalyl acid, ethacrynic acid, furosemide and bumetanide inhibited Ca^{2+} uptake by microsomes. Cysteine abolished the inhibitory effects of mersalyl acid and ethacrynic acid on Ca^{2+} uptake. No appreciable differences in the inhibition were seen between microsomes from kidney cortex and those from medulla. Acetazolamide had no significant effect on Ca^{2+} uptake; however, hydrochlorothiazide stimulated Ca^{2+} uptake activity. The effects of the diuretics on Ca^{2+} uptake by microsomes were entirely on the process of Ca^{2+} accumulation in microsomes, since release of Ca^{2+} that had been taken up previously was not influenced by any of the diuretics tested. These results provide information concerning the biochemical mechanisms of action of diuretics on Ca^{2+} reabsorption in the kidney.

The effects of diuretics on excretion of Ca^{2+} by the kidney have been widely studied by clearance and micropuncture methods. In an earlier review, Suki *et al.* [1] reported that Ca^{2+} excretion during the administration of furosemide, ethacrynic acid or mercurials is increased and that it is slightly affected by the administration of acetazolamide and thiazides. Our previous work indicated that, in the rat, bumetanide also increased Ca^{2+} excretion, but acetazolamide does not [2]. The increase in Ca^{2+} excretion with furosemide and other diuretics apparently involves inhibition of tubular reabsorption of Ca^{2+} [1]. On the other hand, it has been demonstrated that thiazides decrease Ca^{2+} excretion [3–5]. In a more recent study it was suggested that chlorothiazide enhances renal Ca^{2+} reabsorption [6]. However, the mechanisms of action of diuretics on the renal tubule are still incompletely known in terms of Ca^{2+} reabsorption.

Experiments with both plasma membrane and microsomal fractions of kidney indicate that ATP-dependent Ca^{2+} pump activity occurs in these fractions, suggesting that such activity plays an important role in Ca^{2+} transport and in the regulation of intracellular Ca^{2+} [7–10]. Harada *et al.* [11] showed that Ca^{2+} uptake by renal microsomes may be useful as a model system for studying Ca^{2+} transport in the kidney. In this present paper we examine the effects of diuretics on ATP-dependent Ca^{2+} uptake and Ca^{2+} release by microsomes isolated from rat kidneys in order to obtain information relating to the mechanism of action of diuretics on Ca^{2+} transport in renal plasma membrane.

MATERIALS AND METHODS

Materials. Furosemide (Lasix, Hoechst Japan Ltd., Tokyo, Japan) and acetazolamide (Diamox, Lederle Japan Ltd., Tokyo, Japan) were dissolved in redistilled water. Mersalyl acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), ethacrynic acid (Japan Merk Banyu Co., Tokyo, Japan), and bumetanide (Sankyo Co. Ltd., Tokyo, Japan) were dissolved in Tris and adjusted to pH 6.6 with HCl. The final concentration of Tris in the assay system was less than 32 mM. The concentration of Tris used did not affect microsomal Ca^{2+} uptake and release. Hydrochlorothiazide (Sandoz Pharmaceuticals Ltd., Tokyo, Japan) and the divalent cation ionophore A 23187 (Lilly Research Corp., Indianapolis, IN, U.S.A.) were diluted in 50% ethyl alcohol. The final concentration of ethyl alcohol was 2.5% in the assay medium. EGTA (glycoletherdiamine tetra-acetic acid) and ATP (Tris salt, Sigma Chemical Co., St. Louis, MO, U.S.A.) were adjusted to pH 6.6 with Tris.

Preparation of microsomes from rat renal cortex and medulla. Male Wistar rats, weighing 200–250 g, were used in all experiments. After decapitation, both kidneys were removed immediately, decapsulated and cooled in ice-cold 0.25 M sucrose. Thereafter the kidneys were divided with scissors into cortex and medulla. Homogenization was performed in 0.25 M sucrose containing 10 mM imidazole–HCl buffer (pH 6.6) at 0°. The procedure used for preparation of microsomes was the same as that reported previously, except that final centrifugation at 105,000 *g* was omitted [2]. The final precipitate was used as the microsomal fraction after resuspension in 0.25 M sucrose containing 10 mM imidazole–HCl buffer (pH 6.6). The microsomes were used immediately after preparation for assay of Ca^{2+} uptake and release.

Assay of Ca^{2+} *uptake.* Ca^{2+} uptake studies were carried out at 37° in a standard medium containing the components specified in the legends of the figures and tables. The standard medium contained 100 mM KCl, 30 mM imidazole–HCl buffer (pH 6.6), 5 mM NaN_3, 20 mM ammonium oxalate, 5 mM ATP, 50 μM $CaCl_2$, 5 mM $MgCl_2$, $^{45}CaCl_2$ (0.1 μCi/ml),

* To whom reprint requests should be addressed.

25 mM sucrose and microsomes (0.2–0.3 mg protein/ml) as final concentrations. The assay was initiated by the addition of microsomal fraction. Aliquots (0.5 ml) of the assay were filtered through 0.3 μm membrane filters (Sartorius SM 11325) after incubation, and the separated microsomes on the filters were washed with 0.25 M sucrose (3 ml). Accumulated $^{45}Ca^{2+}$ in microsomes was determined by liquid scintillation spectrometry.

Assay of Ca^{2+} *release.* Calcium release was measured after loading microsomes with Ca^{2+} for 20 min at 37° in 3 ml of the standard medium for Ca^{2+} uptake studies. At zero time, 0.5-ml aliquots were removed for determination of $^{45}Ca^{2+}$ loaded in microsomes; then Ca^{2+} release was initiated by the addition of 0.5 ml of 100 mM KCl plus EGTA solution containing reagents specified in the legends of the figures and tables. The final concentration of EGTA was 2 mM. The rate of Ca^{2+} release was determined by measuring filtered $^{45}Ca^{2+}$ aliquots at the times indicated.

Protein assay. Protein was determined by the method of Lowry *et al.* [12] using bovine serum albumin as standard.

Calculations. ATP-dependent Ca^{2+} uptake was calculated by subtracting Ca^{2+} binding of microsomes in the absence of ATP in the medium from total Ca^{2+} uptake in its presence. Linear regression of a Hofstee plot, computed by least squares analysis, was used to obtain values for apparent K_m and V_{max} for Ca^{2+} uptake. K_i values, concentrations of diuretics producing 50 per cent inhibition of Ca^{2+} uptake, were also calculated using linear regressions.

RESULTS

Properties of Ca^{2+} *uptake by renal microsomes.* Calcium uptake by microsomes isolated from rabbit, rat or cat kidney has been studied by other workers [7, 8, 11]. Their results, however, show a variety of values for Ca^{2+} uptake and sensitivity to oxalate. Therefore the properties of Ca^{2+} uptake were first re-examined with microsomes isolated from rats.

Calcium was accumulated by microsomes of the renal cortex in the presence of ATP but not in its absence (Fig. 1). In the standard medium, the initial rate of Ca^{2+} uptake was maintained at least for 30 min. In the absence of ATP there was no change in Ca^{2+} content of microsomes during incubation. The residual activity in its absence probably represents binding of Ca^{2+} to microsomal membranes; the experimental conditions in the following did not affect Ca^{2+} binding. Therefore, the Ca^{2+} uptake activity of microsomes described in the following has been calculated after subtracting Ca^{2+} binding from the total Ca^{2+} uptake, and the term—Ca^{2+} uptake—signifies ATP-dependent Ca^{2+} uptake.

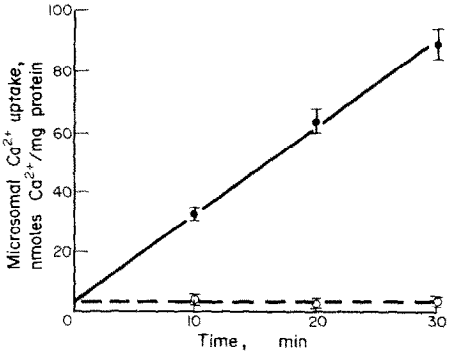

Fig. 1. Time course for total Ca^{2+} uptake. Microsomes of renal cortex were incubated in the standard medium with (●—●) or without (○– – –○) 5 mM ATP. Each point represents the mean of four experiments. Vertical bars indicate S.E.M.

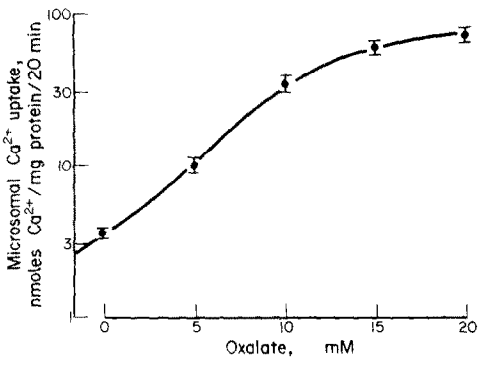

Fig. 2. Dependence of microsomal Ca^{2+} uptake of renal cortex on oxalate. Assays of Ca^{2+} uptake were performed in the standard medium containing various concentrations of ammonium oxalate. Each point represents the mean of four experiments. Vertical bars indicate S.E.M.

The dependence of Ca^{2+} uptake on oxalate is shown in Fig. 2. Ca^{2+} uptake by microsomes was increased in a concentration-dependent way. At 20 mM oxalate, Ca^{2+} uptake was almost maximal. The increased Ca^{2+} uptake was probably due to the precipitation of calcium oxalate in the interior of the microsomal vesicles.

Figure 3 illustrates the effect of the Ca^{2+} concentration of the medium on Ca^{2+} uptake by micro-

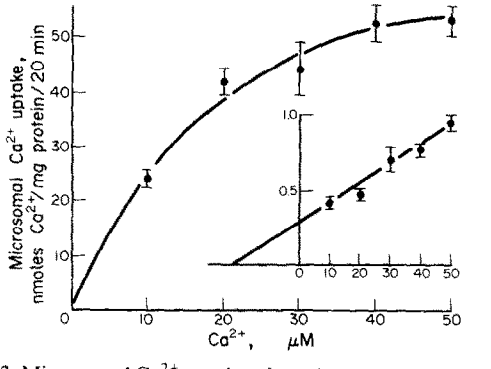

Fig. 3. Microsomal Ca^{2+} uptake of renal cortex as a function of the concentration of Ca^{2+}. $CaCl_2$ was added to the standard medium to give final concentrations ranging from 10 to 50 μM. The inset shows the data plotted according to the Hofstee equation. The ordinate of the inset represents Ca^{2+} concentration/microsomal Ca^{2+} uptake (μM Ca^{2+}·mg protein·20 min/nmoles Ca^{2+}) and the abscissa represents Ca^{2+} concentration (μM). Each point represents the mean of four experiments. Vertical bars indicate S.E.M.

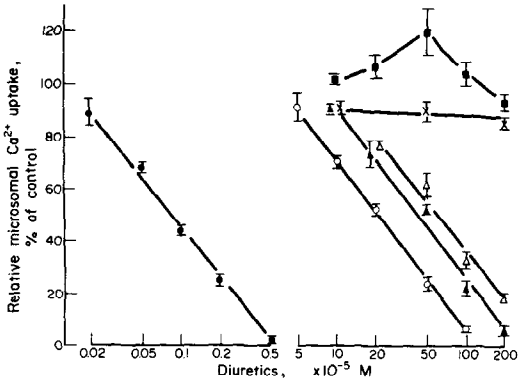

Fig. 4. Dose-response relationship between microsomal Ca^{2+} uptake of renal cortex and various diuretics. Key: mersalyl acid (●—●), ethacrynic acid (○—○), bumetanide (▲—▲), furosemide (△—△), acetazolamide (×—×) and hydrochlorothiazide (■—■). Control uptake values in the standard medium without or with 2.5% ethyl alcohol were 49.03 ± 2.88 or 35.78 ± 9.40 nmoles Ca^{2+}/mg protein/20 min, respectively. Vertical bars indicate S.E.M.

somes. The stimulation of Ca^{2+} uptake was dependent on the Ca^{2+} concentration of the medium. A Hofstee plot of the data is presented in the inset. The apparent V_{max} was 77.1 ± 8.2 nmoles Ca^{2+}/mg protein/20 min at 37°. The K_m for Ca^{2+} in the medium was 20.8 ± 4.4 μM.

Omission of sodium azide from the incubation medium had no effect on the Ca^{2+} uptake (data not shown). Therefore, Ca^{2+} uptake by the microsomal fraction was not ascribable to a contamination with Ca^{2+} uptake by mitochondria.

Effects of diuretics on microsomal Ca^{2+} uptake. The effects of several diuretics on Ca^{2+} uptake by renal cortical microsomes are represented in Fig. 4. Mersalyl acid, ethacrynic acid, bumetanide and furosemide inhibited Ca^{2+} uptake. The 50 per cent inhibitory concentrations (K_i) of these diuretics were calculated by least-squares regression analysis: 0.85 μM mersalyl acid, 0.21 mM ethacrynic acid, 0.44 mM bumetanide and 0.66 mM furosemide. Acetazolamide did not affect Ca^{2+} uptake in the range of concentrations between 0.1 and 2 mM. The rate of Ca^{2+} uptake, however, was increased significantly by hydrochlorothiazide, with an optimum concentration of 0.5 mM.

The possibility that cysteine modifies the inhibitory action of diuretics on Ca^{2+} uptake was examined (Table 1). Cysteine (2 mM) increased Ca^{2+} uptake in the standard medium (see legend) and markedly diminished the inhibitory effects of mersalyl acid and ethacrynic acid on Ca^{2+} uptake. Bumetanide and furosemide, however, decreased the Ca^{2+} uptake activity of renal cortical microsomes in both the presence and the absence of cysteine.

It is well known that mercurials, ethacrynic acid, furosemide and bumetanide act on the loop of Henle in addition to the proximal tubule [1, 13], so the effects of these diuretics on Ca^{2+} uptake by microsomes from the renal medulla were tested. As shown in Table 2, no significant differences in Ca^{2+} uptake activity were observed in microsomes isolated from renal cortex and medulla in the medium without diuretics. The inhibitory effects of diuretics on Ca^{2+} uptake were also observed in microsomes of kidney medulla as well as in those of cortex.

Calcium release from microsomes and the effects of diuretics. Calcium uptake measured in the presence of oxalate is believed to represent active Ca^{2+} transport alone, because oxalate reduces the passive outflow of Ca^{2+} by precipitating the Ca^{2+} taken up [8, 14]. The divalent cation ionophore A 23187 (0.5 μM) inhibited completely Ca^{2+} uptake in the presence of 20 mM oxalate (data not shown). After Ca^{2+} uptake had progressed in the standard medium with oxalate for 20 min, Ca^{2+} release was initiated by the addition of 2 mM EGTA as a final concentration. As seen in Fig. 5, gradual Ca^{2+} release from microsomes was observed during 30 min of incubation. Further addition of A 23187 (0.5 μM) caused rapid Ca^{2+} release. On the basis of the above data, it is suggested that A 23187 enhances the permeability to Ca^{2+} of the microsomal membranes of kidney. Therefore, the effect of the diuretics on Ca^{2+} release from renal microsomes was studied (Fig. 5). If the inhibitory effects of diuretics on Ca^{2+} uptake were due to an increase in the rate of Ca^{2+} release such as with A 23187, Ca^{2+} release with EGTA would be changed by these diuretics.

Table 1. Effects of cysteine on the inhibition by diuretics of Ca^{2+} uptake by renal cortical microsomes*

Diuretics	Concn (M)	Relative Ca^{2+} uptake† Cysteine 0 mM	Cysteine 2 mM
None		100‡	100‡
Mersalyl acid	1×10^{-6}	42.5 ± 4.0	109.9 ± 4.5
Ethacrynic acid	2×10^{-4}	44.7 ± 1.6	83.8 ± 2.6
Bumetanide	5×10^{-4}	41.9 ± 1.5	45.4 ± 1.4
Furosemide	1×10^{-3}	43.8 ± 1.2	43.6 ± 0.9

* Calcium uptake was measured after incubation for 20 min at 37° in the standard medium with or without 2 mM cysteine. Diuretics were added at the concentrations indicated.

† Data are the means ± S.E.M. of four experiments.

‡ Calcium uptake activities in the standard medium in the absence and presence of cysteine were 58.29 ± 9.00 and 72.33 ± 11.07 nmoles Ca^{2+}/mg protein/20 min, respectively.

Table 2. Effects of diuretics on Ca^{2+} uptake by microsomes of renal cortex and medulla*

Diuretics	Concn (M)	Microsomal Ca^{2+} uptake† (nmoles Ca^{2+}/mg protein/20 min) Cortex	Medulla
None		58.29 ± 9.00	51.40 ± 11.84
Mersalyl acid	1×10^{-6}	22.98 ± 2.63	18.45 ± 2.79
Ethacrynic acid	2×10^{-4}	24.96 ± 3.67	26.12 ± 5.10
Bumetanide	5×10^{-4}	23.95 ± 4.37	26.90 ± 6.50
Furosemide	1×10^{-3}	24.58 ± 3.80	25.38 ± 5.98

* Microsomes were incubated in the standard medium with diuretics as indicated.

† Data are the means ± S.E.M. of four experiments.

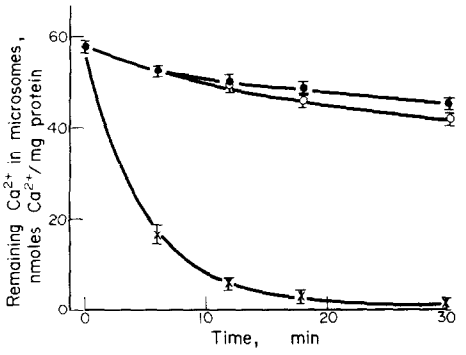

Fig. 5. Time course for Ca^{2+} release from renal cortical microsomes. At zero time, Ca^{2+} release was initiated by addition of EGTA (●—●), EGTA plus ethyl alcohol (○—○) and EGTA, ethyl alcohol plus A 23187 (×—×). The final concentrations of EGTA, ethyl alcohol and A 23187 were 2 mM, 2.5% and 0.5 μM, respectively. Each point represents the mean of four experiments. Vertical bars indicate S.E.M.

As seen in Table 3, no diuretic influenced the rate of Ca^{2+} release from microsomes at concentrations that significantly affected Ca^{2+} uptake of microsomes.

DISCUSSION

The present study demonstrates that Ca^{2+} uptake by renal cortical microsomes is differentially sensitive to various diuretics. Mersalyl acid, in μM concentrations, strongly inhibited Ca^{2+} uptake. The high ceiling diuretics, ethacrynic acid, furosemide and bumetanide, also decreased Ca^{2+} uptake, but acetazolamide did not. Mersalyl acid and the high ceiling diuretics also inhibited Ca^{2+} uptake by microsomes isolated from renal medulla. On the other hand, hydrochlorothiazide significantly increased Ca^{2+} uptake activity, with an optimum concentration of 0.5 mM.

It is well established that cysteine and dithiothreitol abolish the effects of ethacrynic acid and mercurials on mitochondria, cell membranes or cell-free preparations [15–19]. As shown in Table 1, cysteine prevented the inhibitory effects of mersalyl acid and ethacrynic acid on Ca^{2+} uptake by renal cortical microsomes, indicating that both diuretics exert their actions on Ca^{2+} uptake by reacting with sulfhydryl groups of microsomal membranes. However, cysteine could not block the effects of furosemide and bumetanide. The results suggest that the mechanisms of inhibition by furosemide and bumetanide of Ca^{2+} uptake are different from the actions of mersalyl acid and ethacrynic acid.

It has been demonstrated that the divalent cation ionophore A 23187 makes biological membranes permeable to Ca^{2+} [20, 21]. The present study also showed that A 23187 made kidney microsomal membrane permeable to Ca^{2+} (Fig. 5), but diuretics did not affect at all the rate of Ca^{2+} release from renal cortical microsomes (Table 3). More recently, Moore and Landon [22] have demonstrated that the high ceiling diuretics, ethacrynic acid and furosemide, inhibit the Ca^{2+} pump activity of microsomes isolated from whole kidneys and that furosemide acts as a reversible, non-competitive inhibitor. Although their light microsomal fraction was slightly different from our microsomal preparation, their results of inhibitory effects of both drugs were very similar to the data obtained in the present study. However, on the basis of our results it seems that mersalyl acid and high ceiling diuretics inhibit Ca^{2+} accumulation and hydrochlorothiazide accelerates it.

In earlier findings obtained *in vivo*, it was demonstrated that mersalyl acid and high ceiling diuretics increase urinary Ca^{2+} excretion but that thiazides decrease Ca^{2+} excretion [1–6]. The effects of diuretics on urinary Ca^{2+} excretion involve their action on tubular reabsorption of Ca^{2+}. There may exist a causal relationship between the effects of diuretics on Ca^{2+} reabsorption by renal tubules and their influence on Ca^{2+} uptake by kidney microsomes. We do not have any data on the renal cytoplasmic levels of diuretics after administering the drugs to rats *in vivo*. The K_i values for the diuretics, except for mersalyl acid, were found not to be very small. Therefore, further study is needed to determine the effects of diuretics on Ca^{2+} uptake by microsomes isolated from kidney after the injection of pharmacologically active dosages.

An ATP-dependent Ca^{2+} uptake system has been demonstrated in the microsomes of renal cortex, suggesting the importance of the system in the regulation of cytosol Ca^{2+} [8]. This concept is

Table 3. Effects of diuretics on Ca^{2+} release from renal cortical microsomes*

Diuretics	Concn (M)	Ca^{2+} release† (nmoles Ca^{2+}/mg protein/20 min)
None		11.53 ± 0.96
Mersalyl acid	1×10^{-6}	10.98 ± 1.44
Ethacrynic acid	2×10^{-4}	13.36 ± 0.94
Bumetanide	5×10^{-4}	12.25 ± 1.44
Furosemide	1×10^{-3}	13.68 ± 1.39
Hydrochlorothiazide	5×10^{-4}	11.17 ± 0.51

* Microsomes were preincubated in the standard medium for 20 min before addition of 2 mM EGTA and diuretics as indicated. Calcium release from the microsomes was measured after subsequent incubation for 20 min at 37°.

† Data are the means ± S.E.M. of four experiments.

strengthened by the finding that Ca^{2+} uptake by cortical and papillary microsomes is enhanced by perfusion with parathyroid hormone [11]. In addition, the present data indicate that Ca^{2+} uptake by renal microsomes is responsive to diuretics and that the effects of the drugs on this uptake are similar to their effects on Ca^{2+} reabsorption *in vivo*. These data provide further support for renal microsomes being a useful model system to study Ca^{2+} transport in the kidney. Although it is not clear how microsomal Ca^{2+} uptake relates to overall transepithelial Ca^{2+} transport in kidney, one may speculate that the direct action of diuretics on Ca^{2+} uptake by renal microsomes probably participates in their mechanisms of action on tubular Ca^{2+} reabsorption.

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