J. Membrane Biol. 180, 111–121 (2001)
DOI: 10.1007/s002320010063

The Journal of
Membrane
Biology

L-Arginine Effects on Na^+ Transport in M-1 Mouse Cortical Collecting Duct Cells—A Cationic Amino Acid Absorbing Epithelium

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Received: 11 September 2000/Revised: 6 December 2000

Abstract. The effect of L-arginine on transepithelial ion transport was examined in cultured M-1 mouse renal cortical collecting duct (CCD) cells using continuous short circuit current (I_{SC}) measurements in HCO_3^-/CO_2 buffered solution. Steady state I_{SC} averaged 73.8 ± 3.2 $\mu A/cm^2$ ($n = 126$) and was reduced by 94 ± 0.6% ($n = 16$) by the apical addition of 100 μM amiloride. This confirms that the predominant electrogenic ion transport in M-1 cells is Na^+ absorption via the epithelial sodium channel (ENaC). Experiments using the cationic amino acid L-lysine (radiolabeled) as a stable arginine analogue show that the combined activity of an apical system y^+ and a basal amino acid transport system y^+L are responsible for most cationic amino acid transport across M-1 cells. Together they generate net absorptive cationic amino acid flux. Application of L-arginine (10 mM) either apically or basolaterally induced a transient peak increase in I_{SC} averaging 36.6 ± 5.4 $\mu A/cm^2$ ($n = 19$) and 32.0 ± 7.2 $\mu A/cm^2$ ($n = 8$), respectively. The response was preserved in the absence of bath Cl^- ($n = 4$), but was abolished either in the absence of apical Na^+ ($n = 4$) or by apical addition of 100 μM amiloride ($n = 6$). L-lysine, which cannot serve as a precursor of NO, caused a response similar to that of L-arginine ($n = 4$); neither L-NMMA (100 μM; $n = 3$) nor L-NAME (1 mM; $n = 4$) (both NO-synthase inhibitors) affected the I_{SC} response to L-arginine. The effects of arginine or lysine were replicated by alkalinization that mimicked the transient alkalinization of the bath solution upon addition of these amino acids. We conclude that in M-1 cells L-arginine stimulates Na^+ absorption via a pH-dependent, but NO-independent mechanism. The observed net cationic amino acid absorption will counteract passive cationic amino acid leak into the CCD in the presence of electrogenic Na^+ transport, consistent with reports of stimulated expression of Na^+ and cationic amino acid transporters by aldosterone.

Key words: Renal collecting duct — Amiloride — Epithelial sodium channel (ENaC) — Nitric oxide (NO) — pH — Cationic amino acid transport — System y^+ — System y^+L

Introduction

Cationic amino acids are transported across the apical and basal membranes of epithelial cells by a variety of asymmetrically distributed transport systems. Originally resolved by their functional properties, more recently these have been defined at a molecular level (Devés & Boyd, 1998). Of topical interest is the role of these transporters in the delivery of L-arginine as a substrate for intracellular nitric oxide (NO) synthesis. Various cell types, including endothelium, vascular smooth muscle, macrophages, and neuronal tissue, are able to generate NO from L-arginine using the enzyme NO synthase (Moncada & Higgs, 1993).

In the kidney NO has effects both on hemodynamics and on tubular transport (Kone & Baylis, 1997). Indeed, NO has been reported to inhibit sodium reabsorption in the cortical collecting duct (CCD) (Stoos et al., 1992; Stoos, Carretero & Gavien, 1994; Stoos, Garcia & Gavin, 1995) and may be involved in the fine control of cross-talk between apical Na^+ transport and basolateral K^+ conductance in CCD principal cells known to express neuronal nitric oxide synthase (nNOS) (Lu, Giebisch & Wang, 1997*a,b;* Wang et al., 1998). The rate-limiting

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Part of this work has been published in abstract form (Korbmacher, Bertog & Boyd, 1999).

step of Na^+ reabsorption in this nephron segment is the amiloride-sensitive epithelial Na^+ channel (ENaC) and many signaling pathways are involved in its regulation (Garty & Palmer, 1997). A regulatory effect of NO on amiloride-sensitive Na^+ transport in the CCD may therefore be important for Na^+ homeostasis and hence long-term control of blood pressure.

Since L-arginine can be considered as a precursor for NO production (Palmer et al., 1988*b*), the presence of arginine transporters may be indicative of this process. Therefore, in this investigation we have examined cationic amino acid transport in cultured M-1 mouse CCD cells (Stoos et al., 1991) and have also studied the effects of extracellular arginine on transepithelial amiloride-sensitive Na^+ transport measured electrophysiologically. Cultured M-1 cells express morphological and functional properties typical of CCD principal cells including amiloride-sensitive Na^+ transport via ENaC (Korbmacher et al., 1993; Letz et al., 1995; Chalfant et al., 1996). We show that there is net (absorptive) arginine transport across this tight epithelium, that this is well explained by the combined activity of apical system y^+ and basal system y^+L transporters, and that addition of arginine does markedly stimulate amiloride-sensitive Na^+ transport. However, we find that the ability of the added cationic amino acid to stimulate sodium transport is not dependent on nitric oxide synthesis, and we have therefore further investigated the underlying mechanism.

Materials and Methods

Cell Culture

The M-1 cell line (ATCC 2038-CRL, American Type Culture Collection, Rockville, MD) was originally obtained from Dr. G. Fejes-Tóth (Stoos et al., 1991). Cells were handled as previously described (Korbmacher et al., 1993; Letz et al., 1995; Bertog et al., 1999). For transepithelial studies cells were seeded onto 12 mm diameter Millicell-HA culture plate inserts (Millipore, Bedford, MA). Cells were grown to confluence and transepithelial voltage (V_{te}) and resistance (R_{te}) of the cultures were routinely checked using a EVOM™ epithelial volt-ohmmeter (World Precision Instruments, Sarasota, FL).

Short-Circuit Current Measurements

Eleven or twelve days after seeding the Millicell inserts with confluent M-1 cells were transferred to purpose-built Ussing chambers for continuous equivalent I_{SC} measurements, as described (Bertog et al., 1999; Cuffe et al., 2000). R_{te} was evaluated every second by measuring the voltage deflections induced by 100 msec symmetrical square current pulses of ±10 $\mu A/cm^2$. Open circuit V_{te} was also measured and I_{SC} was calculated according to Ohm's law. Conventionally, a lumen negative V_{te} corresponds to a positive I_{SC} which may be due to electrogenic cation absorption or electrogenic anion secretion or a combination of both. A standard bath solution containing (in mM): 140 Na^+, 4 K^+, 1 Ca^{2+}, 1 Mg^{2+}, 124 Cl^-, 24 HCO_3^-, 5 D-glucose, was used on both the apical and basolateral side of the epithelial monolayer. Bath solution was maintained at 37°C and gassed with a mixture of 5% CO_2/95% O_2 maintaining pH at 7.4. Chloride-free solutions were achieved by replacing Cl^- by gluconate and contained 6 mM Ca^{2+} gluconate to compensate for the Ca^{2+} buffering properties of gluconate. Na^+-free solution was obtained by replacing 120 mM Na^+ by NMDG (N-methyl-D-glucamine) and 24 mM Na^+ by choline.

Lysine Flux Measurements

Radioactive lysine (Amersham International) was used to measure cationic amino acid fluxes as previously described (Devés, Chavez & Boyd, 1992). It was used at a low (2 μM) concentration so that the contribution of both high and low affinity transporters could be determined. For the flux studies cells were transferred into a medium containing (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (adjusted to pH 7.5 with NaOH). For the effects of the open-circuit transepithelial potential on electrogenic transepithelial cationic amino acid fluxes to be appropriately controlled, all flux experiments were carried out in the presence of a concentration of amiloride (10 μM) sufficient to abolish this potential (*see* below). Inhibitory amino acids were used as described in Devés and Boyd (1998) to isolate the contribution of the four potential transport systems (y^+, y^+L, B^{0+} and b^{0+}) known to translocate cationic amino acids across mammalian cell membranes. Filters were set up in such a way that there was an apical compartment volume of 500 μl and a basal compartment volume of 1 ml with minimal transepithelial hydrostatic pressure gradient. Aliquots (30 μl) for estimation of fluxes were removed from each compartment at times indicated and were replaced with the same volume of fresh medium. At the end of the experiment filters were briefly washed of extracellular isotope by rapid passage through three wash solutions containing ice-cold incubation medium. The cells were then lysed and an aliquot of the lysis solution counted for determination of intracellular radioactivity. Transport studies were performed in a humidified, temperature-controlled shaking chamber at 37°C.

Drugs Used

Amiloride hydrochloride, S-nitroso-N-acetyl-penicillamine (SNAP), N^G-L-nitro-arginine methyl ester (L-NAME), and the various amino acids were purchased from Sigma-Aldrich (Poole, Dorset, UK). N(G)-monomethyl-L-arginine (L-NMMA) was supplied by Calbiochem-Novabiochem (Nottingham, UK). All stock solutions were freshly prepared on the day of the experiment and all other chemicals used were of the highest grade commercially available.

Data Analysis

Data are expressed as mean ± SE and significance of difference was estimated using the appropriate version of Student's *t* test.

Results

Flux Studies Using ^3H and ^{14}C L-Lysine

We investigated the pathways available for cationic amino acid transport in M-1 cells using ^3H lysine (2 μM) as a stable arginine analogue. All studies were carried

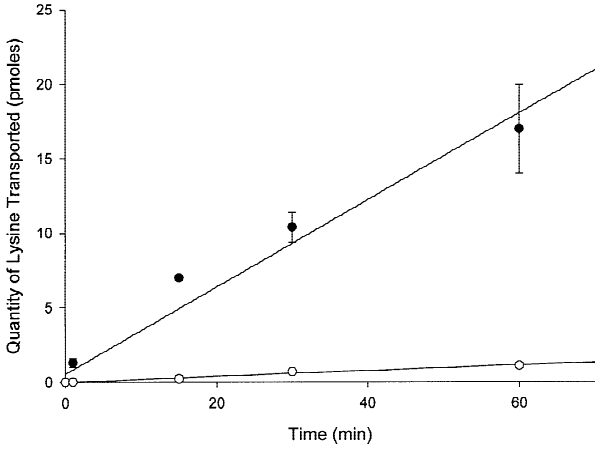

Fig. 1. Unidirectional transepithelial lysine fluxes across M-1 cells monolayers. Fluxes were measured simultaneously from apical to basal (●) and from basal to apical (○) using 2 μM substrate in each compartment. Results are shown as means ±SE ($n = 3$ filters for each time point).

out using cells grown to confluence as proven by their electrophysiological properties. (In these experiments the mean values of V_{te} and of R_{te} were 41 ± 4 mV and 1422 ± 39 Ωcm^2, respectively; the high transepithelial resistance was found to be preserved in a sample filter that was rechecked following the transport assay protocol). To establish whether there was net transport, bidirectional flux experiments were carried out using tracer (2 μM) lysine added simultaneously to both compartments, ^{14}C basally and ^{3}H apically. Results (Fig. 1) show the individual unidirectional fluxes to be linear over the time course of the experiment (60 min). The observed absorptive (apical to basal) flux was substantially greater than that in the opposite (basal to apical) direction; in a further series of experiments the mean flux ratio (absorptive to secretory) was 6.4 ± 1.4 ($n = 5$). The net absorptive flux was 35.8 ± 5.8 ($n = 5$) pmoles/cm^2/hr.

To elucidate the individual transport systems in the apical and basal membranes generating this net flux, experiments were carried out using a variety of competing amino acids to distinguish between different candidate pathways. As shown in Table 1, addition of unlabeled lysine (10 mM) to the apical compartment reduced apical to basal but did not alter basal to apical flux. The presence of 5 mM apical BCH (a substrate for system B^{0+}) influenced neither flux, excluding a role for this transporter in lysine movement across the apical membrane. Similarly cystine added apically (1 mM) had little effect excluding a role for system b^{0+} in this membrane. In contrast apical leucine (100 μM) stimulated apical to basal flux of lysine while inhibiting basal to apical flux; consequently the transepithelial flux ratio for lysine was markedly greater in the presence of 100 μM apical leucine than in its absence. Since precisely similar observations (stimulation of transepithelial lysine fluxes by leucine) are found in other epithelia (Munck & Schultz, 1969) and the modern explanation of this phenomenon relates (*see* Devés & Boyd, 1998) to the presence in the basal membrane of transport system y^+L, we therefore examined in more detail processes involved in the cellular (rather than transepithelial) uptake of lysine from the basal compartment while also manipulating its amino acid composition.

Table 1. Transepithelial lysine flux (pmol/cm^2/hr)

Inhibitor amino acid	Location	Apical to basal	Basal to apical	Flux ratio
Nil	—	41.6 ± 6.7	6.7 ± 0.8	6.4
Lysine (10 mM)	Apical	25.0 ± 6.7	6.7 ± 1.0	3.6
Leucine (0.1 mM)	Apical	58.3 ± 11.0	5.8 ± 0.7	10.0
Cystine (1 mM)	Apical	35.0 ± 4.3	4.7 ± 0.5	7.5
BCH (5 mM)	Apical	41.7 ± 0.7	4.7 ± 1.5	8.7

Apical membrane uptake of lysine was substantially slower than basal membrane uptake (19.6 ± 1.5 compared to 50.8 ± 5.7 pmoles/cm^2/hr), suggesting that the cationic amino acid permeability of the basal membrane is greater. Basal membrane lysine influx (Table 2) was substantially inhibited by addition of unlabeled lysine (10 mM), defining mediated flux to be 37.3 ± 6.5 pmoles/cm^2/hr. A low concentration of leucine (0.1 mM) inhibited more than 70% of this, but neither BCH nor cystine had an effect. These results show that system y^+L is the major pathway for lysine movement into the cell across the basal membrane. In keeping with this, other experiments showed that the intracellular accumulation of lysine, entering the cell from the apical compartment, was reduced (from 11.8 ± 0.9 to 6.8 ± 0.2 pmoles per filter) by addition of basal leucine (0.1 mM), but was not altered by addition of cystine or BCH.

These findings suggest that an apical system y^+ transporter and a basal y^+L transport system are responsible for the observed cationic amino acid fluxes in these cells. Since in the presence of extracellular arginine these systems could provide substrate for NO synthesis in M-1 cells and since NO may affect transepithelial ion transport, we went on to study this possibility.

Baseline Electrophysiological Parameters

After an equilibration period of about 30 min following transfer into Ussing type chambers, confluent monolayers of M-1 cells displayed mean transepithelial voltage (V_{te}), resistance (R_{te}), and equivalent short-circuit current (I_{SC}) values of −46.4 ± 1.2 mV (lumen negative), 704 ± 21 Ωcm^2, and 73.8 ± 3.2 μA/cm^2 ($n = 126$), respectively. Apical application of 100 μM amiloride reduced

Table 2. Basal membrane lysine flux (pmol/cm^2/hr)

Inhibitor amino acid	Location	Influx	N number	Significance
Nil		50.8 ± 5.7	3	
Lysine (10 mM)	Basal	13.5 ± 1.3	3	$P < 0.01$
Leucine (0.1 mM)	Basal	26.2 ± 3.3	3	$P < 0.01$
Cystine (1 mM)	Basal	48.5 ± 1.5	3	NS
BCH (5 mM)	Basal	49.8 ± 1.5	3	NS

V_{te} from −38.6 ± 3.2 to −3.4 ± 0.3 mV and I_{SC} from 63.6 ± 10.2 to 2.9 ± 0.1 μA/cm^2, while R_{te} rose from 694 ± 54 to 1172 ± 89 Ωcm^2 ($n = 16$). This corresponds to a 94 ± 0.6% inhibition of I_{SC}. This confirms that the predominant electrogenic ion transport across M-1 monolayers is Na^+ absorption via the amiloride-sensitive epithelial Na^+ channel (ENaC) known to be expressed in M-1 cells (Korbmacher et al., 1993; Letz et al., 1995; Chalfant et al., 1996). In contrast, in the presence of amiloride apical addition of the chloride channel blocker DPC (1 mM) had only a minor effect demonstrating the absence of Cl^- secretion under baseline conditions (Bertog et al., 1999; Cuffe et al., 2000). To assess the Na^+ absorptive and Cl^- secretory component of I_{SC} under various experimental conditions, amiloride (100 μM) and DPC (1 mM) were routinely added at the end of most experiments.

Apical or Basolateral Application of L-arginine Stimulates I_{SC}

Figure 2*A* shows an experiment in which the effect of apical application of L-arginine on I_{SC} was tested. L-arginine (10 mM) induced a transient increase in I_{SC} with a peak reaching its maximum within ≈2 min after addition. The peak amplitude averaged 36.6 ± 5.4 μA/cm^2, changing the I_{SC} from 72.8 ± 7.6 to 109.4 ± 12.6 μA/cm^2 ($n = 19$). The shape of the L-arginine-induced I_{SC} increase was very consistent. Apical application of 1 mM L-arginine caused a similar but smaller I_{SC} response with an average peak amplitude of 5.7 ± 0.7 μA/cm^2 ($n = 5$) while 0.1 mM L-arginine had no significant effect ($n = 5$). Figure 2*B* shows that basolateral application of L-arginine (10 mM) elicited an almost identical I_{SC} response to that observed with apical L-arginine. Following basolateral application of 10 mM L-arginine the peak I_{SC} increase averaged 32.0 ± 7.2 μA/cm^2, changing from 82.4 ± 11.5 to 114.4 ± 18.0 μA/cm^2 ($n = 8$). The shape and time course of the response was similar for both apical and basolateral L-arginine.

Cl^- Dependence of the Stimulatory L-Arginine Response

The stimulatory response to L-arginine may be due to an increase in electrogenic cation absorption or anion secretion. Indeed, the peak response to L-arginine is reminiscent of Cl^- secretory responses mediated by purinoreceptors ($P2Y_2$) located in the apical and basolateral membrane of M-1 cells or by basolateral proteinase-activated receptor (PAR-2) (Bertog et al., 1999; Cuffe et al., 2000).

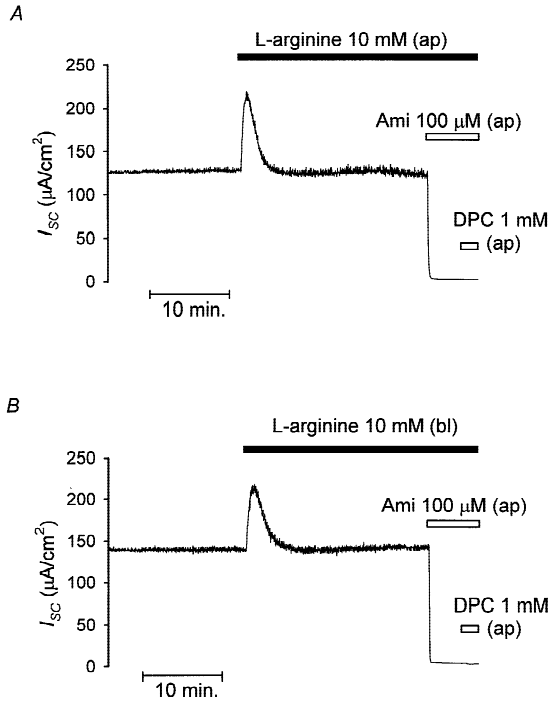

Fig. 2. Apical or basolateral addition of L-arginine induces a transient increase in I_{SC}. Typical experimental traces where L-arginine (10 mM) was applied to *A* the apical bath (ap) and *B* the basolateral bath (bl) followed by the additions of apical amiloride (Ami; 100 μM) and DPC (1 mM).

To test whether the L-arginine-induced increase in I_{SC} was due to Cl^- secretion, we examined the Cl^--dependence of the L-arginine response by replacing extracellular Cl^- ions (apically and basolaterally) with gluconate in experiments as shown in Fig. 3. In the absence of extracellular Cl^-, L-arginine (10 mM) induced a peak response averaging 28.2 ± 4.0 μA/cm^2, changing from 41.5 ± 1.9 to 69.7 ± 2.4 μA/cm^2 ($n = 4$). Note that the return to baseline I_{SC} was somewhat delayed in the absence of extracellular Cl^- and that baseline I_{SC} was lower in the absence of Cl^- than in the presence of Cl^- (Fig. 3). The effect of Cl^- removal on baseline I_{SC} was not further investigated. The finding that the peak response to L-arginine was preserved in the absence of extracellular Cl^- indicates that the I_{SC} response is Cl^- independent and does not involve a Cl^- secretory component.

Amiloride-Sensitive Component of the L-Arginine Response

To establish whether the L-arginine peak response is mediated by transepithelial Na^+ absorption, amiloride was

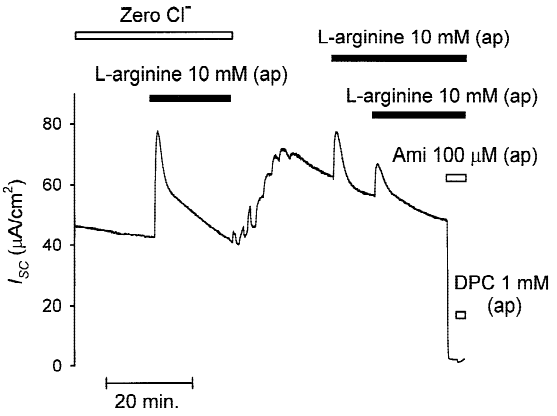

Fig. 3. The stimulatory response to L-arginine is preserved in the absence of extracellular Cl^- (Zero Cl^-). L-arginine (10 mM) was applied in the absence of extracellular Cl^- and subsequently in its presence.

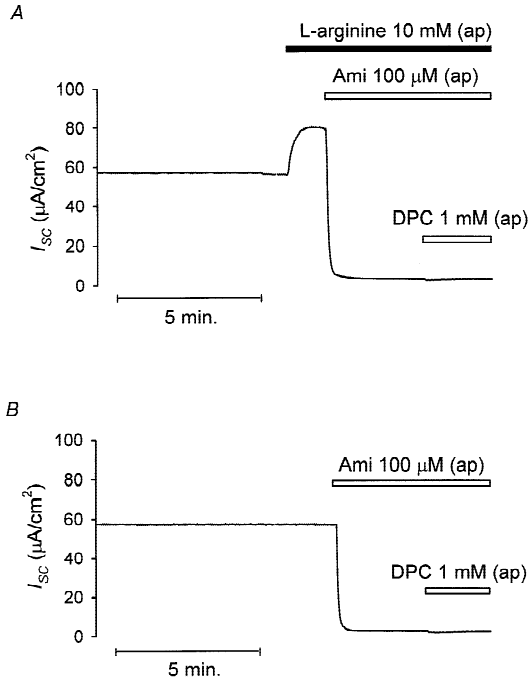

Fig. 4. Amiloride addition completely inhibits the L-arginine peak I_{SC} response. In *A* L-arginine (10 mM) was applied to the apical bath and amiloride (100 μM) was added when the resulting response reached its maximum. Once a plateau was established, DPC (1 mM) was added. (*B*) Shows a control experiment performed on a monolayer from the same batch of cells.

added at the peak of the L-arginine response (Fig. 4*A*). The apical addition of amiloride (100 μM) during the peak response to L-arginine reduced I_{SC} from 82.2 ± 5.9 to 3.7 ± 0.1 μA/cm^2 (n = 5), compared to time-matched controls (Fig. 4*B*) where amiloride reduced I_{SC} from 51.2 ± 3.0 to 2.7 ± 0.2 μA/cm^2 (n = 5). These results indicate that the I_{SC} component stimulated by addition of L-arginine is amiloride-sensitive and involves the stimulation of transepithelial Na^+ absorption via ENaC.

The Effect of L-Arginine on V_{te} and R_{te}

The application of apical L-arginine induced a hyperpolarization of V_{te} averaging 5.6 ± 0.4 mV, changing from −44.6 ± 2.5 mV to −50.2 ± 2.6 mV (n = 19). The corresponding R_{te} traces showed reductions in resistance averaging 161 ± 19 Ωcm^2, changing from 683 ± 56 Ωcm^2 to 522 ± 41 Ωcm^2 (n = 19). As mentioned earlier, the transepithelial voltage and current across this cortical collecting duct cell type is mainly a result of transepithelial Na^+ absorption. The hyperpolarization of V_{te} and decrease in R_{te} caused by L-arginine addition are consistent with an increased activity of ENaC.

The Stimulatory Effect of L-Arginine is Prevented in the Presence of Amiloride

To further confirm that the stimulatory response to L-arginine is mediated by the activation of ENaC, experiments were performed in which L-arginine was applied in the presence of amiloride. As shown in Fig. 5*A* the I_{SC} peak induced by L-arginine was almost completely suppressed in the presence of 100 μM apical amiloride with an average response of 3.4 ± 1.2 μA/cm^2, changing I_{SC} from 3.5 ± 0.3 μA/cm^2 to 6.8 ± 1.2 (n = 6). This confirms that the transient I_{SC} increase induced by L-arginine is amiloride-sensitive. In addition, the experiment shown in Fig. 5*A* demonstrates that after washout of amiloride a usual response to L-arginine may be elicited, causing an increase in I_{SC} of 16.5 ± 4.6 μA/cm^2, changing it from 53.5 ± 3.5 to 70.0 ± 7.3 μA/cm^2 (n = 6). Amiloride added during the peak increase elicited by L-arginine almost completely abolished I_{SC}, consistent with the interpretation that the stimulated I_{SC} component is indeed mediated by ENaC.

The Effect of L-Arginine is Prevented by Removal of Sodium from the Bathing Solution

If the response to L-arginine is mediated by an activation of ENaC the response should be dependent on the presence of extracellular Na^+ on the apical side. Thus, to investigate the sodium dependence of the L-arginine response, the effect of removing Na^+ from the apical bathing solution was examined. In the experiment shown in Fig. 5*B*, NaCl in the apical bath was replaced by N-methyl-D-glucamine (NMDG) chloride and $NaHCO_3$ was replaced by choline bicarbonate. As the graph shows, the stimulatory I_{SC} response to L-arginine was completely blocked by removal of apical Na^+, in fact a small decrease in I_{SC} averaging 2.1 ± 0.3 μA/cm^2 was

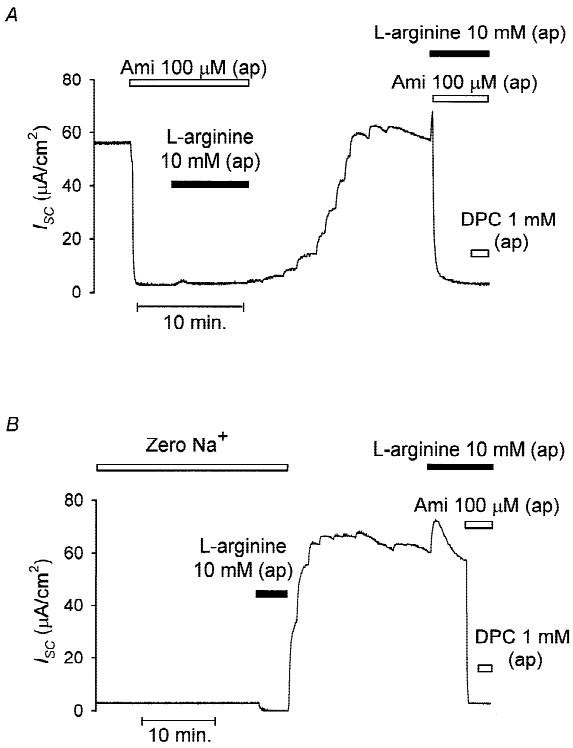

Fig. 5. The stimulatory L-arginine response is blocked in the presence of amiloride and in the absence of extracellular Na^+. (*A*) L-arginine (10 mM) was applied in the presence and absence of amiloride (100 μM). (*B*) L-arginine (10 mM) was applied in the absence (Zero Na^+) and presence of extracellular sodium.

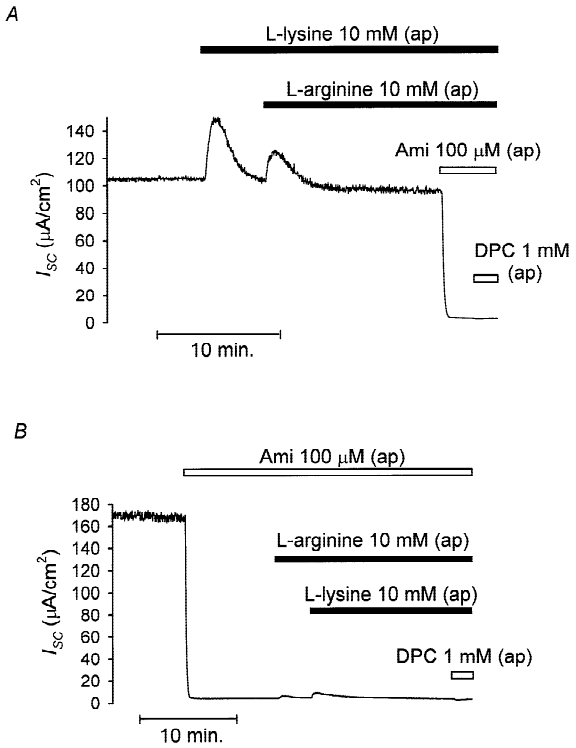

Fig. 6. L-lysine produces a similar I_{SC} response as L-arginine. In *A* the addition of apical L-lysine (10 mM) was followed by apical application of L-arginine (10 mM). In *B* L-arginine (10 mM) and L-lysine (10 mM) were applied in the presence of apical amiloride (100 μM).

observed upon L-arginine addition ($n = 4$). At present we have no explanation for this small decrease of I_{SC} induced by L-arginine in the absence of extracellular Na^+. After re-addition of Na^+ into the apical bathing solution, a second application of L-arginine could elicit the usual stimulatory response increasing I_{SC} on average by 9.1 ± 1.4 μA/cm^2, changing from 57.9 ± 1.0 μA/cm^2 to 67.0 ± 2.3 μA/cm^2 ($n = 4$). Taken together these data provide evidence that the increase in I_{SC} caused by L-arginine is mediated by stimulation of electrogenic Na^+ absorption.

L-Lysine Stimulates I_{SC} in Similar Fashion to L-Arginine

The effect of L-arginine may be mediated through its conversion to nitric oxide (NO) and the subsequent NO stimulation of amiloride-sensitive I_{SC}. Since the cationic amino acid L-lysine is known to be transported similarly to L-arginine (Devés & Boyd, 1998) but cannot be converted to NO, the effect of L-lysine on I_{SC} was tested. Fig. 6*A* shows an experiment where L-lysine (10 mM) and L-arginine (10 mM) were applied apically in succession. L-lysine produced a stimulation of I_{SC} similar to that of L-arginine when applied either apically or basolaterally. The peak I_{SC} increase caused by apical L-lysine averaged 42.2 ± 6.7 μA/cm^2, changing from 100.7 ± 7.7 to 142.8 ± 13.6 μA/cm^2 ($n = 4$). The subsequent L-arginine application stimulated I_{SC} on average by 22.9 ± 2.6 μA/cm^2, changing from 104.7 ± 8.0 to 127.6 ± 10.6 μA/cm^2 ($n = 4$). As shown in Fig. 6*B* the L-lysine effect, as with the L-arginine effect, is almost completely prevented in the presence of amiloride ($n = 2$). Thus, the L-lysine effect is also due to stimulation of electrogenic Na^+ absorption.

NO Synthase Inhibitors had no Effect on the L-Arginine Response

The finding that L-lysine and L-arginine had similar effects on I_{SC} suggests that the L-arginine response is not mediated by increased NO synthesis due to L-arginine uptake and NO synthase activity in M-1 cells. To confirm this, the effect of L-arginine was tested in M-1 cells treated with various NO synthase inhibitors. As shown in Fig. 7*A* application of 100 μM L-NMMA had no effect on the L-arginine response. The peak I_{SC} increase caused, in the presence of basolateral L-NMMA (100 μM), averaged 14.8 ± 1.6 μA/cm^2 ($n = 3$), as compared

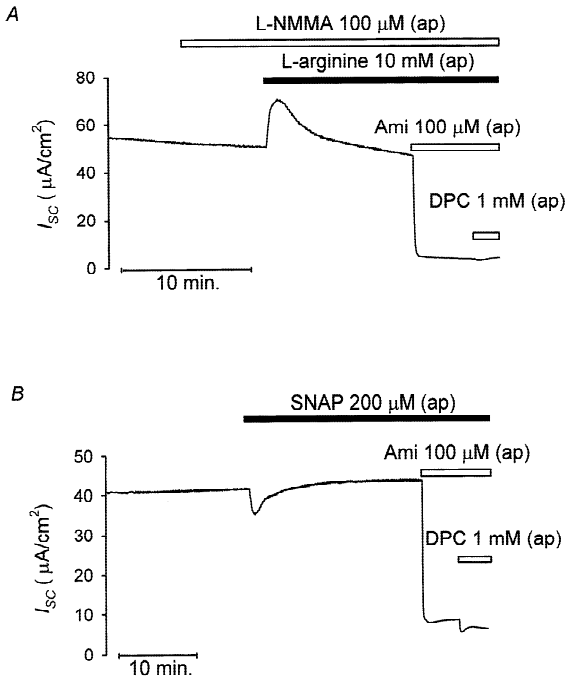

Fig. 7. The stimulatory response to L-arginine is maintained in the presence of a NOS inhibitor while the effect of the NO donor SNAP is different from that of L-arginine. (*A*) L-arginine (10 mM) was applied to the apical bath in the presence of a specific inhibitor for the inducible form of NOS, L-NMMA (100 μM). In *B* the NO donor SNAP (200 μM) was applied to the apical bath.

with time-matched controls of 19.9 ± 2.1 μA/cm² (n = 3). Likewise, in the presence of basolateral L-NAME (1 mM), the I_{SC} increase was preserved averaging 20.1 ± 1.1 μA/cm² (n = 4). These findings are consistent with the interpretation that the L-arginine response is not mediated by NO. Fig. 7*B* further corroborates the suggestion that the effect of L-arginine is independent of NO actions in M-1 cells. In this experiment the application of 200 μM SNAP, a nitric oxide donor, had a different effect on the I_{SC} compared to L-arginine, causing a slight and transient decrease in I_{SC} averaging 4.6 ± 1.0 μA/cm² (n = 3).

Effects of L-Arginine and L-Lysine are Mediated by pH Changes

The similarity of the responses to L-arginine and L-lysine suggested a common mechanism mediating stimulation of amiloride-sensitive Na^+ transport by these two cationic amino acids. L-arginine and L-lysine were used in free base form and therefore will tend to alkalinize solutions to which they are added. Since changes of bath pH may affect the amiloride-sensitive Na^+ transport, the extent of the pH changes elicited by the addition of L-arginine or L-lysine were determined using a pH-sensitive electrode placed in HCO_3^-/CO_2 buffered solution which was continuously gassed with a mixture of 5% CO_2/95% O_2 and was of the same volume and composition as those used in the Ussing chamber experiments (final volume 10 ml; final amino acid concentration 10 mM). Addition of L-arginine produced a rapid transient pH increase from pH 7.4 to about pH 9 with subsequent return towards baseline pH. Addition of L-lysine had a similar effect (*data not shown*).

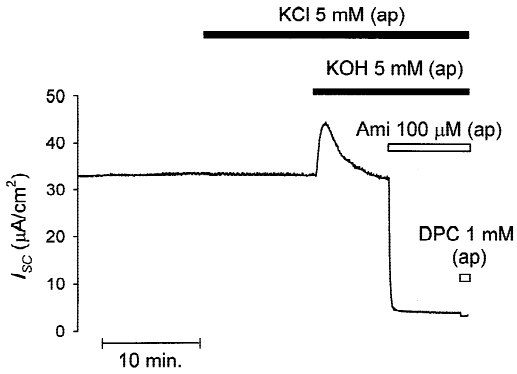

Fig. 8. Alkalinization mimics the response elicited by cationic amino acids. Addition of apical KCl (5 mM) was followed by apical KOH (5 mM) application.

Interestingly, the pH changes induced by the addition of L-arginine or L-lysine had a similar shape and time course as the I_{SC} responses observed upon addition of these amino acids. To test whether a change in bath pH was responsible for the I_{SC} response to L-arginine the effect of KOH addition on I_{SC} was investigated. Figure 8 shows that the addition of KOH (5 mM), which closely mimicked the pH effect of L-arginine (*data not shown*), stimulates a transient increase in I_{SC} similar to that caused by L-arginine, with a peak averaging 8.5 ± 1.4 μA/cm² (n = 4). This response is not due to the addition of potassium ions since addition of KCl (5 mM) had no effect on I_{SC} (Fig. 8). The similarity between the responses of KOH and L-arginine suggests that a concomitant transient alkalinization is the most likely candidate for mediating the I_{SC} response upon addition of L-arginine and L-lysine.

To confirm that pH mediates the effects of L-arginine and L-lysine, 10 mM L-histidine, an amino acid with a pK value of 6.8 was tested. Since L-histidine acts as a weak acid in solution, it transiently acidifies the HCO_3^-/CO_2 buffered solution to about pH 6.7 (*data not shown*) and consequently we would expect an I_{SC} response opposite to that observed with L-arginine or L-lysine. Indeed, Fig. 9*A* shows that L-histidine produced a rapid transient decrease in the I_{SC} which recovered back to baseline within ~10 min. The trough of the I_{SC} decrease averaged 25.9 ± 2.4 μA/cm² (n = 10). Exposure to L-histidine did not affect the subsequent response to L-arginine which increased I_{SC} by 24.8 ± 2.8 μA/cm² (n

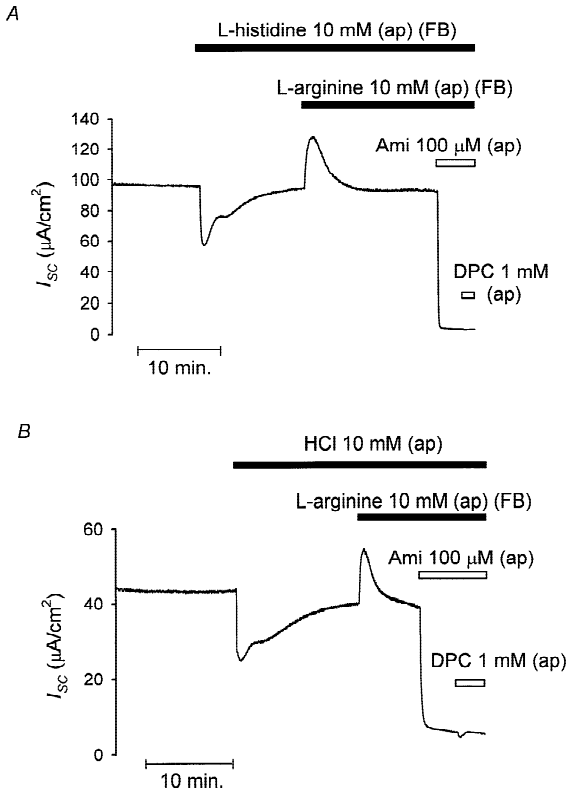

Fig. 9. L-histidine and acidification by HCl has an effect on I_{SC} that is opposite to that elicited by L-arginine. In *A* and *B* responses to apical application of L-histidine (10 mM) and HCl (10 mM) are shown, respectively. Apical L-arginine (10 mM) was subsequently applied for comparison.

= 10). An equivalent HCl (10 mM) addition caused a very similar response to that observed with L-histidine, with a trough I_{SC} decrease averaging 17.5 ± 0.6 μA/cm^2 (n = 4) (Fig. 10*B*). The similarity between the L-histidine and HCl responses further corroborates that the amino acid effects are mediated by changes in pH. Transient alkalinization of the bath solution stimulates amiloride-sensitive Na^+ absorption in M-1 cells while acidification has the opposite effect.

It is well established that changing extracellular P_{CO2} is an effective way of changing pH_i. To establish whether the L-arginine effect was possibly mediated by a CO_2-induced change in pH_i, experiments were performed in which extracellular P_{CO2} was acutely lowered by changing the gas with which the apical solution was bubbled from a mixture of 5% CO_2/95% O_2 to pure O_2 or room air. This maneuver results in an increase of pH_o from 7.4 to about 8.6 and will cause CO_2 efflux and consequently an intracellular alkalinization. A transient increase in I_{SC} by 15.8 ± 1.8 μA/cm^2 (n = 10, $P < 0.01$) was observed upon lowering extracellular P_{CO2} consistent with stimulation of I_{SC} by intracellular alkalinization due to CO_2 efflux. Subsequent return to a gas mixture of 5% CO_2/95% O_2 resulted in a corresponding transient decrease of I_{SC} by 19.9 ± 1.4 μA/cm^2 (n = 6, $P < 0.01$) consistent with inhibition of I_{SC} by intracellular acidification due to CO_2 influx. The I_{SC} component stimulated by lowering extracellular P_{CO2} was amiloride sensitive (n = 5) which indicates that it is due to stimulation of electrogenic Na^+ absorption via ENaC. Taken together these results suggest that addition of L-arginine stimulates I_{SC} by causing a transient intracellular alkalinization and activation of ENaC.

Finally, experiments were performed in which L-arginine was applied while preventing a concomitant change in bath pH. This was accomplished by using a bath solution and an L-arginine stock solution (1 M) which were nominally HCO_3^-/CO_2 free and contained 10 mM HEPES (adjusted to pH 7.4). Under these conditions application of 10 mM L-arginine had no effect on I_{SC} when added either apically (n = 3) or basolaterally (n = 3). These findings demonstrate that in the absence of a pH change addition of L-arginine has no affect on I_{SC}.

Discussion

The two major findings of this study are that M-1 CCD cells express specific apical and basal cationic amino acid transport pathways and that addition of L-arginine stimulates transepithelial sodium absorption via a pH-dependent but nitric-oxide-independent mechanism. Additionally, it was found that NO synthase inhibitors or NO-donors induced only small effects on baseline I_{SC} suggesting that tonic regulation of sodium transport by NO plays but a minor role in these cultured CCD cells.

The amino acid transport systems identified (an apical electrogenic system y^+, and a basal electroneutral system y^+L) act together to generate net (re)absorption of cationic amino acids across this epithelial model of cortical collecting duct. This process is stimulated by the presence of luminal leucine. Physiologically these systems may act to retrieve basic amino acids remaining in the tubular lumen of the CCD, and may also provide substrate for NO synthesis in these cells. We can reasonably infer that the driving force for the net reabsorption we see is the sodium gradient across the basal membrane (created by the basal membrane sodium potassium pump). This will drive the electroneutral exit of cationic amino acid in exchange for sodium coupled neutral amino acid entry into the cell through system y^+L (Kanai et al., 2000).

The second finding of this study is that L-arginine can stimulate amiloride-sensitive Na^+ absorption in M-1 cells. Evidence that L-arginine alters the rate of Na^+ absorption in M-1 cells comes from the finding that the response to the amino acid was maintained in the absence of extracellular Cl^-, but is abolished in the absence

of Na^+ in the apical bath. Furthermore, the response to L-arginine was prevented in the presence of amiloride, a known inhibitor of CCD Na^+ absorption (O'Neil & Boulpaep, 1979), and application of amiloride at the height of the response to L-arginine essentially abolished I_{SC}.

L-arginine is well established as the precursor of NO synthesis (Moncada & Higgs, 1993; Palmer et al., 1988*a,b*). Thus, in many tissues including CCD and thick ascending limb, L-arginine effects have been interpreted as being mediated by NO (Lu et al., 1997*a;* Plato, Shesely & Garvin, 2000). However, the mechanism whereby supplying high concentrations of this amino acid boosts NO production remains controversial (McDonald et al., 1997; Musialek, Paterson & Casadei, 1999).

This study suggests that in M-1 CCD cells, L-arginine does not act via the NO pathway. Firstly L-lysine, a similar cationic amino acid as L-arginine but not involved in the NO pathway, produced a very similar stimulatory I_{SC} response to that of L-arginine. Secondly, the NO donor, SNAP, produced a transient decrease in the baseline I_{SC}. The effect of SNAP suggests that NO causes a different effect in M-1 cells to that of L-arginine, possibly an inhibition of amiloride-sensitive Na^+ absorption consistent with previous studies investigating the action of NO in the CCD (Stoos et al., 1992, 1994, 1995). Thirdly, the application of NOS inhibitors, L-NMMA and L-NAME, did not affect the I_{SC} response to L-arginine.

The finding that L-lysine and L-arginine caused similar responses in M-1 cells suggested that they mediate their effects via the same mechanism. Electrogenic transepithelial amino acid transport is unlikely to contribute to the observed effects since similar I_{SC} responses were elicited when the amino acids were applied either to the apical or basolateral bath. Moreover, the stimulatory effect of L-arginine and L-lysine was dependent on the presence of apical Na^+ and essentially abolished by amiloride, whereas sytem y^+, the apical cationic amino acid transporter in M-1 cells, is not Na^+ dependent (Devés & Boyd, 1998) and is not sensitive to amiloride (Galietta et al., 1998). Quantitatively, the currents generated by electrogenic amino acid transport will be at least three orders of magnitude smaller than those observed after addition of the cationic amino acids. Thus, the observed transient I_{SC} response is probably exclusively due to a stimulation of amiloride-sensitive Na^+ absorption. This stimulatory effect may be mediated by an activation of the basolateral Na^+/K^+-ATPase, or of basolateral K^+ channels, or of ENaC, or by a combination of all three factors.

In this context a pH-mediated stimulation of Na^+ transport is the most likely explanation for the effects of L-arginine and L-lysine which were shown to cause a transient alkalinization upon addition to the bath solution. Indeed, the finding that equivalent additions of alkali (KOH) mimicked the stimulatory effect of L-arginine and of L-lysine support the interpretation that the effect is mediated by transient alkalinization. Furthermore, the inhibitory effect of L-histidine, which causes a transient acidification of the bath solution and inhibition of I_{SC}, was replicated by an equivalent acid (HCl) addition to the bath solution. Taken together these findings indicate that the observed effects of L-arginine and L-lysine on I_{SC} are pH-mediated.

Extracellular or intracellular alkalinization may increase the intracellular calcium concentration by increasing calcium influx via pH-sensitive ECaC channels known to be expressed in the distal tubule (Hoenderop, Willems & Bindels, 2000) or by Ca^{2+} release from intracellular stores (Speake & Elliott, 1998). However, an intracellular calcium increase is unlikely to explain the stimulatory effect of alkalinization on I_{SC} since there is considerable evidence that a rise in intracellular calcium inhibits ENaC possibly via an activation of PKC (Garty & Palmer, 1997).

Patch-clamp experiments using excised inside-out patches of microdissected rat CCD demonstrated a marked reduction of open state probability for ENaC upon cytosolic acidification (Palmer & Frindt, 1987). Recent studies performed in *Xenopus laevis* oocytes, confirmed the pH-sensitivity of heterologously expressed ENaC and showed this to depend on changes in intracellular rather than extracellular pH (Chalfant et al., 1999; Konstas, Mavrelos & Korbmacher, 2000). Addition of L-arginine, L-lysine or KOH to the bath solution alters primarily extracellular pH. However, the removal of protons shifts the HCO_3^-/CO_2 buffering equilibrium ($H_2O + CO_2 \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+$) to the right. The resultant transient reduction in extracellular CO_2 partial pressure leads to CO_2 efflux from the intracellular compartment causing consequent intracellular alkalinization. Since the bath solution is continuously gassed with 5% CO_2, extracellular and intracellular pH subsequently will recover towards baseline values. The same equilibrium mechanism, but in reverse, is active when the acidic amino acid, L-histidine, or HCl is applied extracellularly. The HCO_3^-/CO_2 buffering equilibrium, thus, enables the amino acids to induce intracellular pH changes, which then elicit the observed alterations in transepithelial sodium transport. The effects are consistent with a direct modulation of ENaC by intracellular pH, consistent with the finding that the effect was identical with apical and basal application of cationic amino acids. This interpretation was confirmed by experiments in which extracellular P_{CO2} was acutely lowered by changing the composition of the gas mixture with which the bath solution was bubbled. This maneuver, which results in CO_2 efflux and intracellular alkalinization, was also shown to stimulate I_{SC}.

The pH sensitivity of CCD Na^+ transport is highlighted by this study. It is likely that intracellular pH is an important regulator of the apical Na^+ conductance and may mediate the effects of several hormones (Lyall, Feldman & Biber, 1995). Precautions must be taken when investigating the actions of various agents on Na^+ transport in the CCD, as it is possible that responses seen are due to secondary changes in pH rather than the direct action of the agent. This appears to be the case with L-arginine in M-1 CCD cells leading to the more general conclusion that L-arginine cannot always be relied upon as a tool to investigate the role of the NO pathway. Indeed, in pancreatic beta cells it has been suggested that L-arginine potentiation of glucose-induced insulin secretion occurs independently of NO (Thams & Capito, 1999) and a recent study in the heart demonstrated that changes in extracellular pH rather than NO mediate the chronotropic responses to L-arginine (Musialek et al., 1999).

An interesting aspect of the coexistence of absorptive cationic amino acid transport and amiloride-sensitive sodium absorption in M-1 cells is the recent discovery that a homologue of the early aldosterone-regulated gene product, ASUR4 (Verrey et al., 1999), has been found to be a component of system y^+L (Devés & Boyd, 2000), which we have shown here to be present in the basolateral membrane of M-1 cells. Up to now little attention has been paid to the possibility of, and consequences resulting from, substantial paracellular cationic amino acid leak into the tubular lumen driven by the lumen negative transepithelial potential difference set up by electrogenic sodium reabsorption in the CCD. The fact, that when aldosterone acts to increase Na^+ transport it may also simultaneously increase the capacity for transcellular cationic amino acid retrieval from the lumen, will counteract the net secretion of cationic amino acid that otherwise necessarily would occur. This coordinated balance of cationic amino acid retrieval depending on the rate of aldosterone-regulated Na^+ reabsorption may account for the otherwise unexplained lack of cationic amino acid secretion in the CCD, a nephron segment previously considered to lack amino acid transport (Dantzler & Silbernagl, 1988).

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