A Calcium Binding Component of Dog Kidney Cortex and Its Relationship to Calcium Transport[1] (35770)

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The mechanism by which calcium is actively transported in kidney cortex is poorly understood. It has been suggested that one of the components which participates in active transport is protein in nature. Proteins have been implicated in transport of sugars in bacteria (1, 2). Regarding ion transport, Wasserman *et al.* has purified a protein from chick intestinal mucosa which binds calcium and is thought to be related to calcium transport (3, 4). The quantity of this mucosal protein was correlated with the calcium absorptive capacity of chick intestine. The present study was undertaken to investigate whether or not a protein binding component might be implicated in calcium reabsorption in kidney. We have found a component in dog renal cortex which binds calcium and which may play a physiological role in calcium transport.

Methods and Materials. Mongrel dogs, weighing between 14 and 20 kg, were anesthetized by intravenous administration of pentobarbital. The ureters were catheterized at the point of their junction with the bladder. Arterial blood was obtained through an indwelling needle placed in a femoral artery. Following urine and blood collections for clearance determinations, the left kidney and spleen were removed. Immediately after removal the kidneys were perfused via the renal artery with 50 ml of chilled 0.3 *M* sucrose, and 0.014 *M* Tris at pH 7.4. Tissues were assayed within 2 hr of removal of the kidney or were stored at −20°. Stored tissue was used for most of the studies on the nature of the binding component.

Spleen, renal cortex, and inner medulla were separated, minced and homogenized in cold, buffered sucrose solution (4 ml of buffer/g tissue) in a Virtis homogenizer at 45,000 rpm, for 1 min. The homogenate was centrifuged at 750*g* for 10 min to remove cellular debris. The supernatant fluid was either passed through two layers of gauze or recentrifuged at 750*g* for 10 min. The resulting fluid was centrifuged at 16,000*g* for 20 min. The microsomal rich supernatant was used in the following studies unless otherwise indicated. All preparative procedures were carried out at 0–4°. Calcium binding capacity was measured by a modification of the assays of Wasserman *et al.* (3, 4) and Harmeyer and DeLuca (5). All procedures were carried out at room temperature. One ml of each fraction derived from differential centrifugation of the tissue homogenate was added to 1 ml of a solution containing 0.014 *M* Tris, 0.12 *M* NaCl and 4.74 m*M* KCl and 0.1 ml of solid Chelex-100 resin (100-200 mesh, Biorad) at pH 7.4. To this was added approximately 10^6 cpm of ^{45}Ca (0.5 μCi in 0.1 ml) and the mixture was agitated for 20 min in a wrist action shaker. The samples were then centrifuged at 1000*g* for 10 min. One ml of the supernatant fluid was added to 1 ml of buffer containing Chelex resin and the above procedure was repeated. After centrifugation, the supernatant fluid was filtered through Whatman No. 2 filter paper to prevent the transfer to the counting vial of ^{45}Ca bound to Chelex particles.

Radioactivity in the soluble fraction after this procedure was determined by liquid scintillation counting of 0.5 ml of the filtrate in 10 ml of 10% Beckman BBS-3 in a toluene solution. The counting efficiency was approximately 80–85%. Radioactivity represents calcium bound preferentially to a soluble com-

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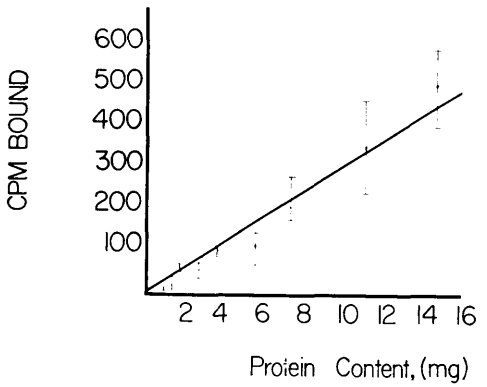

FIG. 1. The 16,000*g* supernatant fluid from kidney cortex was diluted with sucrose–Tris buffer and assayed for binding activity. Each dilution was assayed in triplicate. The mean of the triplicate determination and the range of values are shown.

ponent in competition with Chelex resin.

Protein was assayed by the method of Lowry *et al.* (6). Urine calcium content was determined by atomic absorption spectrophotometry. Calcium binding capacity of the binding component is expressed as net counts per minute bound to the soluble component per milligram of protein. Net counts bound to the soluble component are defined as counts per minute in the assay tube containing tissue protein minus counts per minute in assay tubes containing only buffer.

Results are analyzed by Student's *t* test taking $p<0.05$ to be the level of significance.

Results. The two-step procedure for the assay of calcium binding by fractions of dog kidney was necessary because of the lack of reproducibility of the previously published assays (3–5) when applied to this tissue. Many variations of the Chelex assay were tried and the procedure reported in this paper combined the essential features of the Wasserman *et al.* (3, 4), and the Harmeyer and DeLuca assay (5). This combination resulted in the best reproducibility of the determinations of many variations tried. The linearity of the assay system when the 16,000*g* supernatant fluid is diluted with buffered sucrose is illustrated in Fig. 1. Each point represents the mean of triplicate determinations and brackets encompass the range of values. Under our conditions, one obtains a linear relationship between the counts which remained soluble and the protein content of the tissue fractions. To assure linearity, most assays were done with sufficient protein to bind 100 cpm or more. All assays were done in triplicate. Because the assay uses a double exposure to the Chelex resin it measures binding of calcium which is extremely tight and which may be irreversible under the conditions used.

Table I compares the calcium binding capacity of three different tissues—kidney cortex, kidney medulla, and spleen as obtained from "hypotonic saline"-loaded animals. These dogs were given 1 liter of 0.45% sodium chloride intravenously at a rate of 16 ml/min and a sustaining infusion of 0.45% sodium chloride at a rate of 5 ml/min. Additional isotonic saline was administered, if necessary, to assure a urine flow of approximately 1 ml/min.

The binding capacity, as well as its subcellular distribution, in these three tissues was different. In the cortex, the binding capacity was greatest in the 16,000*g* supernatant fluid and further centrifugation reduced the binding capacity. Little binding capacity was found in the 100,000*g* supernatant fluid. Attempts to recover the activity from the 100,000*g* pellet resulted in a solution of moderately high binding capacity but very poor total recovery of the activity. In other words,

TABLE I. Calcium Binding Capacity (cpm/mg of protein).

	Supernatant fluid, 750*g*	Pellet, 16,000*g*	Supernatant fluid	
			16,000*g*	100,000*g*
Renal				
cortex ($n=8$)	439 ± 61	327 ± 27	617 ± 94	92 ± 34
medulla ($n=8$)	117 ± 18	126 ± 32	66 ± 13	12 ± 7
Spleen ($n=6$)	85 ± 20	76 ± 21	94 ± 27	47 ± 12

TABLE II. Cortex Binding Capacity (cpm/mg of protein).

	n	UV_{Ca} (μg/min)	Supernatant, 750g	Pellet, 16,000g	Supernatant 16,000g	Supernatant 100,000g	Pellet, 100,000g
Hypotonic saline	6	52.3 ± 14.0	434 ± 61	343 ± 34	578 ± 123	59 ± 15	—
Thyroid-parathyroidectomy + hypotonic saline (PTX)	4	33.7 ± 7.0	834 ± 90	1078 ± 252	687 ± 68	110 ± 12	—
Hydropenia	10	19.5 ± 7.1	389 ± 34	297 ± 27	865 ± 140	104 ± 14	1444 ± 141
DOCA + hydropenia	4	35.1 ± 5.2	235 ± 24	205 ± 49	212 ± 19	58 ± 13	903 ± 174
				p values			
Saline vs PTX		ns	<<0.01	0.01	ns	0.025	—
Saline vs Hydro		0.025	ns	ns	0.05	0.05	—
Hydro vs DOCA		0.05	<<0.01	ns	<<0.01	0.05	0.01

a significant portion of the calcium binding component was lost by the ultracentrifugation. The medulla had considerably less binding capacity than cortex. As an additional distinguishing factor, the binding capacity of the medulla was greatest in the 16,000*g* pellet. The binding capacity of the spleen differed greatly from both kidney cortex and medulla. The binding capacity of spleen was distributed equally in most fractions.

Electron microscopic photographs[2] showed the 16,000*g* pellet of both cortex and medulla to contain mitochondria while the 100,000*g* pellet was rich in microsomes and free of mitochondria.

To determine if there was a calcium binding component of dog kidney which participates in the reabsorptive process, the calcium binding capacities of kidney cortex and medulla were studied after various treatments. A decrease in the reabsorption of calcium results in an increase in the rate of calcium excretion [UV_{Ca}] and might be accompanied by a decrease in the binding capacity if the binding component were related to reabsorption. Calcium excretion was altered by four maneuvers.

1. Hypotonic saline. One liter of 0.45% sodium chloride was given intravenously to each of eight dogs. Of these, six had urine flow rates greater than 1.0 ml/min. The calcium binding capacities of the cortical and medullary fractions obtained from these six dogs are included in Tables II and III. The mean and SEM for UV_{Ca} in this series was 52.3 ± 14.0 μg/min. Comparisons with dogs treated by other physiological maneuvers are shown below.

2. Thyroid-parathyroidectomy. Six dogs were thyroid-parathyroidectomized 3 to 6 days prior to the experiment. Mean serum calcium levels were reduced by about 25% in comparison with group 1. Dogs in this series were given infusions as in group 1. The data from the four dogs with urine flow rates of 1.0 ml/min and greater are included in Tables II and III. The UV_{Ca} in this series was not significantly different from the UV_{Ca} of the "hypotonic saline" series. However, there were marked differences in the calcium binding capacities of the cortex of the two groups. The binding capacity of the 750*g* supernatant fluid, the 16,000*g* pellet, and the 100,000*g* supernatant fluid were markedly increased by thyroid-parathyroidectomy. The calcium binding capacity of the medulla showed no significant changes from that of the hypotonic saline series.

3. Hydropenia. Ten hydropenic dogs were given a sustaining infusion of 1 ml/min of 0.9% saline. All dogs in this series had urine flow rates less than 1.0 ml/min. The data are included in Tables II and III. The mean UV_{Ca} of this group was 19.5 ± 7.1 μg/min as compared to 52.3 ± 14.0 μg/min found in

[2] Electron microscopic photographs were generously provided by Dr. J. Hammond of the VA Research Hospital.

TABLE III. Medulla Binding Capacity (cpm/mg of protein).

	n	UV_{Ca} (μg/min)	Supernatant, 750g	Pellet, 16,000g	Supernatant 16,000g	Supernatant 100,000g	Pellet, 100,000g
Hypotonic saline	6	52.3 ± 14.0	97 ± 15	117 ± 42	57 ± 14	15 ± 10	—
Thyroid-parathyroidectomy + hypotonic saline (PTX)	4	33.7 ± 7.0	121 ± 48	151 ± 20	125 ± 39	9 ± 4	—
Hydropenia	10	19.5 ± 7.1	152 ± 8	165 ± 15	110 ± 20	16 ± 6	402 ± 182
DOCA + hydropenia	4	35.1 ± 5.2	113 ± 6	139 ± 19	69 ± 6	16 ± 8	282 ± 48
				p values			
Saline vs PTX		ns	ns	ns	ns	ns	—
Saline vs Hydro		0.025	0.01	ns	0.05	ns	—
Hydro vs DOCA		0.05	<0.01	ns	0.05	ns	ns

the hypotonic saline series. Concomitant with the decrease in the rate of excretion of calcium was an increase in the calcium binding capacity of the cortex found in the 16,000g supernatant fluid and the 100,000g pellet. Similar changes in the calcium binding capacity of the medulla were seen in the 750g supernatant fluid and the 16,000g supernatant fluid.

4. DOCA. In order to increase the UV_{Ca} of the hydropenic dogs deoxycorticosterone acetate (DOCA) was injected for 7 days (20 mg/day). The six dogs in the DOCA series fell into two distinct groups based on their rate of calcium excretion. Four dogs had UV_{Ca} greater than 20 μg/min. These data are included in Tables II and III. DOCA significantly reduced the calcium binding capacity of all fractions of the cortex except the 16,000g pellet. In the medulla the 750g and 16,000g supernatant fluids also had reduced binding capacity in comparison to the hydropenic series (group 3).

There is no definitive association between UV_{Ca} and calcium binding data in the data of Tables II and III. However, when all the experimental data in the four series were pooled and the calcium binding data was divided into two groups based on the UV_{Ca}, a correlation was uncovered. A UV_{Ca} greater than 20 μg/min is associated with a decrease in the cortical binding capacity of the 16,000g supernatant fluid (Table IV).

To determine if the cortical binding component was associated with a protein, the effects of dialysis, heat, and proteolytic enzymes were studied. Four dialysis bags containing 4 ml of the 16,000g supernatant fluid were dialyzed in the cold against 500 ml of the sucrose–Tris buffer. At 30-min intervals the dialysis fluid was changed and one bag was removed for assay. Dialysis up to 2 hr had no effect on the calcium binding capacity. Upon heating to 60° for 10 min the component lost 70% of its activity.

The effect of various digestive enzymes on the calcium binding capacity are summarized in Fig. 2. The 16,000g supernatant fluid was incubated at room temperature for 4 hr with

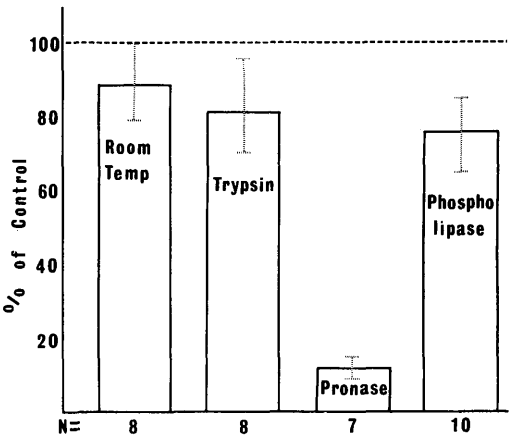

FIG. 2. The 16,000g supernatant fluid from kidney cortex was incubated at room temperature with 20% of each of the indicated enzymes over 4 hr. The results are reported as the percentage of binding activity of an aliquot kept at 0°. N = number of observations.

TABLE IV. Pooled Data—30 Dogs.

	UV_{Ca} (μg/min)		
	<20	>20	p
UV_{Ca}	10.4 ± 1.6	50.8 ± 6.6	<<0.01
UV_{Na}	35.8 ± 11.6	121.6 ± 22.0	0.01
Binding capacity			
Cortex			
Supernatant, 750*g*	465 ± 47	393 ± 41	ns
16,000*g*	781 ± 99	526 ± 83	0.05
Pellet, 16,000*g*	616 ±163	396 ± 65	ns
Supernatant, 100,000*g*	108 ± 16	92 ± 17	ns
Medulla			
Supernatant, 750*g*	179 ± 27	126 ± 9	0.05
16,000*g*	116 ± 21	82 ± 12	ns
Pellet, 16,000*g*	200 ± 33	142 ± 19	ns
Supernatant, 100,000*g*	17 ± 6	11 ± 4	ns
Distribution			
Hypotonic saline	2	6	
Hydropenia	7	3	
Saline + PTX	3	3	
Hydropenia + DOCA	2	4	
Total	14	16	

the digestive enzymes. The amount of enzymes added was 20% by weight of the protein content of the kidney extract. The reaction took place in the sucrose–Tris buffer at pH 7.4. Four hr at room temperature reduced the binding capacity by only 12%. Addition of pronase reduced the calcium binding capacity by 88%. Trypsin and phospholipase C had no significant effect on the calcium binding component.

The calcium binding component appears to be associated with particulate fractions of the cell. Thirty percent of the activity sediments at 30,000*g* for 20 min and almost no activity was found in the 100,000*g* supernatant fluid (data not shown). Attempts at recovering the activity from the 100,000*g* sediment have not been successful. A fraction of high specific activity could be obtained when the 100,000*g* pellet is resuspended; however, the recovery of the binding capacity was usually less than 25%. Attempts to solubilize the binding capacity of the 100,000*g* pellet by treatment with Triton X-100 or sonication has resulted in a further loss of calcium binding capacity.

Discussion. About 5 g of calcium are filtered by the glomeruli of a canine kidney in the course of a day. Of this, 98% is normally reabsorbed by the renal tubules. The mechanism of this reabsorption process is virtually unknown. An interest in the mechanism of calcium reabsorption goes beyond calcium transport because of the interrelationship between the handling of calcium and sodium by the renal tubule. For example, in diuretic dogs the clearance of the free calcium ion equals the clearance of sodium (7), loading with saline produces an increase in calcium excretion (8, 9), a calcium infusion enhances natriuresis (10), a mercurial diuretic drug increases calciuresis (11), the bulk of calcium, as well as sodium, is reabsorbed in the proximal tubules (12).

Wasserman and co-workers (3, 4) have contributed to the understanding of calcium transport in the chick intestinal mucosa, as well as elucidating an action of vitamin D. They identified a calcium binding protein in the chick intestine that was lacking in rachitic chicks and was restored after administration of vitamin D. In addition, they have reported the presence of a calcium binding protein in the chick kidney. The amount of

the kidney protein also was influenced by vitamin D (13).

The purpose of the present investigation was to apply the technique of Wasserman in a search for a specific calcium binding component in dog kidney; and, if it were found, to relate this component's binding capacity to the renal handling of calcium. Were calcium binding related to transport, a decrease in binding should be accompanied by an increase in UV_{Ca}.

The assay used in this study detects calcium binding to cellular components. The binding is exceptionally tight since it can not be removed by the double and prolonged exposure with Chelex as described. Chelex is capable of complexing all of the labeled calcium when buffer or albumin is assayed for calcium binding capacity. The physiological significance of the binding component is suggested because of the greater binding capacity of tissue with calcium transport function. The cortex has five times the calcium binding capacity of the medulla, a fact which agrees with the observation that the greatest amount of calcium reabsorption occurs in cortical segments. To correlate excretion of calcium with calcium binding capacity, procedures were employed to alter calcium excretion. Because of the interrelationship of sodium and calcium reabsorption, hydropenic and hypotonic saline-loaded dogs were compared. The increased UV_{Ca} of the saline-loaded dogs was accompanied by a decrease in binding capacity. Massry *et al.* (14) reported that long-term DOCA administration increased UV_{Ca}. We found that this procedure increased UV_{Ca} and was accompanied by a decrease in the binding capacity. Parathyroid hormone and thyrocalcitonin have been implicated in the control of renal calcium handling (15, 16). Thyroid-parathyroidectomy in this study did not significantly alter the UV_{Ca}, perhaps due to the removal of both glands. This procedure did produce a marked increase in the binding capacity which is unrelated to changes in the reabsorption of calcium. This increased binding capacity was in the mitochondrial fraction of the cortex and thyroid-parathyroidectomy was the only maneuver which effected that fraction. DeLuca *et al.* (17) have demonstrated that parathyroid hormone stimulates the release of calcium from mitochondria of rats fed vitamin D. Perhaps the increase in binding of calcium by the mitochondrial fraction of dog renal cortex after thyroid-parathyroidectomy is a related phenomenon.

A division of the pooled data according to the rate of calcium excretion was suggested by the results of DOCA administration. Massry *et al.* (14), in their study of the effects of DOCA, also found that all non-treated dogs had a UV_{Ca} less than 20 μg/min. Inspection of our data showed this to be an appropriate division. We found that an increase in UV_{Ca} (above 20 μg/min) was associated with a significant decrease in the binding capacity of the 16,000*g* supernatant fluid of the cortex. A slight increase is also seen in the 750*g* supernatant fluid of the medulla but we conclude that the 16,000*g* supernatant from the cortex is of major physiological significance because the cortex is the major site of calcium transport. It also contains more of the binding component than the medulla, as shown above.

The data from this study suggest that there are at least two mechanisms which affect calcium binding capacity of kidney cortex components. One is exposed by throid-parathyroidectomy which results in an increase in binding by the whole homogenate and the mitochondrial fraction and no change in UV_{Ca} and the binding capacity of the 16,000*g* supernatant fluid. The second can be related to the UV_{Ca} and appears when the UV_{Ca} is used as the criteria for the division of dogs into groups of reduced and increased excretion of calcium. In the groups with the high UV_{Ca}, the binding capacity of the 16,000*g* supernatant fluid was reduced while the opposite was true of the groups with the reduced UV_{Ca}.

Changes in calcium binding capacity could have resulted from a change in the number of binding sites or from a change in the intracellular pool of calcium and the number of sites occupied by calcium before assay. A decrease in the calcium pool size would have saturated fewer binding components and

would have appeared as an increased binding capacity. The data needed to evaluate the two possibilities were not available. However, the differences in the calcium concentration in the several procedures are probably small, since the tissues are all treated to the same extensive washings and dilutions. The difference in calcium content between cortex and medulla may be as high as 1:4, due to the osmotic gradient in the kidney. This may partially account for the lower binding capacity found in the medulla.

The binding capacity is associated with a protein component of the cell since it is nondialyzable, heat labile, and pronase sensitive. However, the calcium binding component of dog kidney cortex differs in many respects from that of the chick intestine. It is destroyed by heating to 60°, while the chick protein is not. The chick intestinal mucosal enzyme is soluble; whereas, that of dog kidney is precipated by centrifugal forces of 30,000*g* or greater. The dog kidney cortex binding component is also resistant to trypsin digestion; whereas the calcium binding of the chick is destroyed by this enzyme. The pronase sensitivity and trypsin resistance of the calcium binding component of dog kidney is similar to the acetylcholine receptor reported by Changeux *et al.* (18) and the calcium binding protein of dog intestine reported by Taylor and Wasserman (19). However, the latter is heat stable.

The fact that the calcium binding capacity can be precipitated by moderate *g* forces suggests that the activity is in particulate form. The loss of activity after treatment with Triton X-100 or sonication supports this concept. However, binding is not due to calcium uptake by mitochondria, since the fractions with the greatest binding capacity are those that are free of mitochondria, but may be due to binding by the microsomal vesicles.

In 1970, Palmer and Posey (20) reported calcium binding to rabbit renal cortical membranes that is enhanced at ATP and Mg ion. Preliminary experiments have indicated that the binding in our assay system of the various tissue fractions is not affected by the presence or absence of ATP and Mg ion. Further comparisons of the two preparative techniques and assays would be in order, although it is unlikely that binding, as measured in the present work, is dependent upon ATP or Mg ion.

Summary. We have identified a calcium binding component of dog kidney cortex which is in part protein and the capacity of which is related to the excretion of calcium by the kidney.

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