Calcium Transport in the Thick Ascending Limb of Henle

HETEROGENEITY OF FUNCTION IN THE MEDULLARY AND CORTICAL SEGMENTS

WADI N. SUKI, DIANE ROUSE, ROLAND C. K. NG, and JUHA P. KOKKO, *Renal Section, Department of Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030; Department of Internal Medicine, University of Texas Health Science Center at Dallas, Texas 75235*

ABSTRACT Calcium transport was studied in medullary and cortical segments of the thick ascending limb of Henle perfused in vitro. ^{45}Ca was added to the perfusate for measuring lumen-to-bath flux (J_{lb}^{Ca}), to the bath for measuring bath-to-lumen flux (J_{bl}^{Ca}), or to both perfusate and bath for measuring net flux (J_{net}^{Ca}). In the medullary segment J_{lb}^{Ca} exceeded J_{bl}^{Ca} and the efflux:influx coefficient ratio was not different from the value predicted from the observed potential difference (PD). In the cortical segments, however, efflux:influx coefficient ratio was greater than the value predicted from the PD, suggesting that calcium transport in this segment may be active, while it is passive in the medullary segment. Furosemide, which reversibly decreases PD in both cortical and medullary segments, inhibited J_{lb}^{Ca} only in the medullary segment. Parathyroid hormone (PTH), on the other hand, had no effect on J_{net}^{Ca} in the medullary segment, but it significantly augmented J_{net}^{Ca} in the cortical segment. These results indicate that calcium transport in the thick ascending limb is heterogeneous. In the medullary segment it is passive, inhibited by furosemide and not influenced by PTH. In the cortical segment, however, calcium transport appears to be active, not inhibited by furosemide and stimulated by PTH.

INTRODUCTION

Micropuncture studies indicate that ~55% of the filtered calcium is reabsorbed in the proximal convoluted tubule, and 25–40% is reabsorbed between the end of the proximal convoluted tubule and early distal convoluted tubule (1–6). Calcium absorption beyond the proximal convoluted tubule has been attributed to the thick ascending limb of Henle because furosemide, which inhibits chloride transport in this segment (7), also inhibits the absorption of calcium (5, 8). Direct evidence for calcium absorption in the cortical segment of the thick ascending limb has been advanced from four different laboratories using isolated rabbit tubule segments perfused in vitro (9–12). The conclusions reached concerning the nature of calcium transport in this segment, however, have not been unanimous. Both Rocha et al. (9) and Imai (11) advanced evidence for active calcium transport in the cortical thick ascending limb. On the other hand, Shareghi and Stoner (10) found calcium transport in this segment to be passive, and Bourdeau and Burg (12) invoked single-file diffusion to explain the voltage-dependent calcium efflux which they observed.

The present studies were undertaken to further characterize calcium transport across the thick ascending limb, to investigate calcium transport in the medullary thick ascending limb that previously had not been investigated, and to examine the hormonal regulation of calcium transport in these two segments of the thick ascending limb of Henle.

METHODS

Normal female New Zealand white rabbits weighing 1.5–2.5 kg, maintained on standard rabbit chow and allowed free access to water, were killed by guillotine. The left flank was opened quickly, the kidney excised and decapsulated, and 1-mm thick transverse sections removed and placed in a chilled artificial medium ("A" solution, millimolar concentrations: Na^+, 145.5; K^+, 5.0; Ca^{++}, 1.8; Mg^{++}, 1.0; Cl^-, 118.0; HCO_3^-, 26.0; $PO_4^=$, 2.3; acetate, 10.0; L-alanine, 5.0; glucose, 8.3; total osmolality 300 mOsmol/kg H_2O) to which 5% by volume fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) was added. This, and

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all other solutions, was gassed with a 5% CO_2 and 95% O_2 gas mixture. Thick ascending limb segments were dissected under a binocular dissecting microscope with careful attention to anatomical appearance and cortical or medullary origin. The tubule segments were then transferred to a thermostatically controlled lucite perfusion chamber resting on the stage of an inverted microscope, and perfused according to the method of Burg et al. (13). The perfusion solution differed from A solution only in its concentrations of HCO_3^- (1.0 mM) and Cl^- (143.0 mM). The perfusate was driven hydraulically at the desired rate. All studies were carried out at 37°C, and at pH 7.4; A solution with fetal calf serum was used to bathe the tubule and was exchanged at a constant rate of 0.5 ml/min in order to minimize compositional changes resulting from evaporative water loss. Transtubular potential difference (PD)[1] was measured using agarose-Ringer's bridges and calomel half-cells (Beckman Instruments, Inc., Fullerton, Calif.) according to a circuit previously described by Rocha and Kokko (14). The recorded PD was corrected for the liquid junction potential resulting from the asymmetry of the perfusion and bathing solutions (15). After an equilibration period of 30–60 min, timed fluid collections were made under a layer of oil using calibrated constant-volume pipettes.

Perfusion rate (Vi) was verified using exhaustively dialyzed (16) [*methoxy*-^{3}H]inulin (New England Nuclear, Boston, Mass.) added to the perfusate (100 μCi in 3–5 ml of perfusate). Calcium flux (J^{Ca}) was measured with $^{45}CaCl_2$ (New England Nuclear) added to the perfusate and/or to the bath in a concentration of 2.5–10.0 μCi of ^{45}Ca per ml. The specific activity of ^{45}Ca was 20–30 Ci/g, and the quantity of isotope added would have increased the concentration of calcium by only 0.15–0.7%. Samples of fluid were expelled into counting vials containing 1 ml of water; 10 ml of Biofluor (New England Nuclear) was then added and each vial shaken vigorously. Isotopic concentrations were measured in a liquid-scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.).

To prevent loss of CO_2 alkalinization of fluid samples, and precipitation of calcium salts, all oils used were equilibrated with water saturated with CO_2. Also, to prevent binding of calcium to glass, all pipettes coming in contact with the perfusion solution and the collected fluid were siliconized with a 6% solution of SC-87 (Pierce Chemical Co., Rockford, Ill.) in chloroform, flushed with toluene, and air dried.

Three types of experiments were performed: (*a*) In five medullary (mean length 1.46±0.16 mm) and six cortical (mean length 0.97±0.07 mm) thick ascending limb segments perfused at mean rates of 10–13 nl/min, J^{Ca} was measured from lumen-to-bath (lb) and from bath-to-lumen (bl) sequentially in each tubule, and alternately in different tubules. For lb (efflux) measurements ^{45}Ca was added only to the perfusate. For bl (influx) measurements ^{45}Ca was added only to the bath. At least 30 min were allowed between changes in bath or perfusate and commencement of fluid sample collection. (*b*) In six medullary (mean length 1.7±0.15 mm) and seven cortical (mean length 1.2±0.16 mm) thick ascending limb segments perfused at mean rates of 2.5–3.8 nl/min, only J_{lb}^{Ca} was measured. Two to four control samples first were collected; the perfusion solution was then exchanged with one to which 50 μm furosemide was added. After at least 30 min, two or more collections were made. This was followed by again exchanging the perfusate with the control perfusate, allowing 30–45 min for recovery and collecting an additional two or more samples. (*c*) In four medullary (mean length 1.41±0.11 mm) and five cortical (mean length 1.26±0.19 mm) thick ascending limb segments perfused at mean rates of 10.5–14.5 nl/min, only net calcium flux (J_{net}^{Ca}) was measured. In these experiments the chemical concentration of calcium and the concentration of ^{45}Ca were identical in both the perfusate and the bathing medium. After two to four control samples were collected, purified parathyroid hormone (PTH) (Inolex Corp., Biomedical Div., Glenwood, Ill.) was added to the bath in a concentration of 0.1 U/ml. 30 min were allowed before an additional two to four collections were made. The bathing solution was then exchanged with control bathing solution, 30 min were allowed to elapse, and two to four samples were collected during the recovery period. As a control for this group of experiments six tubules (three cortical and three medullary), averaging 1.0±0.13 mm in length and perfused at mean rates of 9.1–10.2 nl/min, were studied in an identical fashion and for a comparable duration with the exception that PTH was not added to the bath. Because all six tubules behaved in a similar fashion, they shall be considered as one group for the purposes of data analysis.

Calculations and measurements. Vi (nanoliters per minute) was measured from the volume (P_V in nanoliters) of the calibrated constant-volume pipette and the time (t in minutes) of collection and verified by the following formula:

$$Vi = \frac{P_V}{t}(C_o^I/C_i^I) \quad (1)$$

where C_o^I and C_i^I are the concentrations of inulin in the collected fluid and perfusate, respectively. There was no significant difference between the values of Vi derived by these two different methods in any of the studies reported.

At rapid perfusion rates (>10 nl/min) J_{lb}^{Ca} and J_{bl}^{Ca} (peq·min^{-1}·cm^{-1}) were calculated by the following mass balance equations:

$$J_{lb}^{Ca} = \frac{Vi}{L}(Ci - Co)([Ca]i/Ci) \quad (2)$$

and

$$J_{bl}^{Ca} = \left(\frac{Vi\ Co}{L}\right)([Ca]b/Cb) \quad (3)$$

where Ci, Co, and Cb are the counts per minute per nanoliter of ^{45}Ca in the perfusate, collected fluid, and bath, respectively; [Ca]i and [Ca]b are the chemical concentrations of calcium in the perfusate and bath, respectively; and L is the length of the tubule measured with a reticle in the eyepiece of the microscope. In 13 tubules calcium efflux measured by the method represented in Eq. 2 was compared to the flux of calcium as measured by the appearance of calcium in the bath. There was excellent correlation between these two methods ($y = 1.2x - 14$, $r = 0.91$, slope not significantly different from unity and intercept not significantly different from zero, $P > 0.1$, where y is the calculated calcium flux from lumen to bath and x is the measured appearance of calcium in the bath in peq/30 min). J_{net}^{Ca} was calculated according to Eq. 2.

At slow perfusion rates (<10 nl/min) J_{lb}^{Ca} was calculated by the following formula:

$$J_{lb}^{Ca} = K_e^{Ca} \times \bar{C} \quad (4)$$

where $K_e^{Ca}(\times 10^{-6}$ cm·s$^{-1})$ is the efflux coefficient for cal-

[1] *Abbreviations used in this paper*: J_{lb}^{Ca}, calcium lumen-to-bath flux; J_{bl}^{Ca}, calcium bath-to-lumen flux; J_{net}^{Ca}, net calcium flux; lb, lumen to bath; bl, bath-to-lumen; PD, potential difference; PTH, parathyroid hormone.

cium and $\bar{C}$ is the mean luminal calcium concentration calculated approximately from the following formula:

$$\bar{C} = [Ca]i \left(\frac{1 + \frac{Co}{Ci}}{2} \right) \quad (5)$$

K_e^{Ca} and the influx coefficient for calcium, K_i^{Ca} ($\times 10^{-6}$ cm·s^{-1}), were calculated as follows:

$$K_e^{Ca} = \frac{Vi}{A} \ln \frac{Ci}{Co} \quad (6),$$

and

$$K_i^{Ca} = \frac{Co}{Cb} \left(\frac{K_e^{Ca}}{1 - e^{-(AK_e^{Ca}/Vi)}} \right) \quad (7),$$

where A is the area of the tubule calculated from the measured length and the inside diameter of the tubule. The mechanism of calcium transport was examined by a modification of the Ussing flux ratio analysis (17) wherein calcium transport may be assumed to be by passive diffusion when the flux coefficient ratio equals the value predicted from the PD as follows:

$$K_e^{Ca}/K_i^{Ca} = e^{zFE/RT} \quad (8),$$

where z is the valence of calcium, F is the Faraday constant, E is the PD, R is the gas constant and T the absolute temperature.

Statistical analysis was performed by analysis of variance, paired t statistic or t statistic as indicated (18).

RESULTS

J_{lb}^{Ca} and J_{bl}^{Ca} measurements. The results for the medullary and cortical segments are presented in Table I. In the medullary segments J_{lb}^{Ca} was 25.0 ±12.9, J_{bl}^{Ca} was 16.0±9.8, and the net transport calculated as the mean of the difference between these values was 9.0±2.8 peq·cm^{-1}·min^{-1}. The PD during the two collection periods was similar and averaged 5.5±1.3 mV. K_e was 20.7±10.5 and K_i was 13.9±8.6 $\times 10^{-6}$ cm·s^{-1}, giving a ratio of 2.01±0.20. This did not differ significantly ($P > 0.2$) from the value of 1.60±0.20 that was predicted from the PD.

In the cortical segments J_{lb}^{Ca} was 14.8±3.9, J_{bl}^{Ca} was 3.9±2.9, and the net transport was 10.9±2.6 peq·cm^{-1}·min^{-1}. The PD during the two collection periods was 4.6±0.2 and 4.5±0.6 mV, respectively. K_e was 11.5±2.6 and K_i was 2.6±0.8 $\times 10^{-6}$ cm·s^{-1}, giving a ratio of 5.41 ±1.26. This measured flux coefficient ratio was significantly greater ($P < 0.05$) than the value 1.50±0.21 predicted from the PD.

Effect of furosemide on J_{lb}^{Ca}. The results for the medullary and cortical segments are shown in Table II. In the medullary segments J_{lb}^{Ca} fell significantly after the addition of furosemide to the perfusate from a value of 11.2±2.8 to 6.2±1.2 peq·cm^{-1}·min^{-1} ($P < 0.01$), and recovered to 8.8±2.3 peq·cm^{-1}·min^{-1} when furosemide was removed, a value not significantly different from control. These changes were accompanied by parallel changes in PD, which was 4.2±0.7 mV in control, 1.4±0.3 mV after furosemide ($P < 0.005$), and 3.9±1.0 mV during recovery.

In the cortical segments PD also fell significantly after furosemide from 3.3±0.5 mV to 1.7±0.2 mV ($P < 0.005$), and rose to 2.7±0.4 mV during recovery. J_{lb}^{Ca}, however, was not significantly changed at 12.0±3.2, 18.4±4.0, and 10.0±2.7 peq·cm^{-1}·min^{-1} in the control, furosemide, and recovery periods, respectively.

Effect of PTH on J_{net}^{Ca}. The results for medullary and cortical segments are depicted in Table III. In the medullary segments J_{net}^{Ca} was 9.9±1.8 in the control period, 10.7±3.4 after the addition of PTH to the bath, and 10.7±1.7 peq·cm^{-1}·min^{-1} after removal of PTH. None of these values is significantly different from the other. The PD also was not significantly changed at 3.2±0.5, 2.3±0.4, and 2.9±0.9 mV in the three periods, respectively.

In the cortical segments, the addition of PTH to the bath raised J_{net}^{Ca} significantly from 13.8±1.2 to 23.0 ±1.5 peq·cm^{-1}·min^{-1} ($P < 0.025$), and its removal resulted in the return of J_{net}^{Ca} to a value of 17.5±4.1 peq·cm^{-1}·min^{-1}, which is not significantly different from control. These changes were not accompanied by significant changes in PD, which was stable at 4.3

TABLE I
Unidirectional Flux of Calcium in the Thick Ascending Limb

Segment	J^{Ca}			PD				Ke/Ki‡		
	lb	bl	net*	lb	bl	Ke	Ki	Measured	Predicted	*P* value
	peq/cm·min			*mV*		*10^{-6} cm/s*				
Medullary	25.0	16.0	9.0	5.5	5.5	20.7	13.9	2.01	1.60	<0.2
SEM	12.9	9.8	2.8	1.3	1.3	10.5	8.6	0.20	0.20	
Cortical	14.8	3.9	10.9	4.6	4.5	11.5	2.6	5.41	1.50	<0.05
SEM	3.2	2.9	2.6	0.2	0.6	2.6	0.8	1.26	0.21	

* Calculated as ($J_{lb}^{Ca} - J_{bl}^{Ca}$) in each experiment.
‡ Efflux:influx coefficient ratio.

TABLE II

Effect of Furosemide on Calcium Efflux in the Thick Ascending Limb

Segment	J_{lb}^{Ca}			PD		
	Control	Furosemide	Recovery	Control	Furosemide	Recovery
	peq/cm·min			*mV*		
Medullary	11.2	6.2	8.8	4.2	1.4	3.9
SEM	2.8	1.2	2.3	0.7	0.3	1.0
P C vs. F		<0.01			<0.005	
C vs. R		NS			NS	
Cortical	12.0	18.4	10.0	3.3	1.7	2.7
SEM	3.2	4.0	2.7	0.5	0.2	0.4
P C vs. F		NS			<0.005	
C vs. R		NS			NS	

C, control; F, furosemide; R, recovery.

±1.1, 3.5±1.0, and 2.6±0.2 mV in the three periods, respectively. In contrast to these results, the control group of tubules showed no significant changes in calcium flux or in voltage. J_{net}^{Ca} was 15.9±3.3, 11.7±3.1, and 11.3±2.1 peq·cm^{-1}·min^{-1}, and the voltage was 4.1±0.7, 2.1±2.0, and 3.3±0.7 mV in the three corresponding periods.

DISCUSSION

The present studies demonstrate for the first time that calcium transport in the thick ascending limb of Henle's loop is heterogeneous. In the medullary segment the flux coefficient ratio was not significantly different from that predicted from the PD using the Ussing equation (17), suggesting that calcium transport in this segment is passive and is driven by the positive luminal potential generated by the active chloride efflux (14). Predictably, therefore, the addition of furosemide to the perfusate, which is known to inhibit chloride transport and decrease the PD (7), resulted in significant inhibition of calcium efflux from this segment. By contrast, in the cortical segment the flux coefficient ratio was significantly higher than that predicted from the PD, suggesting that calcium transport in this segment is active. It is possible that as the perfusate travels along the length of the tubule, the concentration of sodium is reduced progressively and the positive luminal potential increases. The PD measured at the tip of the perfusing pipette, therefore, may not accurately reflect the PD downstream. It easily can be calculated, however, that the flux coefficient ratio observed in the cortical segments requires that a PD > +20 mV prevail throughout the tubular length to account for the observed calcium flux by passive diffusion alone. This, in turn, requires a degree of dilution of the tubular fluid not likely to be accomplished at these perfusion rates even at the end of the tubular segment. It is more likely that a component of active calcium transport exists in the cortical segment of the

TABLE III

Effect of Parathyroid Hormone on Net Calcium Efflux in the Thick Ascending Limb

Segment	J_{net}^{Ca}			PD		
	Control	PTH	Recovery	Control	PTH	Recovery
	peq/cm·min			*mV*		
Medullary	9.9	10.7	10.7	3.2	2.3	2.9
SEM	1.8	3.4	1.7	0.5	0.4	0.9
P C vs. PTH		NS			NS	
C vs. R		NS			NS	
Cortical	13.8	23.0	17.5	4.3	3.5	2.6
SEM	1.2	1.5	4.1	1.1	1.0	0.2
P C vs. PTH		<0.025			NS	
C vs. R		NS			NS	

For meaning of symbols refer to Table II.

thick ascending limb of Henle, a feature that distinguishes it from the medullary segment. In support of this suggestion was the observation that furosemide added to the perfusate reduced the PD but did not decrease calcium efflux in this segment.

The suggestion that the thick ascending limb may be functionally heterogeneous in its medullary and cortical segments is strengthened by a number of anatomical, biochemical, and functional studies. Allen and Tisher (19) have demonstrated by scanning electron microscopy that the epithelial lining of the medullary thick ascending limb differs from that of the cortical segment. Two distinct cell types were described, smooth- and rough-surfaced cells. The smooth-surfaced cells predominate in the medullary, whereas the rough-surfaced cells predominate in the cortical segment. Schmidt and Dubach (20) have demonstrated a higher concentration of Na^+, K^+-activated adenosine triphosphatase in the medullary than in the cortical segment, and Morel and co-workers (21, 22) have also shown different enzyme activity patterns with a PTH-sensitive adenylate cyclase activity present in the cortical segment and an antidiuretic hormone-sensitive adenylate cyclase activity present in the medullary segment. Finally, Stokes (23) has shown that prostaglandin E_2 inhibits chloride transport in the medullary segment of the thick ascending limb but not in the cortical segment. Our studies of the effect of PTH on calcium transport in the medullary and cortical segments represent another example of the functional heterogeneity of these two segments, and provide a functional parallel to the enzymatic studies of Chabardès et al. (22). PTH stimulated net calcium efflux in the cortical thick ascending limb without raising PD, presumably by stimulating an active transport process, but had no effect on the medullary segment. Also, a control group of tubules exhibited no significant change in net calcium efflux or in PD over a similar period of study. This observation is consonant with the finding by Chabardès et al. (22) of PTH-sensitive adenylate cyclase activity only in the cortical segment but not in the medullary segment. Our findings also suggest that in addition to the granular segments of the distal convoluted tubule and the collecting tubule (10), the cortical thick ascending limb may be one site where PTH exerts its known hypocalciuric effect.

Several investigators have examined calcium transport in the cortical thick ascending limb and reached different conclusions. Rocha et al. (9) and Imai (11) found flux ratios greater than can be accounted for by the PD, and that ouabain (9, 11) and furosemide (11) produced small inhibition or no change in calcium transport despite a major inhibition of the PD. Our findings are qualitatively similar to those of Rocha et al. (9) and Imai (11). Bourdeau and Burg (12) also found a flux ratio at high PD greater than that predicted for simple passive diffusion. However, at zero PD the flux ratio was unity and they invoked single-file pore diffusion of calcium (12). Shareghi and Stoner (10), on the other hand, concluded that calcium transport in their studies was by simple passive diffusion, although they admit that of the tubules they studied some "may have been medullary." The findings of Shareghi and Stoner (10) resemble our findings in the medullary segments. The reason(s) for the differences between the findings of different investigators are not apparent.

There has only been one previous examination of the effects of PTH on the cortical thick ascending limb reported (10), and with negative results. However, those investigators used a PTH concentration of only 0.01–0.02 U/ml. Chabardès et al. (22) reported a threshold concentration of 0.05 U/ml for this segment of the rabbit nephron. In our studies we used a concentration twofold the threshold concentration and observed a clear-cut augmentation of net calcium transport in the cortical segment.

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