Calcium transport in canine renal basolateral membrane vesicles. Effects of parathyroid hormone.

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Research Article

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Calcium Transport in Canine Renal Basolateral Membrane Vesicles

Effects of Parathyroid Hormone

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Abstract

The effects of parathyroid hormone were studied on Ca^{2+} fluxes in canine renal proximal tubular basolateral membrane vesicles (BLMV). Efflux of Ca^{2+} from preloaded BLMV was found to be stimulated by an external Na^+ gradient, and this was inhibited by the Na^+ ionophore, monensin, and enhanced by intravesicular negative electrical potentials, which indicated electrogenic Na^+/Ca^{2+} exchange activity. There was a Na^+ gradient independent Ca^{2+} flux, but membrane binding of Ca^{2+} was excluded from contributing to the Na^+ gradient-dependent efflux. The Na^+ gradient-dependent flux of Ca^{2+} was very rapid, and even 2- and 5-s points may not fully represent absolute initial rates. It was saturable with respect to the interaction of Ca^{2+} and Na^+ with an apparent (5 s) K_m for Na^+-dependent Ca^{2+} uptake of 10 μM, and an apparent (5 s) V_{max} of 0.33 nmol/mg protein per 5 s. The Na^+ concentration that yielded half maximal Ca^{2+} efflux (2 s) was 11 mM, and the Hill coefficient was two or greater. Both Na^+ gradient dependent and independent Ca^{2+} efflux were decreased in BLMV prepared from kidneys of thyroparathyroidectomized (TPTX) dogs, and both were stimulated by parathyroid hormone (PTH) infusion to TPTX dogs. BLMV from TPTX dogs exhibited significantly reduced maximal stimulation of Na^+ gradient-dependent Ca^{2+} uptake with an apparent (5 s) V_{max} of 0.23 nmol/mg protein per 5 s, but the apparent K_m was 8 μM, which was unchanged from normal. The Na^+ gradient independent Ca^{2+} uptake was also reduced in BLMV from TPTX dogs compared with normal. Thus, PTH stimulated both Na^+/Ca^{2+} exchange activity and Na^+ independent Ca^{2+} flux. In vivo, the latter could result in an elevation of cytosolic Ca^{2+} by PTH, and this might contribute to the observed decrease in solute transport in the proximal tubule.

Parts of these data were presented to the American Society of Nephrology, Washington, DC, 1983, and were published in abstract form in *Kidney Int.*, 1984, 25:144. While this manuscript was under review for publication, Drs. A. Jayakumar, L. Cheng, C. T. Liang, and B. Sacktor have published work showing Na/Ca exchanger activity in BLMV *J. Biol. Chem.* 259:10827–10833, 1984. Their kinetics were based on longer incubation times but gave similar results to these. They reported an effect of PTH but no kinetic data for this.

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Introduction

Approximately 60% of the filtered Ca^{2+} is reabsorbed in the proximal convoluted tubule (1). The bulk of Ca^{2+} reabsorption in this segment parallels the reabsorption of Na^+, such that tubular fluid (TF) to plasma ultrafiltrate (UF) $TF/UF_{Na^+}/TF/UF_{Ca^{2+}}$ concentration is nearly a unity under nondiuretic conditions (1, 2). In situ microperfusion experiments demonstrate high permeability to Ca^{2+} in this segment, such that the unidirectional efflux of Ca^{2+} is 2–3 times larger than the net flux (3–5). These observations have been interpreted to indicate that the bulk of Ca^{2+} reabsorption in the proximal convoluted tubule is a passive process, and some investigators (6) have speculated that a paracellular Ca^{2+} flux through the tight junctions is the major path of Ca^{2+} reabsorption in the proximal convoluted tubule. However, Ullrich et al. (4) found that more Ca^{2+} was transported than could be accounted for by the electrochemical gradient when equilibrium solutions were present in the lumen and the bath of isolated perfused proximal segments. This suggested that an element of Ca^{2+} reabsorption was active in this segment. These findings have been criticized on technical grounds (6). However, recent findings of Bomsztyk and Wright (7, 8) confirm the presence of net Ca^{2+} absorption in the absence of transepithelial electrochemical driving forces which indicate active Ca^{2+} transport. In these studies, there was also clear dissociation between Na^+ and Ca^{2+} transport rates in certain experimental settings. Recent studies, as reviewed by Schaefer (9), have also demonstrated that transepithelial volume flow in the proximal tubule is established through transcellular solute transport (9). Thus, transcellular Ca^{2+} transport is an important mechanism of reabsorption in the proximal convoluted tubule.

The fact that parathyroid hormone (PTH)[1] stimulates renal Ca^{2+} reabsorption is well established (10–15). In the proximal convoluted tubule, where Ca^{2+} transport is isotonic and generally associated with fluid and Na^+ fluxes (1–4), PTH has been shown to inhibit solute reabsorption isotonically (16–19), or alternatively to have no effect on Ca^{2+} reabsorption (20, 21). In the hamster, Harris et al. (13) have reported stimulatory effects of PTH on Ca^{2+} transport in the proximal convoluted tubule, but this may be a species-specific effect. Only a preliminary report (22) supports PTH stimulation of proximal tubular Ca^{2+} transport in other rodents. In summary, the effect of PTH on Ca^{2+} reabsorption in the mammalian proximal

1. *Abbreviations used in this paper:* BLMV, basolateral membrane vesicles; BBMV, brush border membrane vesicles; DOC, deoxycholate; IOV, inside out vesicle; PTH, parathyroid hormone; ROV, right side out vesicle; TPP^+, tetraphenylphosphonium; TPTX, thyroparathyroidectomy.

nephron is unclear. In some respects, the effect of PTH on proximal tubular solute reabsorption is similar to procedures which decrease peritubular Na^+ concentrations or are presumed to increase cytosolic Ca^{2+} (23).

Recently, Khalifa et al. (24) demonstrated that PTH stimulates Ca^{2+} uptake through binding and translocation in brush border membrane vesicles (BBMV) of the proximal tubule. The relationship of this effect to apical membrane Ca^{2+} permeability or Ca^{2+} flux has not been established, but it potentially represents an entry step for Ca^{2+} through the apical membrane in transcellular transport of Ca^{2+}.

The presence of a sodium/calcium (Na^+/Ca^{2+}) exchanger in the basolateral membrane of rat kidney was established by Gmaj et al. (25). Chase and Al-Awqati (26) studied its characteristics in basolateral membranes of amphibian bladder. This exchanger has been postulated to account for active Ca^{2+} extrusion from the cell in the "transcellular model" of Ca^{2+} transport (27). The properties of the Na^+/Ca^{2+} exchanger in sarcolemma have been elegantly determined (28–30). It appears to be a 3 Na^+/Ca^{2+} exchange mechanism with a K_m for Ca^{2+} of between 1.5 and 18 μM, depending on whether conditions adjusting for charge development were used (30). Exchange activity has been shown to have a half maximal value at a Na^+ concentration of 30–32 mM, although this was sensitive to the presence of ATP, which reduced this K_m for Na^+ (31). In heart muscle, the Na^+/Ca^{2+} exchanger has a V_{max} more than 30 times higher than the Ca^{2+}-ATPase (30). However, estimates of its activity in mammalian renal epithelia may have been impaired by use of incubation times not truly reflective of initial rates of exchange activity (31a). It transports Ca^{2+} in either direction in vitro, depending on the transmembrane potential, and it is responsible for the large amounts of Ca^{2+} that are required to be effluxed after the action potentials in muscle cells.

The experiments in the studies reported here were designed to further characterize Ca^{2+} fluxes in the basolateral membrane of renal proximal tubular epithelial cells produced by the activity of a Na^+/Ca^{2+} exchanger and to analyze the effects of PTH on the exchange mechanism. The results demonstrate that PTH stimulates Na^+/Ca^{2+} exchange activity without affecting its interactions with Na^+ or Ca^{2+}, and that more rapid determinations of its activity may be required to assess its relative role in Ca^{2+} transport.

Methods

Mongrel dogs weighing 15–20 kg, fed Purina dog chow (Ralston Purina Co., St. Louis, MO), were thyroparathyroidectomized (TPTX) under 25 mg/kg pentobarbital anesthesia 48 h before nephrectomy. On the day of experiments, unilateral nephrectomy (TPTX-kidney) was performed under similar anesthesia. Immediately following the first nephrectomy, a bolus of PTH (bovine PTH 1-84, 2 μg/Kg) was given. The remaining kidney was removed 30 min later (TPTX-PTH kidney). The TPTX and PTH infusion was omitted for normal dogs (*n*-kidney). All kidneys were removed and immediately perfused with ice-cold 0.9% saline. Kidney clearance studies during this protocol have been previously reported (32). In that report, PTH administration produced a decline in Pi reabsorption within 15 min. Calcium excretion ($UV_{Ca^{2+}}$) data were not reported, but $UV_{Ca^{2+}}$ was reduced from 0.45 mg/min in TPTX to 0.18 mg/min in TPTX-PTH. There were no changes in plasma-free Ca^{2+} levels during the experimental period after PTH administration.

BLMV. Membrane suspensions enriched in basolateral membrane vesicles were prepared by an approach modified from Sacktor et al. (33). Renal medullary tissue was removed from the kidneys, and renal cortical tissue was diced and homogenized using a Potter-Elvehjem Teflon homogenizer in homogenizing buffer (250 mM sucrose, 20 mM Tris base, and 1 mM EGTA adjusted to pH 7.6), 20 ml/g of tissue. The suspension was then further homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), setting six for three bursts of 30 s. After samples were taken for analysis of protein and enzyme content, the homogenate was centrifuged at 2,500 *g* for 15 min and the pellet discarded. The suspension was then spun at 24,000 *g* for 20 min and the supernatant decanted. The soft portion of the pellet was resuspended in the homogenizing buffer to a value of 1.3 ml/g initial tissue and hand homogenized for seven strokes with a Dounce homogenizer. The suspension was then diluted and thoroughly mixed with 1.6 ml/g initial tissue of 100% Percoll (Pharmacia Fine Chemicals, Upsala, Sweden) and the homogenizing buffer, 17 ml/g initial tissue (final Percoll concentration 8%). The suspension was then centrifuged at 30,000 *g* for 35 min. This produced a density gradient of Percoll from 1.005 to 1.125 g/ml. The fraction corresponding to an opaque band and density 1.030–1.035 g/ml at volume 50–67% of the total was removed. For each set of experiments, the Percoll band containing BLMV was diluted in the solution for vesicle loading, generally 100 mM KCl, 20 mM Tris-Hepes, pH 7.5, unless otherwise indicated, at 14 ml/g initial tissue. BLMV were washed times two with centrifugation at 30,000 *g* for 30 min and resuspended in loading solution. The soft pellet was resuspended in the appropriate loading solution and aliquoted using a Hamilton syringe fitted with a 27-gauge needle (Hamilton Co., Reno, NV). Samples of suspension were taken for analysis of protein and enzyme content.

In some experiments, the BLMV preparation was further purified by incubation in Sepharose-4B bound ATP (Pharmacia Fine Chemicals, Piscataway, NJ). After 30 min agitation at room temperature, the Sepharose-4B ATP and bound membranes were removed by centrifugation at 5,000 *g*, washed and recentrifuged at 5,000 *g*. The BLMV pellet was reformed by centrifuging the supernatant for 30 min at 30,000 *g*.

Enzyme studies. Characterization of the BLMV by means of specific enzyme markers was performed as described previously (33, 34) using activities of Na^+/K^+-ATPase, a basolateral membrane marker (35); acid phosphatase, a lysosomal marker (36); cytochrome oxidase, a mitochondrial marker (37); maltase, a brush border marker (38); and NADPH-cytochrome-C reductase, an endoplasmic reticulum marker (39). Protein content was measured (40) using a bovine serum albumin standard.

BLMV "sidedness." The orientation of the membrane vesicles was assessed by latency of Na^+/K^+-ATPase activity and its ouabain sensitivity before and after two cycles of freezing and thawing during incubation with 0.4 mg/ml deoxycholate (DOC) (34, 41). Orientation was assessed by these maneuvers since the ouabain binding site of Na^+/K^+-ATPase is on the external membrane face and the ATP binding site is on the internal face of plasma membranes containing Na^+/K^+-ATPase activity. Thus, in a mixture of BLMV, inside out vesicles (IOV) showed Na^+/K^+-ATPase activity not sensitive to ouabain before, but sensitive after DOC, and right side out vesicles (ROV) showed Na^+/K^+-ATPase activity only after DOC. Ouabain-sensitive Na^+/K^+-ATPase activity present before and after DOC was not an assignable orientation and may have represented poorly sealed vesicles or membrane sheets. These studies assumed that sealed BLMV were not permeable to ATP or ouabain and were completely disrupted by the DOC/freeze-thaw treatment.

Electron microscopy. Samples of BLMV for transmission electron microscopy were fixed in 2.5% glutaraldehyde and post fixed in 1% osmium tetroxide. They were then dehydrated in a graded series of ethanols and embedded in D.E.R. 332-732 Embedding Media Formula B (Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA).

Ca^{2+} efflux experiments. Vesicles prepared as described above with

100 mM KCl, 20 mM Tris-Hepes in the intravesicular space were divided into 10-μl aliquots and incubated with 40 μl of a loading solution (100 mM, 20 mM Tris-Hepes, 25 μM $^{45}CaCl_2$, pH adjusted to 7.5) for 30 min at 30°C. Calcium efflux was initiated at 30°C with a solution of either 100 mM KCl (K^+ efflux) or NaCl (Na^+ efflux) with 20 mM Tris-Hepes, and 1 mM EGTA, pH 7.5. To examine the effect of the Na^+ gradient, a NaCl efflux solution containing 5 μM monensin, a Na^+ ionophore, was used in addition to the normal Na^+ efflux solution. The reactions were stopped by dilution with either ice-cold 100 mM KCl or NaCl with 20 mM Tris-Hepes, pH 7.5, depending on the solution used to initiate efflux. The solutions were rapidly filtered through 0.65-μM filters (Millipore; Millipore Corp., Bedford, MA) after addition of stop solutions. Radioactivity trapped on the filters was measured with a scintillation spectrometer (Hewlett-Packard Co., model 460-CD, Downers Grove, IL). All experimental points were done at least in triplicate. Variations from these methods are explained with the relevant figure or table. For all experiments in which ethanolic solutions were used, an identical concentration of ethanol was used in the control solutions.

Lanthanum efflux experiments. In these experiments, a 100-fold dilution of BLMV preincubated in 150 mM KCl, 5 mM Hepes, $^{45}CaCl_2$ 100 μM, pH 7.3, by (*a*) 150 mM NaCl, 5 mM Hepes, pH 7.3; (*b*) 150 mM KCl, 5 mM Hepes, pH 7.3, or (*c*) 150 mM KCl, 5 mM Hepes, 0.5 mM $LaCl_3$, pH 7.3, was used to initiate efflux. The ice-cold stop solution contained 150 mM KCl, 5 mM Hepes, and 0.5 mM $LaCl_3$, pH 7.3. Thus, a fourfold dilution into an EGTA-containing solution was not used to produce an outwardly directed Ca^{2+} gradient, but only a 100-fold dilution of the preincubation medium. The change in pH and buffering of the experimental solutions were made to ensure $LaCl_3$ solubility.

Effect of varying [Na^+]. For these experiments, vesicles (10 μl) were prepared and loaded with Ca^{2+} as described above, except that they contained 150 mM KCl instead of 100 mM KCl. Ca^{2+} efflux was initiated by dilution with 200 μl of efflux solutions with varying concentrations of NaCl and KCl to equal 150 mM, 20 mM Tris-Hepes, and 1 mM EGTA. Efflux was determined at 2 or 5 s as described above for efflux experiments.

Effect of varying [Ca^{2+}]. For these experiments, 150 mM NaCl was substituted for 150 mM KCl in the BLMV loading solution for half of the preparation obtained from the Percoll gradient step. Thus, NaCl and KCl loaded vesicles were obtained, and because of the inability to set intravesicular [Ca^{2+}], the Ca^{2+} kinetics were done for uptake rather than efflux. For determination of Ca^{2+} uptake, 10 μl of the BLMV were suspended in 100 μl of 150 mM KCl, 20 mM Tris-Hepes, with various concentrations of free Ca^{2+}. In view of the recent controversy over the use of the EGTA binding constants and the many problems with calculated Ca^{2+}/EGTA solutions (42, 43), the Ca^{2+} electrode (Orion Research, Inc., Cambridge, MA) was calibrated with Ca^{2+} solutions containing 150 mM K^+Cl^-, 20 mM Tris, and 20 mM Hepes, pH 7.5. The calcium concentrations were determined by atomic absorption spectrometry and the electrode was calibrated to 1 μM. The latter was the concentration of Ca^{2+} in nominally Ca^{2+} free solutions. Varying concentrations of free Ca^{2+}, as measured by the electrode, were then produced from a 90 μM $CaCl_2$ solution by the addition of EGTA to produce the experimental solutions. After 5 s, Ca^{2+} uptake was stopped by ice-cold dilution in 150 mM KCl, 20 mM Tris, 20 mM Hepes, 1 mM EGTA, pH 7.5, and rapid filtration. Thus, nonspecific binding of Ca^{2+} was eliminated by use of an EGTA-containing stop solution.

Control experiments demonstrated that Na^+- or K^+-containing solutions were identical in terms of background adherence of $^{45}Ca^{2+}$ to filters, and this was subtracted from uptakes of $^{45}Ca^{2+}$ by BLMV. Background binding of $^{45}Ca^{2+}$ represented <1% of the uptake in all experiments.

Reagents. $^{45}CaCl_2$ (14–17 Ci/g Ca^{2+}) was obtained from New England Nuclear, Boston, MA. Calcium ionophore A23187 was obtained from Calbiochem-Behring Corp., La Jolla, CA. Bovine 1-84 PTH was kindly provided by Dr. Jeremiah Morrissey, Washington University, St. Louis, MO. All other chemicals were of the highest purity available from commercial sources. All solutions were filtered through 0.45- μM Millipore filters on the day of experiment before use.

Analysis of results was by paired *t* test for the TPTX vs. TPTX-PTH experiments and unpaired *t* for comparison of normal vs. manipulated groups.

Results

BLMV characterization. Electron microscopy of the BLMV preparation revealed irregularly shaped sealed vesicular structures of ~1–1.5 μm diameter (Fig. 1). The activity of various enzyme markers in the basic BLMV preparation is shown in Table I. These results are similar to previous reports of BLMV preparation prepared with this technique (33, 34). There was no significant difference between the marker enzyme activities for BLMV from normal, TPTX, and TPTX-PTH kidneys. The major contaminating membranes were lysosomal and brush border. There were significant decreases in the marker enzyme activity for endoplasmic reticulum and mitochondrial markers which are known to possess Na^+/Ca^{2+} exchange activity. The results of the estimation of BLMV orientation were ROV, 74.4±1.0%, and IOV, 25.6±1.0% (mean±SE, n = 3), of the assignable membranes. Unassignable were 57±0.4% of the total. These results were similar to previous reports (33, 34). There were no effects of TPTX and TPTX + PTH on the orientation of BLMV. Incubation of BLMV with Sepharose-4B bound ATP produced 99% ROV, 1% unassignable, and 0% IOV. The low protein yield after the added purification step of the Sepharose-4B ATP precluded its use in experiments with multiple time points. Its use was to confirm results obtained with the basic BLMV preparation as indicated below.

Other investigators from this division (44), using this BLMV preparation, have shown that the BLMV are of solely proximal tubular origin. Adenylate cyclase activity was stimulated 3.3 times over baseline by PTH, but vasopressin, isoproterenol, and thyrocalcitonin did not stimulate adenylate cyclase activity over baseline. These results were similar to those reported by Scalera et al. (45).

Time course of $^{45}Ca^{2+}$ efflux from BLMV. Fig. 2 shows the time course of Ca^{2+} efflux from Ca^{2+} loaded BLMV in conditions of K^+ equivalency and inwardly directed Na^+ gradients. There were two phases to Ca^{2+} efflux, beginning with an initial rapid phase in the first 2 min after addition of the solutions used to initiate efflux. This first phase was exaggerted by Na^+ gradients. The second phase was slow to reach equilibrium, but equilibrium was reached between 180 and 200 min. In these experiments, loss of calcium from BLMV could represent either a transmembrane flux of intravesicular Ca^{2+} or displacement of externally bound Ca^{2+}. Since Ca^{2+} loss was initiated by solutions containing EGTA, displacement of externally bound calcium would be expected unless the membrane binding sites had a greater affinity for Ca^{2+} than EGTA. When the Ca^{2+} ionophore was added, loss of Ca^{2+} from the BLMV was essentially complete. Thus, in the presence of EGTA and ionophore, calcium binding to BLMV was essentially eliminated. This is in contrast to calcium binding to BBMV, wherein ionophore additions resulted in no greater loss of calcium than the 50–60% at equilibrium without ionophore (inset Fig. 2).

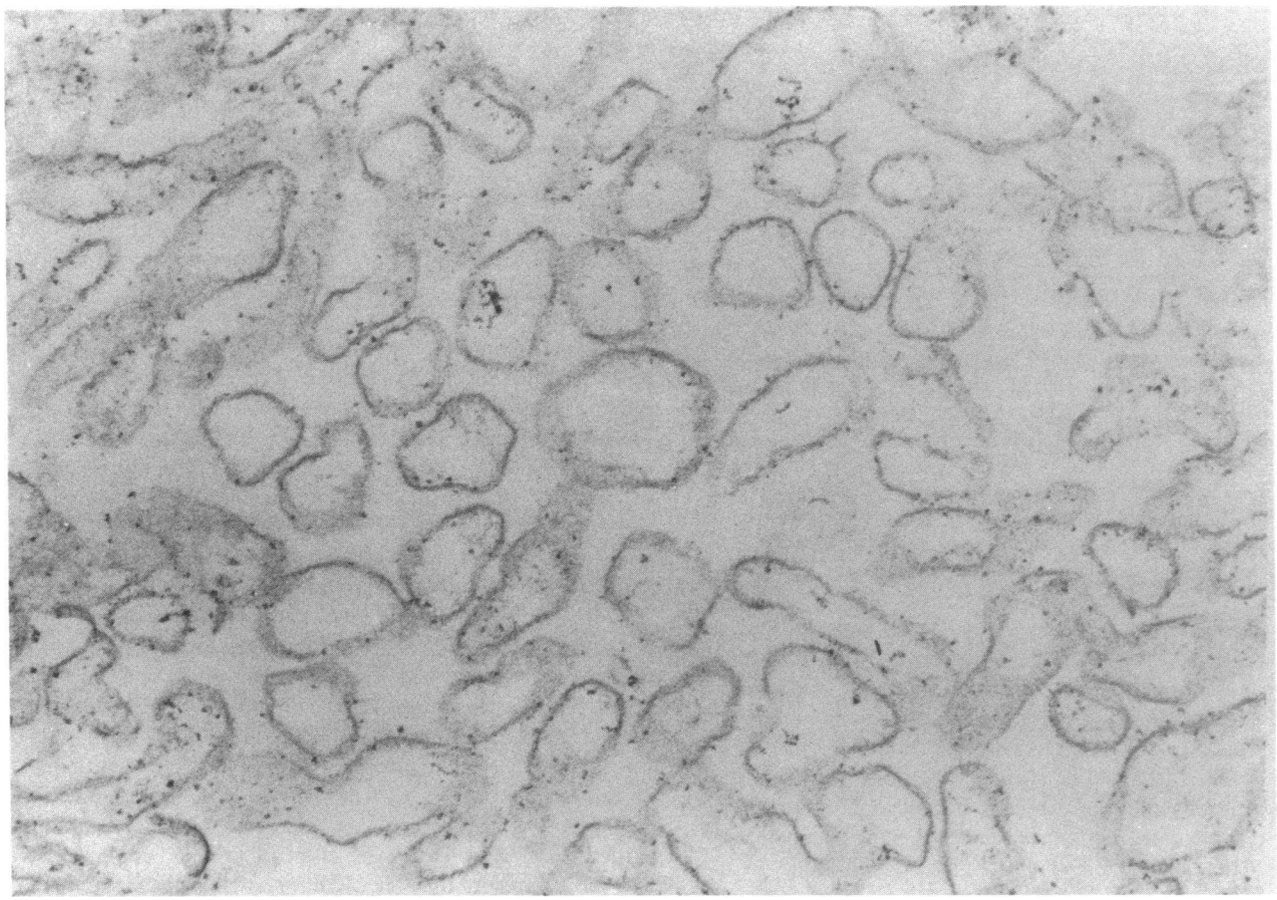

Figure 1. Electron microscopy of the BLMV preparation revealing irregularly shaped sealed vesicular structures of ~1–1.5 μm diameter.

Displacement of calcium binding to BLMV could not account for the stimulation of Ca^{2+} efflux by inwardly directed sodium gradients since displacement of Ca^{2+} binding by the EGTA-containing efflux solution was complete in the K^+ equivalent conditions. Thus, the sodium gradient dependent flux (determined by subtracting Ca^{2+} loss in the presence of

Table I. Specific Activities and Enrichment of Marker Enzymes of Renal Cortical Homogenates and BLMV

Preparation	Na^+/K^+-ATPase	Maltase	NADPH-cytochrome-C reductase	Cytochrome C oxidase	Acid phosphatase
Normal kidneys					
Specific activity					
Homogenate	0.064±0.012	0.064±0.022	0.024±0.005	0.081±0.015	0.046±0.002
Vesicles	0.540±0.065	0.083±0.026	0.020±0.003	0.020±0.002	0.078±0.003
Enrichment	8.44	1.28	0.84	0.22	1.71
TPTX kidneys					
Specific activity					
Homogenate	0.080±0.017	0.086±0.033	0.022±0.003	0.098±0.007	0.040±0.004
Vesicles	0.675±0.178	0.139±0.047	0.017±0.002	0.022±0.007	0.077±0.006
Enrichment	8.42	1.61	0.74	0.24	1.92
TPTX-PTH kidneys					
Specific activity					
Homogenate	0.068±0.012	0.093±0.023	0.021±0.001	0.092±0.007	0.037±0.005
Vesicles	0.580±0.209	0.0942±0.011	0.015±0.003	0.022±0.004	0.061±0.004
Enrichment	8.58	1.02	0.69	0.24	1.65

The specific activities are in $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ and represent the mean±SE for three or more separate determinations. There were no differences between the specific activities or enrichments between the three groups.

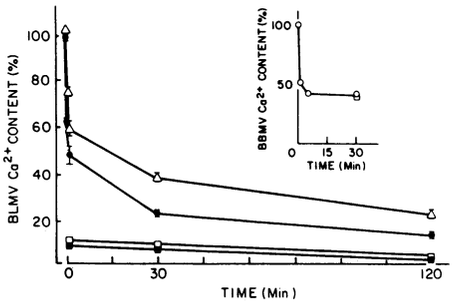

Figure 2. Time course of Ca^{2+} efflux from preloaded BLMV. BLMV were preincubated with 25 μM $^{45}CaCl_2$ as described in Methods. Efflux was initiated with: △, 100 mM KCl, 20 mM Tris/Hepes, and 1 mM EGTA; or, ●, 100 mM NaCl, 20 mM Tris/Hepes, and 1 mM EGTA. For Ca^{2+} ionophore additions (■, NaCl efflux solution, and □, KCl efflux solution), 2.5 μl of 1 mM Ca^{2+} ionophore A23187, final concentration 10 μM, was added 5 min before addition of stop solutions. The inset is modified from Khalifa et al. (22). ○, Ca^{2+} efflux from BBMV preincubated with 25 μM $^{45}CaCl_2$ and efflux initiated with 5 mM EGTA. □, addition of 10 μM A23187 to efflux solution. 100% Ca^{2+} uptake represents 1.01±0.1 nmol/mg^{-1} protein.

sodium gradients from the loss in the presence of potassium equivalent conditions) must represent a transmembrane movement of intravesicular Ca^{2+}. This latter point is further established by the effect of La^{3+}, which when added to solutions used to initiate efflux dramatically inhibited Ca^{2+} efflux (Fig. 3), especially at the 5-s time point which was used for the kinetic data. Since La^{3+} would displace Ca^{2+} bound to the external surface of the BLMV, one would expect an enhancement of calcium loss if displacement of externally bound calcium was the major source of this loss (46). The minor degree of Ca^{2+} loss from BLMV in the presence of La^{3+}, which was equal in KCl or NaCl conditions, further indicates that the Ca^{2+} loss in the presence of potassium equivalent and sodium gradient conditions represents largely a transmembrane flux. No difference in rate of efflux was found when vesicles were loaded with 80 mM KCl, 20 mM NaCl, and 20 mM Tris-Hepes, incubated with 25 μM $^{45}Ca^{2+}$ and efflux initiated with either 80 mM KCl, 20 mM NaCl, 20 mM Tris-Hepes, or 100 mM NaCl. Thus, the presence of Na^+ in nongradient conditions did not alter the Na^+-stimulated efflux that demonstrated the absence of a Na^+-dependent effect on Ca^{2+} binding. This is in agreement with previous data from BBMV (24).

To assess the degree of calcium binding in BLMV, apparent calcium spaces were determined. Calcium uptake into BLMV during the 30 min of preequilibration before initiation of calcium efflux was related to vesicular volumes and the expected calcium uptake at equilibrium. The intravesicular volumes of the BLMV were determined by glucose uptake at equilibrium (24), and they were 2.07±0.02 μl/mg protein. Calcium uptake during the calcium loading preincubation was 15–17 times greater than the expected Ca^{2+} uptake at equilibrium. This degree of calcium binding is similar to the calcium binding in BBMV under similar conditions. The presence of calcium binding similar to that seen in other renal membranes indicated the utility of the experimental design employing Ca^{2+} efflux into EGTA-containing solutions that were sufficient to remove externally bound calcium. The presence of EGTA in the efflux solutions and the effects of lanthenum establish that the Na^+ gradient-dependent increase in calcium efflux could not have been contributed to by calcium binding.

Effect of Na^+ gradients on Ca^{2+} efflux. The rapid effect of inwardly directed added Na^+ gradients on Ca^{2+} efflux is shown in Fig. 4. The zero time points were determined by immediate addition of stop solutions after addition of solutions used to initiate efflux. This procedure, with the KCl efflux solution, was taken as zero. Timing the procedure with a metronome revealed this to take 0.5–1.0 s. With the NaCl efflux solution, a stimulation of Ca^{2+} efflux was already detectable at this earliest point, and by 5 s the effect of the Na^+ gradient was 80–90% of maximum. The addition of the Na^+ ionophore, monensin, resulted in a significant 45% decrease in stimulation of Ca^{2+} efflux by the externally added Na^+ gradient, $P < 0.05$. The stimulation of Ca^{2+} efflux by Na^+ gradients was specific for Na^+. External choline or K^+ gradients, the latter with choline-loaded BLMV were unable to stimulate Ca^{2+} efflux (data not shown). Efflux in choline equilibrated conditions was not different from K^+ equilibrated conditions. Analysis of Ca^{2+} efflux in BLMV incubated with ATP bound to Sepharose-4B revealed patterns of efflux identical to Fig. 4 when efflux was expressed as percent of initial Ca^{2+} uptake remaining.

Effect of transmembrane potential. In BLMV prepared

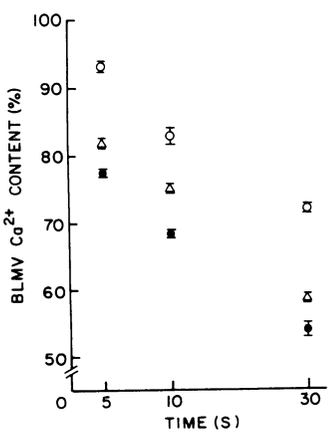

Figure 3. The effect of La^{3+} and Ca^{2+} efflux from BLMV. BLMV were prepared and preincubated as described in Methods for La^{3+} efflux experiments and efflux initiated by 100-fold dilution into: ○, 150 mM KCl, 5 mM Hepes, 0.5 mM $LaCl_3$, pH 7.3; △, 150 mM KCl, 5 mm Hepes, pH 7.3; or ●, 150 mM NaCl, 5 mM Hepes, pH 7.3. The stop solution was 150 mM KCl, 5 mM Hepes, 0.5 mM $LaCl_3$, pH 7.3.

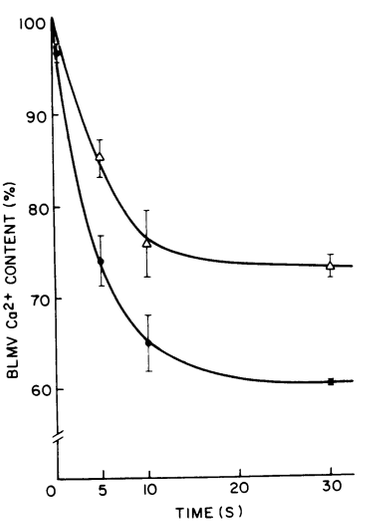

Figure 4. Rapid effect of externally added Na^+ gradients on Ca^{2+} efflux from BLMV. BLMV were preincubated with $^{45}CaCl_2$ and efflux was initiated as described in Methods. △, KCl efflux solution; ●, NaCl efflux solution. Efflux in Na^+ gradient conditions was different from K^+ equivalent conditions $P < 0.005$ at all points. 100% Ca^{2+} uptake represents 0.92±0.09 nmol/mg^{-1} protein.

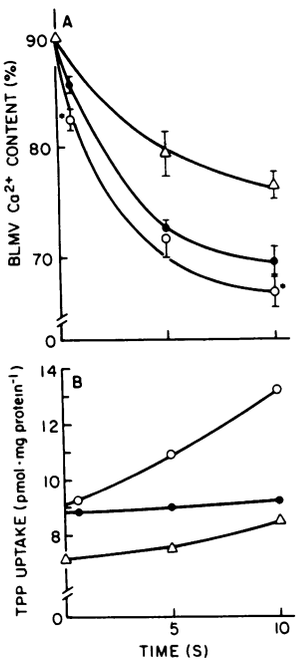

Figure 5. Effect of a transmembrane electrical potential (inside negative) on Ca^{2+} efflux from BLMV. (*A*) BLMV were preincubated as described in Methods with $^{45}CaCl_2$. △, KCl efflux solution; ●, NaCl efflux solution; ○, NaCl efflux solution with 10 μM Valinomycin added during the preincubation. (*B*) Uptake of ^{3}H-labeled TPP^+ by BLMV with: △, KCl efflux solution; ●, NaCl efflux solution; and ○, NaCl efflux solution and BLMV preincubated with 10 μM Valinomycin. *, $P < 0.05$ compared with NaCl gradient conditions. 100% Ca^{2+} uptake represents 1.65±nmol/mg^{-1} protein.

with KCl in the intravesicular space, an intravesicular negative transmembrane electrical potential is created by the addition of the K^+ ionophore, valinomycin, and an external Na^+ gradient. Uptake of the lipid soluble cation, tetraphenylphosphonium (TPP^+), whose distribution reflects the magnitude and direction of the transmembrane electrical potential (28) was used to monitor the membrane potential (Fig. 5 *B*). The high degree of TPP^+ binding in the K^+ equivalent conditions rendered TPP^+ uptake a qualitative means of monitoring the potential. A negative potential was produced immediately, and its magnitude increased for 10 s. This internal negative potential stimulated the initial rates Na^+/Ca^{2+} exchange (Fig. 5 *A*). This suggests that the exchange was electrogenic, and possibly resulted from a stochiometry of 3 Na^+/1 Ca^{2+} or greater for the exchange activity (26, 28–31). It is of interest that the control BLMV in this group had been preincubated with C_2H_5OH as the blank and showed an increase in total calcium after 30 min of incubation.

Effect of PTH on Na^+/Ca^{2+} exchange. Ca^{2+} efflux from BLMV in both the K^+ equivalent (Na^+ independent) and Na^+ gradient conditions was decreased by TPTX 48 h before nephrectomy (Table II). Na^+ dependent Ca^{2+} efflux was significantly reduced at the 5-, 10-, and 30-s time points. Administration of parathyroid hormone 30 min before nephrectomy and preparation of BLMV increased efflux in both K^+ equivalent and Na^+ gradient conditions, and significantly stimulated the initial rates of Na^+-dependent Ca^{2+} efflux. The effects of TPTX and TPTX + PTH on Ca^{2+} flux in K^+ equivalent conditions were also present during the 30-min preincubation period (Ca^{2+} loading of BLMV). Ca^{2+} uptake during the preincubation was significantly decreased by TPTX and increased by TPTX + PTH. Calcium efflux, expressed as nanomole of Ca^{2+} lost/mg protein, was twice that in BLMV incubated with Sepharose-4B ATP as in the basic BLMV preparations. However, Ca^{2+} uptake per milligram protein in the Sepharose-4B ATP treated BLMV during the preincubation period was twice that of each group in Table II, and Ca^{2+} efflux as a percent of initial Ca^{2+} uptake remaining was similar in the two vesicular preparations. This may indicate lack of participation of the membranes whose orientation were unas-

Table II. Effect of PTH on Ca^{2+} Efflux from BLMV

	Ca^{2+} Uptake (30 min preincubation)	Ca^{2+} efflux (s)				
		0.5	5	10	30	1,800
	nmol·mg^{-1}	*% Ca^{2+} remaining in BLMV*				
Efflux in K^+ equivalent conditions						
N (7)	0.91±0.9	100	85.5±2.2	76±3.6	73.4±1.2	26.7±0.3
TPTX (4)	0.74±0.5*	100	92.2±2.5*	85.4±1.4*	72.4±4.2	24.5±1.2
TPTX + PTH (4)	0.96±0.13‡	100	88.9±3.8‡	81.6±1.9‡	71.4±3.2	22.2±0.8
Efflux in Na^+ gradient conditions						
N		96.5±1.1	74.1±2.7	65±3.1	60.5±0.2	15.9±0.5
TPTX		97±2.2	86.7±1.4*	80.8±1.6*	66.4±2.1*	16.8±0.4
TPTX + PTH		92.9±2.1‡	83.9±1.6	76.3±2.3‡	63.2±3.0‡	15.4±0.2
Na^+ minus K^+ (Na^+ dependent efflux)						
N		3.5	11.4	11	12.9	10.8
TPTX		3	5.5*	4.7*	6.0*	7.7*
TPTX + PTH		7.1‡	6.0	5.3	8.2‡	6.8

BLMV were incubated with 25 μM $^{45}CaCl_2$ for 30 min and efflux initiated after the preincubation as described in Methods. N, BLMV from normal dogs; TPTX plus PTH, PTH infusion ½ h before nephrectomy. * $P < 0.05$ compared with normal; ‡ $P < 0.05$ compared with TPTX. Number of experiments in parentheses.

signable during the orientation studies in Ca^{2+} uptake and thus Ca^{2+} efflux.

Effect of PTH on Na^+/Ca^{2+} exchange in the presence of ATP. Since the Na^+/Ca^{2+} exchanger normally functions in the presence of ATP, and since ATP has been reported to directly affect Na^+/Ca^{2+} exchange activity (31, 31a), 10 mM ATP was added to the solutions used for Ca^{2+} loading during the preincubation period. As shown in Fig. 6, Ca^{2+} efflux from the BLMV from kidneys of TPTX dogs was markedly reduced, with a profound effect on Na^+ dependent Ca^{2+} efflux. There was no Mg^{2+} present in the system, which made uptake of Ca^{2+} by IOV due to Mg^{2+}-dependent Ca^{2+} ATPase less likely (25). The effect of ATP on Na^+/Ca^{2+} exchange activity may represent prolongation of the Na^+ gradient by Na^+/K^+ ATPase. However, Reeves et al. (31) have suggested that ATP exerts stimulatory effects on the Na^+/Ca^{2+} exchanger. The data shown in Fig. 6 support this concept, since the maximal effect of inward Na^+ gradients were apparent in the BLMV from kidneys of PTH-treated dogs at the earliest time of observation. The mechanism of ATP's effects on Na^+/Ca^{2+} exchange require further investigation, since the operational kinetics of the system may be the mechanism of the ATP effect. This may affect the interpretation of these kinetics in modeling cellular Ca^{2+} transport (31a).

Effects of Na^+ gradients on Ca^{2+} uptake. In BLMV prepared with KCl in the intravesicular space, the presence of inwardly directly Na^+ gradients decreased Ca^{2+} uptake (Fig. 6). Calcium taken up by the BLMV on the basis of the inwardly directed Ca^{2+} chemical potential was, in part, effluxed uphill in the presence of a Na^+ gradient (26).

Relationship of the Na^+/Ca^{2+} exchange activity to external Na^+ concentration. To further examine the action of trans Na^+ gradients on Ca^{2+} efflux, increasing concentrations of Na^+ were applied to BLMV. For these experiments, the BLMV were preloaded with KCl and incubated with isosmotic solutions of increasing NaCl concentration, the Na^+ replacing external K^+. Since the data in Fig. 4 suggested that 5 s may not represent initial rates of transport, both 2 s and 5 s of efflux were used in these studies. At both time points, the relationship between Ca^{2+} efflux and external Na^+ concentration was sigmoidal, with apparent $[Na^+]_{0.5}$ of 11 mM in BLMV from normal kidneys at 2 s, 15 mM at 5 s, and 19 mM for BLMV from TPTX'd kidneys at 5 s (Fig. 7). The Hill coefficient was

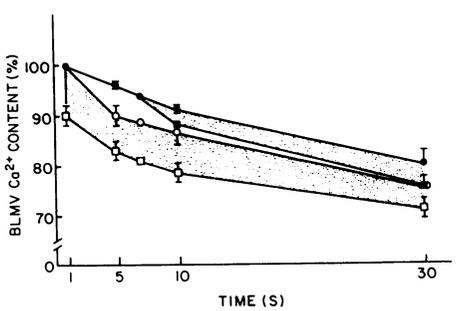

Figure 6. Effect of ATP and PTH on Na^+ gradient dependent Ca^{2+} efflux. Efflux was performed as described in Methods but 10 mM ATP was added during the preincubation with Ca^{2+} loading solutions as described in text: TPTX BLMV ●, KCl efflux solution, and ■, NaCl efflux solution; TPTX-PTH BLMV ○, KCl efflux solution, and □, NaCl efflux solution.

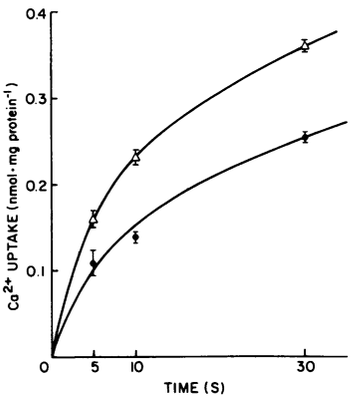

Figure 7. Effects of external Na^+ gradients on BLMV Ca^{2+} uptake. BLMV, prepared as described in the Methods with 100 mM KCl intravesicularly were mixed with 40 μl of either: △, 100 mM KCl, 20 mM Tris-Hepes, 25 μM $^{45}CaCl_2$, pH 7.5; or ●, 100 mM NaCl, 20 mM Tris-Hepes, 25 μM $^{45}CaCl_2$, pH 7.5.

two or greater BLMV from kidneys of both normal and TPTX dogs. For comparative purposes, between BLMV from kidneys of normal and TPTX dogs, 5 s of incubation produced greater intra experimental reproducibility. Time points for estimation of the kinetics of Na^+/Ca^{2+} exchange activity have ranged up to 60 s in some reports (31a). However, the data of Chase and Al-Awqati (26) and our data suggest that the Na^+/Ca^{2+} exchange activity is too rapid for 5 s to represent initial rates. The data in Fig. 4 suggest that 5 s was not an initial rate, since 80% plus of the maximum Na^+-dependent efflux was present at this time. Also, the Ca^{2+} efflux at 2 s was >2/5 of the efflux at 5 s. Thus, accurate determination of the kinetics of Na^+/Ca^{2+} exchange activity will require ≤1-s time points. The data presented as apparent rate constants here are presented for comparative purposes between normal and PTX conditions, since we were unable to accurately assess differences between the groups at shorter time points.

Effects of $[Ca^{2+}]$ on Na^+/Ca^{2+} exchange activity. The relationship between Na^+ gradient stimulated Ca^{2+} uptake and Ca^{2+} concentrations was studied in BLMV prepared either in KCl or NaCl in the intravesicular space as described in Methods. Calcium uptake was measured after the addition of solutions containing $^{45}CaCl_2$ adjusted with EGTA to produce varying free Ca^{2+} concentrations. As shown in Fig. 8, Ca^{2+} uptake at 5 s was a complex integration of several processes. At least three separate processes were discernable from the data. First, in BLMV from kidneys of both normal and TPTX dogs, there was a small nonsaturable increase in Ca^{2+} uptake at higher Ca^{2+} concentrations for both the Na^+ gradient and K^+ equivalent conditions. This is compatible with a simple Ca^{2+} diffusive process in the BLMV. Secondly, there was a saturable component to the Ca^{2+} uptake in the K^+ equivalent conditions. The saturable Ca^{2+} flux in K^+ equivalent conditions was larger than the Na^+-dependent uptake. This contrasts to sarcolemmal vesicles, where there is very little Ca^{2+} flux in the absence of a Na^+ gradient (29). This Ca^{2+} uptake is comprised of Ca^{2+} binding to the BLMV and a form of specialized Ca^{2+} permeability such as a Ca^{2+} channel. Further studies will be required to characterize this flux. Finally, there was a Ca^{2+} uptake stimulated by an outwardly directly Na^+ gradient similar to the stimulation of Ca^{2+} efflux by inwardly directed Na^+ gradients as described above. In the presence of an inside to out gradient of 150 mM Na^+, increasing Ca^{2+} concentrations were associated with a Na^+-dependent stimulation of uptake which was saturated with an apparent K_m for Ca^{2+} of 10 μM in BLMV prepared from kidneys of normal dogs and 8 μM

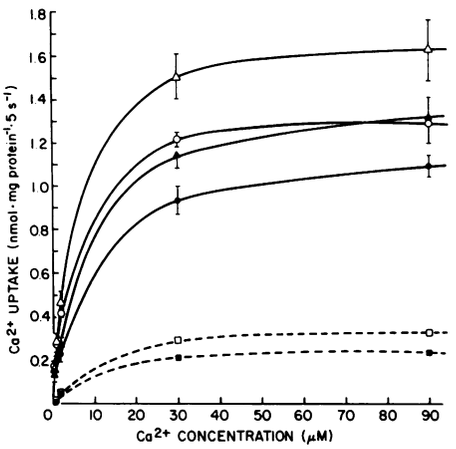

Figure 8. The relationship between Ca^{2+} uptake and external Ca^{2+} concentration in BLMV. BLMV prepared from kidneys of normal or TPTX'd dogs were loaded with either 150 mM KCl, 20 mM Tris-Hepes, pH 7.5 (○, ●), or 150 mM NaCl, 20 mM Tris-Hepes, pH 7.5 (△, ▲). Ca^{2+} uptake was determined as described in Methods. Except at the lowest concentration, Ca^{2+} uptake in presence of Na^+ gradient in BLMV from normal dogs (△) was significantly greater than Ca^{2+} uptake in K^+ equilibrated conditions (○) and in BLMV from TPTX dogs in Na^+ gradient conditions, $P < 0.05$, $n = 4$. Ca^{2+} uptake in BLMV from TPTX dogs was also greater in Na^+ gradient conditions (▲) than K^+ equivalent conditions (●), $P < 0.05$, $n = 4$, except for the lowest concentration. Symbols connected by (– – –) are the differences between Ca^{2+} uptake in the presence of outwardly directed Na^+ gradients and potassium equivalent conditions. (□ – – – □), BLMV from kidneys of normal dogs. (■ – – – ■), BLMV from kidneys of TPTX'd dogs.

in BLMV prepared from kidneys of TPTX'd dogs. The apparent V_{max} of the Na^+-dependent Ca^{2+} uptake was significantly reduced by TPTX from 0.33 nmol/mg protein per 5 s in BLMV from kidneys of normal dogs to 0.23 nmol/mg protein per 5 s in BLMV from kidneys of TPTX'd dogs (Fig. 9).

Discussion

The experiments reported above rely, to a large extent, on Ca^{2+} efflux from preloaded BLMV to study Ca^{2+} fluxes that

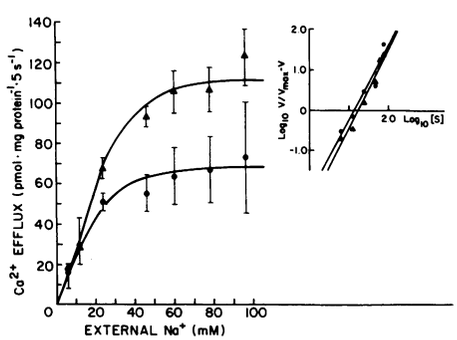

Figure 9. The relationship of external Na^+ concentration to Ca^{2+} flux. BLMV from kidneys of ▲, normal dogs or ●, TPTX'd dogs were prepared as described in Methods except for 150 mmol KCl in the loading solution. BLMV were preincubated with $^{45}CaCl_2$, as in Methods, then Ca^{2+} efflux was initiated by dilution with solutions of varying Na^+ concentrations with osmolality maintained with KCl. Inset is a Hill plot of the data.

avoid the problems associated with membrane Ca^{2+} binding encountered in experiments studying Ca^{2+} uptake (24). The results demonstrate that a Na^+ gradient trans to Ca^{2+} stimulated Ca^{2+} efflux. Substitution for Na^+ removed the stimulation, and dissipation of the gradient by a Na^+ ionophore decreased the stimulation. These results are consistent with the presence of a Na^+/Ca^{2+} exchanger in the basolateral membrane of proximal renal tubular cells. In the presence of an intravesicular negative electrical potential, Na^+/Ca^{2+} exchange activity was stimulated, which suggested that the stoichiometry of the exchange activity was >2:1. This was also supported by the relationship between Na^+ gradient stimulated Ca^{2+} efflux and Na^+ concentration from which the calculated Hill coefficient was two or greater.

The basic BLMV preparation contained both ROV and IOV and unassignable membranes. The heterogeneity in membrane orientation would have been significant if the Na^+/Ca^{2+} exchange activity was asymmetrical and would only respond to a Na^+ gradient applied to the membrane side that was derived from the external cell surface. However, both Na^+ gradients directed from out to in (Ca^{2+} efflux experiments) and from in to out (Ca^{2+} uptake experiments) stimulated Ca^{2+} fluxes. Additionally, removal of IOV and unassignable membranes by Sepharose-4B ATP did not alter either Na^+ gradient dependent or Na^+ independent Ca^{2+} efflux. This was true despite more than doubling Ca^{2+} uptake per milligram membrane protein during the preloading incubation period in the Sepharose-4B ATP BLMV. Thus, neither the orientation of the vesicles nor the presence of unassignable membranes affected Ca^{2+} efflux.

The results presented above demonstate that PTH modulated the Na^+/Ca^{2+} exchange activity in the canine renal proximal tubular BLMV. Analysis of Ca^{2+} efflux demonstrated that Na^+-stimulated efflux was depressed by TPTX and was stimulated by infusion of PTH to TPTX dogs. Analysis of the apparent kinetics of the Na^+/Ca^{2+} exchange activity assessed at 5 s demonstrated that TPTX decreased the maximal stimulation of Ca^{2+} efflux by Na^+ without affecting the Na^+ concentration, and caused half maximal stimulation or the Hill coefficient of the exchange activity, which was two or greater. The absence of PTH also decreased the apparent V_{max} of the exchange activity as a function of Ca^{2+} concentration.

The demonstration of active Ca^{2+} reabsorption in the proximal nephron (7) indicates an important role of transcellular calcium movements in the reabsorptive fluxes of this segment. This implies both an apical influx and basolateral efflux step for calcium in the proximal cell. The entry step at the brush border may involve calcium binding and translocation and is increased by PTH (24). The exit step could either be via the Na^+/Ca^{2+} exchanger or the Ca-Mg-ATPase-Ca^{2+} pump. The best candidate for large calcium fluxes at the basolateral membrane would appear to be the Na^+/Ca^{2+} exchanger. In the cardiac sarcolemma, this exchange mechanism performs the function of a high capacity system with rapid transport of entered calcium. However, the apparent K_m of 8 μM for Na^+ is too high for an effective efflux mechanism in a cell whose cytosolic calcium is probably 100–300 nM (23). Using similar techniques to those described in this paper, the original K_m for the Na^+/Ca^{2+} exchanger in cardiac sarcolemma vesicles was 8 μM (28), but Caroni et al. (30) have shown that with charge equilibration the K_m was in fact 1.5 μM. More recently

they have shown that the degree of membrane phosphorylation mediated by the calcium calmodulin-dependent protein phosphatase system is important, and pretreatment of calf heart sarcolemmal vesicles with ATP, Mg^{2+}, and Ca^{2+} may further reduce the K_m (47). Thus, the in vitro measurements of the kinetic parameters of the Na^+/Ca^{2+} exchanger may not reflect the effective in vivo values, which may be considerably lower. The final proof of the relative importance of the Na^+/Ca^{2+} exchanger and Ca-Mg-ATPase can only be determined by the investigation of their actions in the intact cell using specific inhibitors. These experiments are underway. We postulate that the cytosolic calcium concentration modulates the apical calcium entry step much as has been postulated for the apical sodium entry in toad bladder (48). Thus, in the absence of any change in basolateral membrane calcium permeability, the Na^+/Ca^{2+} exchanger would be the rate-limiting step in transcellular calcium transport. The PTH activation of the Na^+/Ca^{2+} exchanger should increase calcium reabsorption in the proximal tubule. However, in spite of the demonstration of PTH action on the proximal tubule by its stimulation of adenylate cyclase (49), it appears that in this segment it decreases Na^+ and Ca^{2+} reabsorption. This effect, however, would be explained by the concomitant change in basolateral membrane calcium permeability that was demonstrated in this paper. This would tend to increase cytosolic calcium and thus decrease apical entry of calcium and sodium by this model. It may be possible that activation of the Na^+/Ca^{2+} exchanger occurs in response to PTH in all sites where it is present in the renal tubule, but that the change in basolateral calcium permeability is specific to the proximal tubule. This would explain the similarity in action of PTH to experimental protocols that were thought to increase proximal tubular cell cytosolic calcium and decrease the sodium reabsorption (4, 27).

In summary, the data presented here demonstrates that the canine proximal tubular BLMV possess a Na^+/Ca^{2+} exchange activity that has similar properties to the previously described cardiac sarcolemmal Na^+/Ca^{2+} exchanger. This BLMV exchanger is modulated by PTH. The data also demonstrates that the Ca^{2+} permeability of the BLMV is decreased in the TPTX state and increased by infusion of PTH. The assessment of the relative role of the Na^+/Ca^{2+} exchange in transcellular Ca^{2+} transport will require experiments with very short incubation times and analysis of the effects of ATP on exchange activity.

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