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Role of the loop segment in the urinary concentrating defect of hypercalcemia

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Role of the loop segment in the urinary concentrating defect of hypercalcemia. Hypercalcemia is associated with impaired urinary concentrating ability. To explore the mechanism(s) by which hypercalcemia impairs chloride transport in the loop of Henle, we carried out in vivo microperfusion of the loop segment in Sprague–Dawley rats rendered acutely hypercalcemic (12.1 ± 0.1 mg/dliter) by calcium gluconate infusion. Control rats were infused with sodium gluconate and had normal plasma calcium (8.0 ± 0.2 mg/dliter). Compared to control, fractional chloride reabsorption was decreased (61 ± 4 to 50 ± 3%; $P < 0.05$) and early distal chloride increased 74 ± 6 to 98 ± 3 mEq/liter ($P < 0.001$) in hypercalcemia. During hypercalcemia, infusion of verapamil failed to increase fractional chloride reabsorption (49 ± 4%; $P < 0.05$) or decrease early distal chloride (95 ± 2; $P < 0.05$) toward control values. Similarly, indomethacin did not improve fractional chloride reabsorption (48 ± 4%; $P < 0.05$) or distal chloride concentration (93 ± 7; $P < 0.05$). In control rats infused with Ringers HCO_3, the addition of calcium 8.0 mEq/liter to the perfusate increased early distal calcium (0.22 to 3.11 mEq/liter) but was associated with no change in fractional chloride reabsorption (−6 ± 6%) and a slight decrease in early distal chloride (−9 ± 3 mEq/liter; $P < 0.05$). These data are consistent with the hypothesis that an elevated plasma, not luminal calcium, concentration impairs chloride reabsorption in the loop segment, primarily the ADH–stimulated component. This may have an important role in the urinary concentrating defect of hypercalcemia.

Hypercalcemia is associated with impaired renal concentrating ability in humans and experimental animals due to several proposed mechanisms [1, 2]. In the rat, Manitius et al produced hypercalcemia by excess vitamin D administration, and showed decreased papillary sodium and urea content [3]. This decrease in medullary hypertonicity in hypercalcemia has been attributed to a number of functional derangements. First, impaired transport in Henle's loop has been suggested by the observations of reduced free water reabsorption in dogs and rabbits after acute infusions of calcium salts [4–7]. Second, reduction in GFR has been shown in several of these models [3, 4, 8] and has suggested that a decrease in solute delivery to Henle's loop may be an important mechanism. Finally, Brunette et al showed enhanced medullary blood flow which, they suggested, may promote a reduction in papillary solute content [6].

To determine whether and by what mechanism decreased net chloride uptake within the loop of Henle occurs during hypercalcemia, chloride transport in the loop segment (LS) of superficial nephrons was studied by microperfusion techniques in normocalcemic and hypercalcemic rats. The responses to verapamil or indomethacin infusion and the effect of increased luminal calcium concentration without a change in plasma calcium concentration were studied.

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Methods

All studies were carried out in male Sprague–Dawley rats which weighed between 220 and 390 g and were maintained on regular rat chow (Wayne Lab Blox, Chicago, Illinois, USA) and tap water until the day of experiment. Rats were anesthetized with inactin (BYK Gulden Konstanz, FRG) 100 mg/kg body wt (BW) i.p. and placed on a servo-controlled heated table. Rectal temperature was monitored by a telethermometer (Yellow Springs Inst. Corp., Yellow Springs, Ohio, USA) and maintained at 37°C throughout the experiment. A tracheostomy was performed and PE-50 catheters were placed in the jugular vein for the infusion of solutions, in the right femoral artery for the continuous monitoring of blood pressure and intermittent blood sampling, and in the bladder for timed urine collections. Arterial pressure was determined with a model P23ID transducer (Gould–Statham Inst., Inc., Hato Rey, Puerto Rico) and recorded on a model 7D polygraph (Grass Inst. Co., Quincy, Massachusetts, USA). The left kidney was exposed through a flank incision and the perirenal fat was removed. The kidney was placed in a Lucite cup and warmed agar was placed around the kidney to form a well on the surface. Polyfructosan 2% (Inutest, Laevosan Gesellschaft, Linz, Austria) was added to all infusion solutions in sufficient amounts to sustain a plasma concentration of about 1 mg/dliter. The kidney was bathed continually with the infusate without Inutest. After 60 min of equilibration, two 60 min clearance periods were carried out during which microperfusion was performed. Blood was sampled periodically for determination of inulin and chloride concentrations and timed urine collected for determination of volume and inulin concentrations. The experiment was terminated by aortic puncture and plasma concentrations of chloride and calcium were determined.

The degree of hypercalcemia that would produce a defect in concentrating ability was confirmed in a group of rats (HICA-1) which were rendered hypercalcemic by daily subcutaneous injections of calciferol, 175,000 units (Taylor Pharmacal Co., Decatur, Illinois, USA) in oil for three days. Control rats (CON-1) were given sesame oil injections. Animals were al-

lowed free access to food and tap water. Drink was removed in the afternoon of the fourth day and urine was collected until 0700 of the fifth day, and again until 1500 for determination of osmolality. The animals were then sacrificed and plasma calcium concentration was determined on aortic blood.

Microperfusion of the LS was performed as previously described [9]; a brief summary follows. The LS includes a short terminal section of the proximal convoluted tubule (PCT), the proximal straight tubule (PST), the thin descending limb (tDL) of Henle's loop, the medullary and cortical thick ascending limb (TALH) and a short initial segment of the distal convoluted tubule (DCT). A pipet filled with FD and C Green–tinted non-radioactive perfusate was inserted into a proximal tubule at random and a small bolus injected to identify both latest surface proximal and earliest surface distal sites. A perfusion pipet was inserted into the latest accessible surface proximal tubule and the perfusion begun at 22 nliter/min with a precalibrated microperfusion pump (World Precision Instruments, New Haven, Connecticut, USA). The perfusate consisted of (in mM): KCl, 5; $MgSO_4$, 1; $NaHCO_3$, 10; NaH_2PO_4, 1; $CaCl_2$, 1; urea, 4.2; and NaCl, 130. Sufficient methoxy-^3H inulin was added to yield 100 to 150 cpm/nliter. A pipet was inserted proximal to the perfusion pipet to inject a small cast of bone wax. The segment proximal to this block was then vented. After 1 to 2 min of perfusion to insure satisfactory flow, a collection pipet was inserted into the distal tubule site. An oil block was inserted and a complete timed collection obtained. Two to six tubules were perfused in each animal.

Acute hypercalcemia was produced by the infusion of Ringers–bicarbonate and calcium gluconate, 62 mEq/liter at a rate of 3 mliter/100 g BW/hr for 1 hr, and thereafter at 3 mliter/hr. Group HICA-2 (N = 11) received this infusate only. To determine whether antagonism of calcium entry blockade altered the responses to hypercalcemia, verapamil (Searle Pharmaceuticals Inc, Chicago, Illinois, USA) 0.02 mg/kg/hr was added to the infusate in group HICA-3 (N = 8) [10]. To determine the role of prostaglandins on the observed defect in chloride transport, indomethacin (Sigma Co., St. Louis, Missouri, USA) 5 mg/kg was infused as a bolus followed by a sustaining dose of 6 mg/kg/hr in group HICA-4 (N = 9) [11]. The control group (CON-2; N = 9) was infused with Ringers–bicarbonate consisting of: Na, 140; Cl, 100; K, 4.0; HCO_3, 25; acetate, 20; Mg, 1.2; Ca, 1.2; phosphate, 1.2; dextrose, 5.3; and sodium gluconate 62 mEq/liter at 3 mliter/100 g BW/hr for 1 hr and thereafter at 3 mliter/hr.

The effects of elevated luminal calcium concentration were determined in separate groups of rats (group CON-3; N = 7) which were infused with Ringers-bicarbonate at 1.2 mliter/100 g BW/hr. Separate tubules in each animal were perfused with artificial tubule fluid containing calcium at a concentration of either 0.5 or 8.0 mEq/liter and $^{45}Ca^{++}$ 2 μg/mliter.

Analytical techniques

Methods for determination of volume, chloride, and inulin were as described previously [9]. Plasma calcium concentration was determined by a modified method of Kessler and Wolfman [12]. $^{45}Ca^{++}$ activity was determined by liquid scintillation counting (Packard Tri Carb, Model 3255, Packard Instrument Co., Downers Grove, Illinois, USA). Appropriate corrections for crossover counts and background activity were made and quench corrections were determined by an internal standard. In each experiment perfusate and collectate $^{45}Ca^{++}$ activity were determined by the same techniques.

Calculations

Clearance and excretion rates were calculated by standard expressions [9]. In vivo perfusion rate (PR) was calculated by the expression:

$$PR = CR \times {}^3H_2/{}^3H_1 \text{ (nliter/min)}$$

in which CR = distal collection rates, $^3H_1$ = ^3H inulin counts/min/nliter in the perfusate, and $^3H_2$ in collectate. Only perfusions with a calculated rate of >15 and <25 nliter/min were accepted. Fractional chloride reabsorption (FR Cl) was calculated as:

$$FR\ Cl = [(Cl_1 \times PR) - (Cl_2 \times CR)]/(Cl_1 \times PR)$$

in which Cl_1 and Cl_2 are the chloride concentrations in the perfusate and the collectate respectively.

The concentration of calcium in the early distal tubule (ED_{Ca++}) was estimated by the expression:

$$ED_{Ca} = [1-(({}^{45}Ca^{++}{}_1 \times PR) - ({}^{45}Ca^{++}{}_2 \times CR)/({}^{45}Ca_1 \times PR)] \times PF_{Ca} \times {}^3H_1/{}^3H_2$$

in which $^{45}Ca^{++}{}_1$ and $^{45}Ca^{++}{}_2$ are the perfusate and collectate $^{45}Ca^{++}$ counts/min/nliter and PF_{Ca++} is the perfusate calcium concentration.

Values are given as mean ± SEM. For microperfusion data a single mean value is calculated for each variable in each animal and used to calculate the mean value for each group. Statistical significance was determined by an unpaired Student's t-test between groups and a paired t test within groups. Significance was set at the 5% level.

Results

In the vitamin D-treated rats (HICA-1), urine osmolality was 2025 ± 124 on day four and 2367 ± 83 on day five compared with 2752 ± 67 and 3290 ± 134 ($P < 0.02$ and 0.005, respectively) in controls (CON-1). Plasma calcium concentrations on day four were 11.4 ± 0.6 (HICA-1) and 9.8 ± 0.3 mEq/liter (CON-1) ($P < 0.05$).

Body wt, blood pressure, and plasma chloride concentrations did not differ among the groups studied by microperfusion (Table 1). Plasma calcium concentrations in all HICA groups were higher than that in group CON-2 and were in the range shown to produce a decrease in urine osmolality after water deprivation. Inulin clearance in group CON-2 was lower than those in groups HICA-2 and HICA-3; inulin clearances in these latter groups did not differ from that in group CON-3. Tubule perfusion rates did not differ (Table 2).

Compared to group CON-2, hypercalcemia (group HICA-2) was associated with a decrease in fractional chloride reabsorption in the LS (50 ± 3 vs. 61 ± 4%) and an increase in early distal tubule fluid chloride concentration (98 ± 3 vs. 74 ± 6 mEq/liter); the decrease in absolute chloride reabsorption (1422 ± 81 vs. 1686 ± 94 pEq/min) was not significant ($P < 0.07 >$

Table 1. Systemic variables for microperfusion studies.

Group	Body wt *g*	Mean arterial pressure *mm Hg*	Final plasma calcium *mg/dliter*	Final plasma chloride *mEq/liter*	Inulin clearance *μliter/min*
CON-2	288 ± 14	116 ± 3	8.0 ± 0.2	99 ± 2	962 ± 82
HICA-2	264 ± 11	118 ± 6	12.1 ± 0.5[a]	102 ± 2	1483 ± 191[a]
HICA-3	255 ± 14	108 ± 2	11.5 ± 0.6[a]	103 ± 1	1273 ± 176[a]
HICA-4	251 ± 9[a]	118 ± 4	11.2 ± 0.2[a]	104 ± 1	826 ± 108
CON-3	270 ± 11	115 ± 2	—	103 ± 2	1276 ± 179

[a] $P < 0.05$ compared to CON-2.

Table 2. Microperfusion data for hypercalcemic groups.

Group	Perfusion rate nliter/min	Fluid reabsorption %
CON-2	21.2 ± 0.5	34 ± 2
HICA-2	21.2 ± 0.5	33 ± 4
P value[a]	NS	NS
HICA-3	20.1 ± 0.6	29 ± 5
P value[a]	NS	NS
HICA-4	20.6 ± 0.8	22 ± 3
P value[a]	NS	<0.01

[a] Compared to group CON-2.

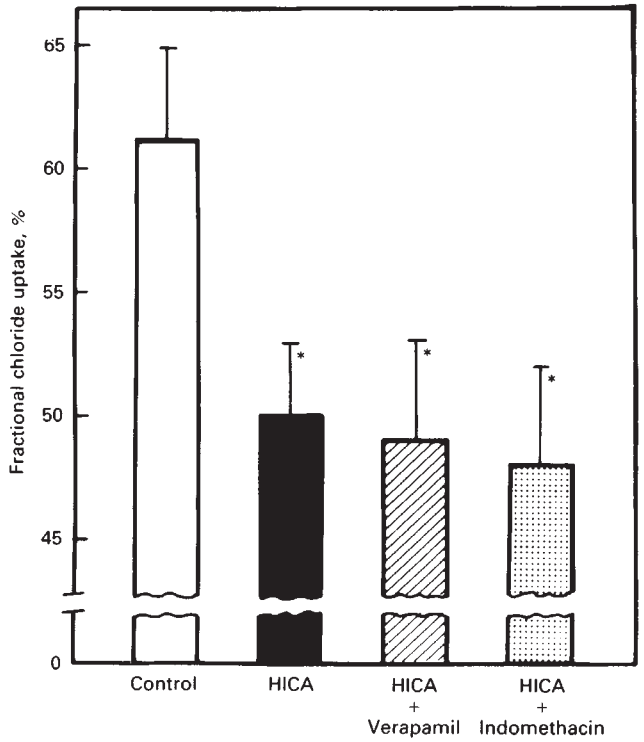

Fig. 1. *Comparison of fractional chloride reabsorption in the perfused loop segment among the hypercalcemia groups.* **P* < 0.05 compared to control.

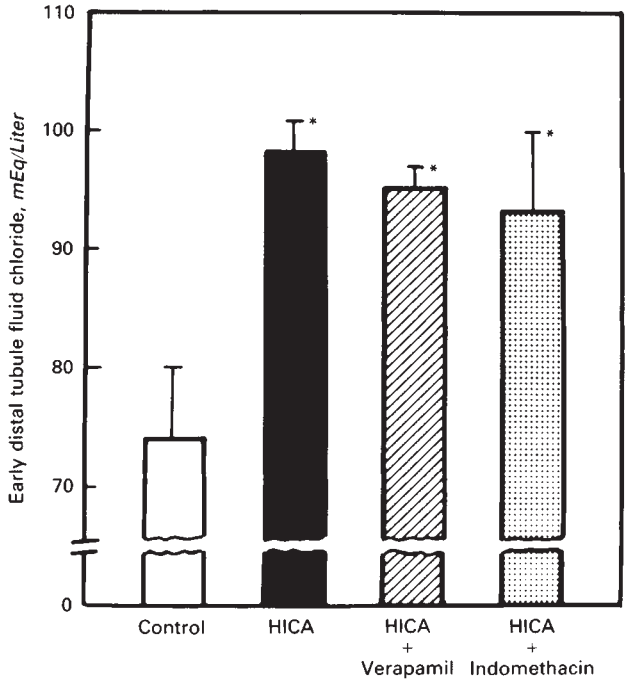

Fig. 2. *Comparison of the early distal tubule fluid chloride concentrations among the hypercalcemic groups in which the loop segment was perfused.* **P* < 0.05 compared to control.

0.05) (Figs. 1–3). In hypercalcemic rats infused with the calcium entry blocker, verapamil (group HICA-3), absolute chloride reabsorption (1292 ± 102 pEq/min), early distal tubule fluid chloride concentration (95 ± 2 mEq/liter) and fractional chloride reabsorption (49 ± 4%) failed to improve (Figs. 1–3). Similarly, indomethacin infusion (group HICA-4) failed to correct absolute (1314 ± 124 pEq/min) or fractional (48 ± 4%) chloride reabsorption or early distal chloride concentration (93 ± 7 mEq/liter) (Figs. 1–3). Fractional fluid reabsorption was lower in group HICA-4 (Table 2).

High perfusate calcium concentrations (8 mEq/liter) increased estimated early distal tubule fluid calcium concentration (Table 3). Nevertheless, absolute and fractional chloride reabsorptions did not differ from control perfusates with 0.5 mEq/liter calcium. Early distal tubule fluid chloride concentration was lower with the 8 mEq/liter perfusate.

Discussion

Our data show that, in the rat, moderate acute hypercalcemia is associated with an almost 20% reduction in fractional chloride reabsorption in the LS and a 25% increase in early distal tubule fluid chloride concentration. A decrease in renal concen-

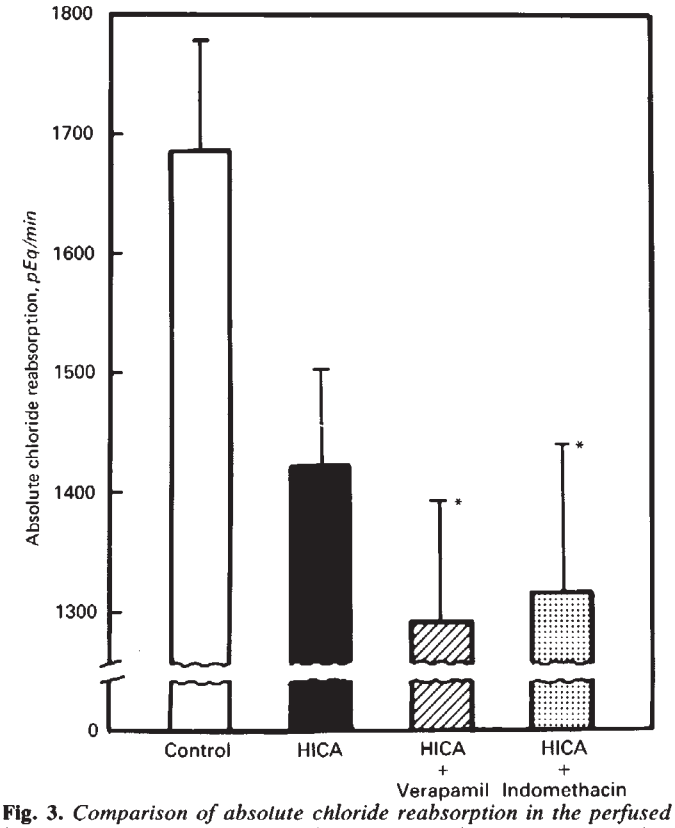

Fig. 3. *Comparison of absolute chloride reabsorption in the perfused loop segment among the hypercalcemic groups.* $*P < 0.05$ compared to control.

trating ability was confirmed for a similar degree of hypercalcemia lasting 72 hr in unanesthetized rats. The approximately 50% increase in plasma calcium concentration in our study is similar to that in other studies in which the acute infusion of calcium salts has been used to induce and investigate the urinary concentrating defect [4, 5, 7]. During acute hypercalcemia, the defect in chloride uptake was not corrected either by calcium entry blockade with verapamil or prostaglandin inhibition with indomethacin.

Although the LS comprises several nephron segments, we interpret our data to relate most directly to active NaCl transport in the TALH for the following reasons: since TALH is virtually impermeant to water, any alteration in net fluid transport in the LS would occur mainly in either the PST or the tDL. If the principal effect of hypercalcemia were in the PST or the tDL, a decrease in fluid reabsorption should accompany the observed decreases in chloride uptake. More severe hypercalcemia than that produced in our study can decrease fluid reabsorption in the PCT [13, 14]; a similar effect could occur in the PST. Medullary washout could also have reduced fluid reabsorption in the tDL which might then induce flow-related changes in chloride uptake in the TALH. Because net LS fluid reabsorption, in fact, did not change in hypercalcemia alone (group HICA-2), it is most likely that Cl uptake was altered by an effect of hypercalcemia in the TALH.

In the isolated perfused rabbit cortical TALH, Shareghi and Agus noted a decrease in transepithelial potential difference (PD) when either the luminal or bath calcium concentration was doubled [15]. However, because they did not directly examine fluid or NaCl transport and we did not determine PD, comparison between these studies is inferential. Quamme [14] perfused the LS of thyroparathyroidectomized Wistar rats in vivo at 25 nliter/min and showed decreased sodium and chloride reabsorption when plasma calcium concentration was increased from 2.2 to 4.2 mM/liter by $CaCl_2$ infusion. Thus, our data are not only in concert with these observations despite the differences between the protocols but also show an effect at a more moderate degree of hypercalcemia which is more often observed clinically.

In contrast, Bank and Aynedjian found no differences in the tubule fluid to plasma osmolality ratios all along the surface DCT between normocalcemic and hypercalcemic rats during mannitol–saline diuresis at an unspecified infusion rate [8]. They inferred that no defect in sodium and, presumably, chloride transport was present in the nephron to the DCT. Differences between our protocol and theirs—especially the effects of an osmotic diuresis—and the marked decrease in GFR in their study may explain, in part, the opposing conclusions.

Hebert and Andreoli [16] have proposed a model of NaCl reabsorption in the TALH in which prostaglandins (PG) modulate ADH-stimulated NaCl co-transport. In support of that model, Andreoli and coworkers [16, 17] showed in the murine medullary TALH that PGE_2 did not alter the transepithelial PD or chloride reabsorption in the absence of ADH and that cAMP could stimulate transepithelial PD and chloride uptake in the presence of ADH. In addition, Torikai and Kurokawa [18] have shown that ADH-stimulated cAMP formation is inhibited by PGE_2 in both the cortical and medullary segments of the isolated TALH of the rat. In the LS of the rat in vivo, Higashihara et al [11] have shown by free-flow micropuncture that indomethacin increases chloride reabsorption, and we have shown that indomethacin enhances primarily the ADH-stimulated component of chloride reabsorption [19]. In our microperfusion study, fractional chloride reabsorption increased and distal chloride concentration decreased ($P < 0.05$) after indomethacin in Sprague–Dawley rats and in Brattleboro rats given ADH but not in Brattleboro rats without ADH replacement, data which further support the model proposed by Hebert and Andreoli.

Hypercalcemia increases urinary PGE_2 excretion and, by inference, renal PGE_2 synthesis, but the extent to which calcium and prostaglandins interact to influence the action of ADH is controversial [20, 21]. In the present study, indomethacin was infused in sufficient doses to inhibit PGE_2 synthesis by 95% [11] but failed to correct the defective LS chloride transport induced by hypercalcemia. Fluid reabsorption also was decreased in the LS in this group (about 2.5 nliter/min) unlike that in group HICA-2 or any of the indomethacin–treated rats previously mentioned [19]. However, PG are not known to influence transport in the proximal tubule [22] and, based on estimates of cortical thickness and rates of fluid reabsorption in the mammalian PST [23], only about 0.75 nliter/min of fluid would be reabsorbed in the PST. Thus, even if transport in the PST were completely inhibited, this segment would not account for the observed small decrease in fluid reabsorption. On the other hand, passive fluid uptake in the tDL is influenced by medullary blood flow which conceivably could have been altered by an interaction between hypercalcemia and PG inhibition in a manner to decrease fluid uptake at this site. However, in this instance, only fluid and not chloride delivery to the

Table 3. Effect of perfusate calcium concentration on loop segment function

	Perfusate Ca		Difference	
	0.5 mEq/liter	8.0 mEq/liter	$N=8$	P value[a]
Collectate Ca^{++} *mEq/liter*	0.22 ± 0.06	3.11 ± 0.43	+2.44 ± 0.48	<0.001
Perfusion rate *nl/min*	19.5 ± 1.0	19.6 ± 0.9	+0.10 ± 1.27	NS
Fluid reabsorption %	42 ± 4	26 ± 7	−16 ± 8	NS
Cl reabsorption *pEq/min*	1558 ± 145	1412 ± 244	−147 ± 236	NS
%	62 ± 4	55 ± 6	−6 ± 6	NS
Collectate Cl *mEq/liter*	81 ± 3	72 ± 4	−9 ± 3	<0.05

[a] Paired Student's *t*-test.

TALH would be increased. Increased delivery (load) should result in increased absolute chloride reabsorption, decreased fractional chloride reabsorption, and increased distal chloride concentration [24–26]. Since no differences in the indices of Cl uptake were observed in group HICA-4 compared to group HICA-2, there were no additional effects of this small increase in fluid delivery. Thus, PG inhibition did not detectably alter Cl uptake in the LS during hypercalcemia. These data suggest that hypercalcemia has already suppressed that component of chloride reabsorption in the LS modulated by PG, that is, the ADH-stimulated component. This interpretation is congruent with the conclusion of Beck et al [27] that the urinary concentrating defect in hypercalcemic rats is due in part to a direct inhibitory effect of calcium on the ADH-responsive cyclic AMP system.

Cytosolic calcium has been shown to modulate sodium transport in several epithelia including toad and turtle bladders, frog skin, and rabbit proximal and collecting tubules [28]. For example, the calcium ionophore, A-23187, inhibits sodium transport in the toad bladder, the magnitude of which is dependent upon extracellular calcium concentration; this inhibition is reversed by ADH [28]. In water–diuresing thyroparathyroidectomized rats, Humes, Simmons, and Brenner [10] showed that verapamil prevented an increase in urinary osmolality that had occurred in response to parathyroid hormone (PTH) infusion. Although it is not known if cytosolic calcium is increased by hypercalcemia [29], they suggested that verapamil had a direct antagonistic effect on a PTH-stimulated event, perhaps an increase in intracellular calcium. While the site of action of the observed effects in their study is also unknown, PTH-responsive adenylate cyclase abounds in the cortical TALH of the rat [30]. Similarly, verapamil did not correct the chloride transport defect in the LS induced by hypercalcemia in the present study. This may indicate that the TALH epithelium does not possess calcium channels that can be blocked by verapamil or that the drug does not alter the defect in chloride uptake. Clearly, further investigation of these interactions is necessary to elucidate their mechanism. In agreement with our observation, Takaichi, Uchida, and Kurokawa [31] recently reported that extracellular calcium per se suppressed ADH-dependent adenylate cyclase in the medullary TALH but not in collecting tubule, and that this effect was not reversed by either verapamil or diltiazem.

In contrast with calcium infusion, no defect in chloride uptake in the LS was observed when the increase in calcium concentration was limited to the tubule lumen (group CON-3). Although fractional fluid reabsorption in the LS of these normocalcemic rats was unchanged as shown by statistical analysis, the decrease appears large. Even if the decrease in fluid reabsorption were significant, none of the expected flow–related changes occurred [24–26]; collectate chloride concentration actually decreased. Conceivably, the high perfusate calcium concentration decreased chloride transport in the PST but increased it in the TALH. Such a possibility would be consistent with our data, but clearly it cannot explain the observed defect in net chloride uptake produced by infused calcium.

We did not examine either impaired collecting duct responses to antidiuretic hormone (ADH) [32] or increased medullary blood flow [6, 33]. Increased blood flow would decrease medullary interstitial hyperosmolality which, in turn, would decrease the transepithelial chloride gradient in the TALH and thus, cannot, per se, explain our findings of impaired chloride reabsorption. However, either of these defects may contribute to the concentrating defect via effects in other nephron segments.

Based on our observations, we conclude that acute hypercalcemia per se and not the consequent increase in luminal calcium concentration inhibits chloride transport in the LS of the rat independently of fluid or chloride load to that segment. This defect may be mediated by an effect of extracellular calcium on ADH-stimulated chloride reabsorption in the TALH. In a recent review of the role of calcium in the action of ADH including a consideration of hypercalcemia, Levine and Schlondorff [30] raised the possibility that calcium inhibits both urinary concentration and sodium (chloride) reabsorption by inhibition of adenylate cyclase in the TALH. Subsequently, Berl et al [34] reported marked inhibition of cAMP accumulation after ADH in TALH but not in medullary collecting ducts from hypercalcemic rats. They concluded that this may importantly contribute to the concentration defect; our data further support this conclusion.

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