Biochimica et Biophysica Acta, 689 (1982) 561–566
Elsevier Biomedical Press

BBA 71292

AMILORIDE STIMULATION OF SODIUM TRANSPORT IN THE PRESENCE OF CALCIUM AND A DIVALENT CATION CHELATOR

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(Received March 3rd, 1982)

Key words: Amiloride stimulation; Short circuit current; Na^+ transport; Epithelial transport; Ca^{2+} dependence; EDTA; Divalent cation chelator; (Toad bladder)

Amiloride in nM to μM concentrations stimulates the short circuit current (I_{sc}) of the toad urinary bladder by as much as 120% when applied in conjunction with apical Ca^{2+} and a divalent cation chelator. A significant decrease in transepithelial resistance (R_t) is observed simultaneously. This response is spontaneously reversible and its amplitude is dependent upon apical sodium concentrations. The stimulated I_{sc} persisted when acetazolamide (1 mM) was introduced, HPO_4^{2-} substituted for HCO_3^- or SO_4^{2-} replaced Cl^-. Consequently, the increase in I_{sc} is not due to the change of Cl^-, H^+ or HCO_3^- flux. This behavior in a 'tight' epithelium may be related to the mechanism controlling apical sodium permeability.

Introduction

Amiloride, calcium and EDTA have marked effects on the transepithelial voltage (V_t) and short circuit current (I_{sc}) of the toad urinary bladder. Calcium ions were first believed to inhibit vasopressin-stimulated H_2O permeability [1] without influencing I_{sc} [2] in the tissue. More recently, it has been demonstrated in frog skin and toad bladder that elevated calcium concentrations in the apical solution inhibit both Na^+ and Cl^- flux [3,4,5]. Increasing calcium in the basolateral solution reduces both basal and hormonal mediated H_2O permeability and Na^+ transport [5,6]. Serosally applied EDTA increases the bidirectional flux of Na^+ in amphibian skins [7]. Toad bladders lose transepithelial resistance as V_t and I_{sc} fall to zero when Ca^{2+} is withdrawn from the mucosa and serosa, causing the luminal cells to separate. The adhesion of mucosal cells to one another is maintained, in part, by a process involving Ca^{2+} [8]. In the electrically 'leaky' rat jejunum, EDTA (5 mM) produced a transient increase in V_t with little change in transepithelial resistance (R_t), and Ca^{2+} (5 mM) potentiated the effect. This enhancement of Na^+ conductance was thought to reflect changes in the epithelial resistance by paracellular pathways [9]. Amiloride inhibits sodium conductance and depolarizes V_t [10]. Although calcium was once thought to be required for amiloride inhibition [11], this dependence varies among species and tissues [12]. In the present case, when serosal Ca^{2+} is kept constant at 0.95 mM, we have observed the concerted effect of apical amiloride, calcium and EDTA in producing a transient increase in V_t and the sodium component of the I_{sc} through the toad urinary bladder. This phenomenon is apparently related to changes in the permeability of the apical barrier to sodium. It should be noted that this response is more pronounced in winter-spring than summer-fall toads.

Abbreviation: EGTA, ethyleneglycol bis(aminoethyl ether)-*N*,*N*'-tetraacetic acid.

0005-2736/82/0000–0000/$02.75

Materials and Methods

Bufo marinus of Mexican origin were purchased from Riverside Biological (Somerset, WI) and maintained on Sanicel moistened with 10% amphibian Ringers. Except for the summer, toads were not fed or kept in the lab for more than one month. During July and August, the anurans were fed minced liver every three weeks.

Urinary bladders were removed from pithed toads and mounted in either a standard Ussing apparatus or a modified chamber that minimized edge damage to the epithelial preparations [13]. In the standard Ussing apparatus, hemibladders were isolated into control and experimental portions. I_{sc} was determined by periodically interrupting open circuit conditions. In the modified chamber, changes in the V_t were observed in response to square pulses of current between 1.0 and 11.0 $\mu A/\pi$ cm^2 (duration = 1.0 s). The I_{sc} and R_t were calculated from V_t displacement. Tissue surface area was 3.4 and π cm^2 for the standard Ussing apparatus and modified chambers, respectively. All results are reported as $\bar{X} \pm$ S.E. Experiments were conducted at room temperature (19 to 25°C).

These preparations were bathed in Ringers consisting of the following (mM): Na^+ (109), K^+ (2.5), Ca^{2+} (0.95), Cl^- (110.9), HCO_3^- (2.5) and glucose (5). They were gassed with 95% O_2/5% CO_2 giving a bathing solution pH between 6.2 and 6.5. Various concentrations of Na^+ were achieved by choline substitution. Sodium salts of HPO_4^{2-} and SO_4^{2-} were used to replace HCO_3^- and Cl^- anions, respectively. To replace apical Ca^{2+}, 0.5 mM EDTA or EGTA was added in its place. Ca^{2+} was always present in the seroal solutions. Usually the disodium salts of the chelators was used, but Na^+-free solutions, the free acid was employed. Acetazolamide was purchased from Sigma Chemical Company. Amiloride was a gift of Merck, Sharp and Dohme.

Results

In Table I, it is shown that calcium levels in the apical medium influence the bioelectric parameters of epithelia recorded in the modified chamber. In the presence of 9.5 mM apical Ca^{2+}, there is a significant ($P \leq 0.001$) reduction in V_t and I_{sc} while R_t is increased. Although the chelators reduce the V_t significantly ($P \leq 0.001$), they have little effect on tissue R_t and I_{sc}. In general, the R_t and I_{sc} of the bladder are not significantly altered if they are recorded in either 0 or 0.95 mM Ca^{2+} or while Ca^{2+} is replaced by EDTA or EGTA in the apical solution. Probit analysis [14] was used to determine the median effective dose (ED_{50}) of amiloride inhibition under each of these Ca^{2+} conditions (Fig. 1). The ED_{50} values did not vary significantly by Student's *t*-test. Consequently, the I_{sc} is inhibited by amiloride in the presence of apical calcium (0, 0.95, 9.5 mM) or a calcium chelator (EDTA or EGTA) with equal efficiency.

On the other hand, if amiloride concentrations between 10^{-9} and 10^{-3} M are applied to the apical membrane in a medium containing both 0.95 mM Ca^{2+} and 0.5 mM chelator, there is a transient increase in V_t and I_{sc} as the R_t is diminished

TABLE I

EFFECTS OF APICAL CALCIUM AND CHELATORS ON THE BIOELECTRIC PROPERTIES OF THE TOAD URINARY BLADDER

Serosal calcium 0.95 mM.

mM	N	V_t (mV)	R_t(k$\Omega \cdot \pi$cm^2)	I_{sc} ($\mu A/\pi$ cm^2)
9.5 Ca^{2+}	6	9.3±3.3	8.67±1.69	11.1±2.1
0.95 Ca^{2+}	7	38.3±8.4	4.85±0.53	25.2±4.2
0	9	36.5±1.5	3.51±0.41	21.1±5.3
0.5 EDTA	4	20.2±5.9	5.40±1.06	22.6±3.4
0.5 EGTA	6	25.9±5.9	4.26±0.73	21.0±5.4
0.5 Chelator+0.95 Ca^{2+} [a]	12	21.5±3.5	5.11±0.30	19.7±2.6

[a] Free Ca^{2+} = 0.4 mM by Orion Ca^{2+}-selective electrode.

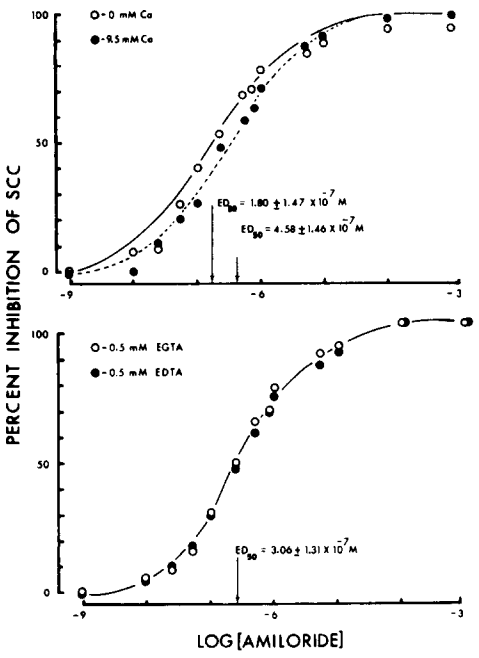

Fig. 1. Response of I_{sc} to apical amiloride in molar concentrations from 10^{-9} to 10^{-3}. Serosal Ca^{2+} = 0.95 M. Upper graph: 0 and 9.5 mM apical calcium. Lower graph: apical EDTA and EGTA in Ca^{2+}-free Ringer. ED_{50} varies between 180 and 450 nM amiloride. SSC, short circuit current.

(Fig. 2). Although not shown in the tracing, this response persists for 5 to 10 min if the solutions are not changed. Afterwards the V_t and I_{sc} decline to baseline. Depolarization and I_{sc} inhibition follow in a short time. If greater amiloride concentrations are applied under identical conditions, depolarization and increased R_t are observed immediately. Both the enhancement and the inhibition of I_{sc} are reversible when amiloride is washed away and fresh Ringers is used to bathe the tissue.

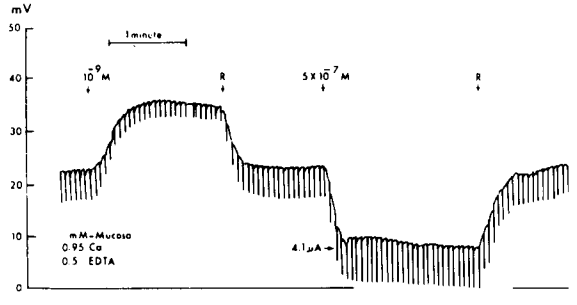

Fig. 2. Recording of amiloride stimulation and inhibition of V_t in toad urinary bladder. In the presence of apical EDTA and Ca^{2+}, V_t is response to applied current of 4.1 $\mu A/\pi$ cm^2. R, rinse with amphibian Ringers without amiloride. Serosal calcium = 0.95 mM.

The response of the epithelium to concentrations of amiloride between 10^{-9} and 10^{-3} M in the presence of 0.95 mM Ca^{2+} and 0.5 mM EDTA is shown in Fig. 3. These data were collected at the peak effect of amiloride stimulation or inhibition of I_{sc}. The ohmic behavior of the epithelia was observed in 10.9 mM Na^+, choline substituted apical Ringers. Apical concentrations of amiloride less than $5 \cdot 10^{-7}$ M increased I_{sc} and V_t above basal. On the average, the V_t and I_{sc} were increased by 120% with 10^{-9} M amiloride. The R_t was always reduced over the same range of amiloride concentrations. Above $5 \cdot 10^{-7}$ M inhibition of V_t and I_{sc} and increased R_t were always noted.

The amplitude of the stimulated I_{sc} is directly related to the concentration of sodium in the apical solution. In Fig. 4, the I_{sc} was recorded in

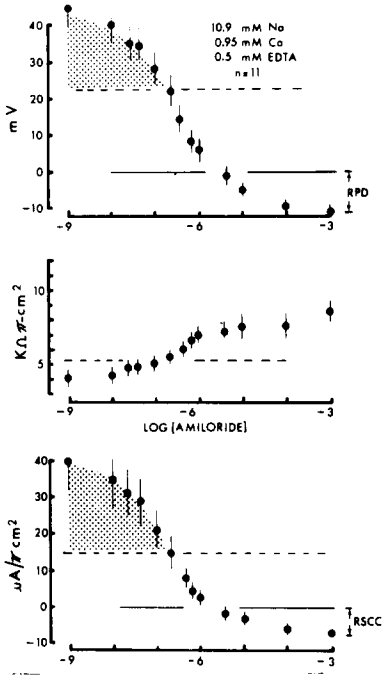

Fig. 3. Electrical response of toad urinary epithelium with apical amiloride, calcium and EDTA. Changes in V_t, R_t and I_{sc} in relation to amiloride concentrations between 10^{-9} and 10^{-3} M. Dashed line, basal V, R, I; stippled line, enhanced region of V, R, I. RPD, reversed polarity p.d. RSCC, reversed polarity I_{sc}. Serosal calcium = 0.95 mM.

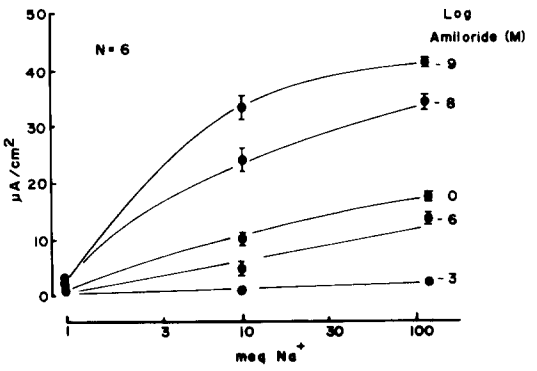

Fig. 4. Dependence of enhanced I_{sc} on apical sodium and amiloride concentration in the presence of acetazolamide 1 mM. Serosa with complete Ringers. See text for complete discussion.

bladders exposed to a sodium regimen of 0, 1, 10, or 109 mM with 0.95 mM Ca^{2+} and 0.5 mM EDTA. The collection of points forming the '0-amiloride' curve represents the basal I_{sc} in the experiments. Inhibition of I_{sc} at each Na^+ concentration is expressed by the 10^{-6} and 10^{-3} M amiloride curves. As long as sodium is present in the apical medium, the I_{sc} is inhibited by amiloride. On the other hand, the amplitude of stimulated I_{sc} observed with 10^{-9} and 10^{-8} M amiloride is dependent upon apical Na^+ concentration. An enhanced I_{sc} is not observed without sodium. This supports the notion that the increased I_{sc} and V_t observed under these conditions are related to the transepithelial movement of sodium. For reasons indicated below, these experiments were conducted in the presence of 1 mM acetazolamide.

To eliminate the possibility that the stimulatory phenomenon is related to changes in the conduction of other ions, inhibitor and replacement experiments were conducted. In the presence of 1 mM acetazolamide, the reversal of I_{sc} is abolished while the stimulation of I_{sc} persists. This is illustrated in Fig. 5 as well as Fig. 4. Likewise, if mucosal Cl^- is replaced by SO_4^{2-} and HPO_4^{2-} substituted for HCO_3^-, the enhanced I_{sc} persists (Fig. 5). Although acetazolamide appears to inhibit I_{sc}, experiments with a standard Ussing chamber revealed that the inhibition of reverse I_{sc} with acetazolamide actually increase net I_{sc} by about 25%. This is shown in Fig. 6. The reason for variance in maximal I_{sc} between experiments in Fig. 5 is not known. Consequently, the amiloride, Ca^{2+},

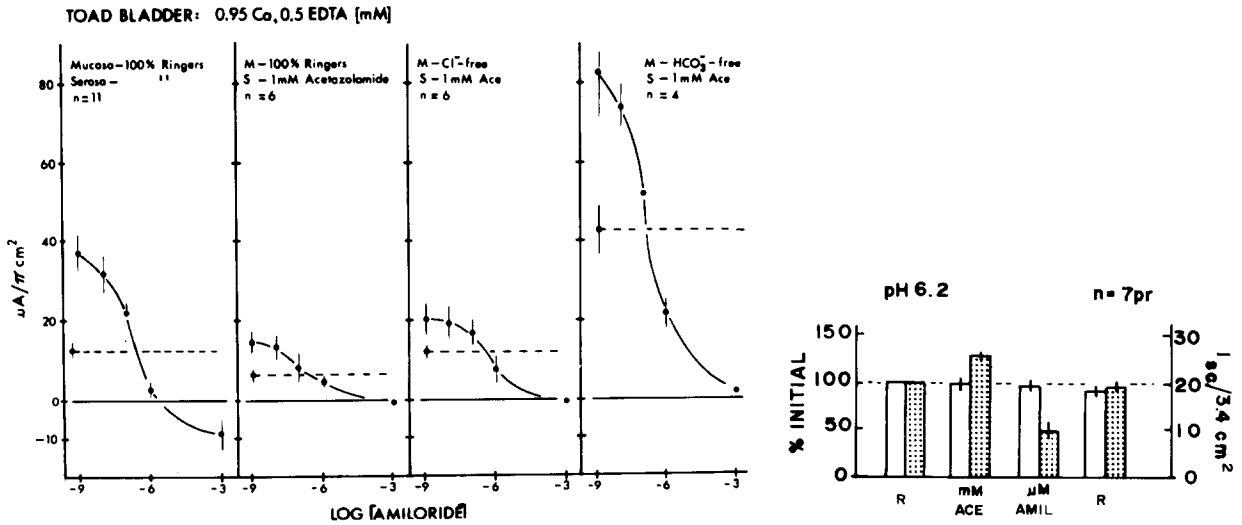

Fig. 5. Effects of acetazolamide treatment and apical ion replacement on stimulated I_{sc}. Dashed line, basal I_{sc}. Ace, acetazolamide. Serosa with complete Ringers.

Fig. 6. Net I_{sc} response to acetazolamide. Ace, 1 mM serosal acetazolamide; Amil, 1 μM apical amiloride; R, amphibian Ringers. Open bars, untreated control portions; stippled bars, experimental portions of toad hemibladders.

EDTA stimulated I_{sc} is not related to changes in Cl^-, H^+ or HCO_3^- flux through the epithelium.

Discussion

Although amiloride [10,12] and Ca^{2+} [3,4,5,6] have long been recognized as inhibitors of I_{sc} in the amphibia epithelia, their concerted effect in the presence of a chelator is to stimulate I_{sc} transiently under the specific conditions reported here. A precise description of the mechanism by which the I_{sc} enhancement occurs is not known. Since Ca^{2+} plays multiple roles in the regulation of cellular activity, several possibilities may be suggested. First, calcium influences directly the molecular architecture and properties of epithelia and cellular membranes [7,8,15]. Treatment of epithelial with EDTA may increase ion permeability. Alterations in extracelular Ca^{2+} concentration may modify plasmalemma structure and change Na^+ permeability. Second, intracellular Ca^{2+} is important in regulating a variety of cytoplasmic functions [16–18]. Consequently, alterations in intra- and extra-cellular Ca^{2+} relationships may lead to modifications in metabolism. Subsequently, the amount of energy available for cellular functions is changed. And third, the $(Na^+ + K^+)$-ATPase activity may be directly related to divalent cations [19,20]. If divalent antagonism of the sodium pump is alleviated by chelation, the ATPase may be activated by lowered concentrations of Ca^{2+} or Zn^{2+}. Any of these may explain the phenomenon observed in these experiments. None of these hypotheses have been examined by detailed experimentation.

Calcium has a profound effect on the structural integrity of amphibian epithelia [7,8]. In the presence of 1.5 to 2.0 mM EDTA, removal of Ca^{2+} from both the mucosal and serosal media reduces cell-cell adhesion and disrupts the epithelial barrier in the toad bladder and has been used to isolate intact cells from the mucosal lining [21]. Under these conditions, as indicated by oxygen consumption experiments, active sodium transport in the separate cells procedes regardless of calcium availability and continues to respond metabolically to vasopressin [8]. Since calcium was always available in the serosal medium to prevent disaggregation of the epithelium in the present investigation, the behavior of the tissue in our experiments is not due either to alterations in intercellular adhesion or to modifications of the sodium pump within the cell. Disruption of intracellular adhesion would have reduced R_t along with V_t and I_{sc} and not been spontaneously reversible.

We tentatively propose that the best explanation for the simulation of I_{sc} under these conditions may lie in the interaction of amiloride with structures on the apical cellular membrane of the tight epithelium. The sodium gating mechanism is envisioned as a integral protein pore [22]. The conformational status of the structure may be regulated by a variety of cations [23]. Initially it was reported that amiloride competition with Na^+ produced transport inhibition at an apical pore site [24]. However, recent kinetic analysis of the inhibition process revealed that a combination of both competitive and non-competitive interference is involved [25]. We believe that in the presence of both Ca^{2+} and EDTA, amiloride inhibition is partially antagonized and transiently converted to stimulation. Both Cl- and NH_2-moieties on the heterocyclic ring of amiloride are required for inhibition of the apical sodium pore to occur. In fact, an analogue with $(CH_3)_2$ substituted for H^+ at 5′-NH_2 stimulates Na^+ flux and I_{sc} in the amphibian skin [26]. In addition, the spacial conformation of the apical Na^+ pore may be enzymatically regulated. Micromolar concentrations of Ca^{2+} induce proteolyis in certain membrane preparations [27] resulting in increased glutamate binding by membrane proteins. These glutamate loci which were previously inaccessible to the substrate are presumed to be unmasked by conformational changes in cytoskeletal-associated proteins. The apical surface of the toad urinary bladder contains a proteolytic enzyme that is inhibited by amiloride [28]. It would appear reasonable to postulate that the increased I_{sc} in the presence of Ca^{2+}, EDTA and amiloride may be due to changes in apical proteins as a result of a chemical equilibria between free Ca^{2+}, EDTA-bound Ca^{2+} and membrane-bound Ca^{2+}. The transient increase in Na^+ conductance may be a response of a proteolytic mechanism which regulates the structure of the Na^+ pore and its amiloride sensitivity (i.e. the Cl- and NH_2-binding loci) to micromolar Ca^{2+} concentrations. From the present investigation, we

conclude that different concentrations of calcium or a divalent cation chelator alone have little or no effect on sodium currents through the toad urinary bladder. However, they appear to alter the specific structure of the sodium gate in such a way that amiloride becomes a stimulator when all three are simultaneously present in mucosal solutions. The transient nature of this phenomenon remains unexplained.

Acknowledgements

This research was supported by USPHS, NIH Grant HL-07357 and the National Kidney Foundation of Northwest Ohio. We gratefully appreciate the technical assistance of Anne M. Trinchero.

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