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Rapid Effects of Aldosterone on Sodium-Hydrogen Exchange in Isolated Colonic Crypts

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Abstract. Aldosterone plays a central role in the homeostatic regulation of extracellular fluid volume by stimulating transepithelial electrolyte transport. These effects involve binding to an intracellular receptor, modification of genomic events and protein synthesis. Rapid cellular responses to steroid hormones have been observed in a variety of nonepithelial tissues. The term "nongenomic" has been proposed for these fast steroid responses since they are unaffected by inhibitors of protein synthesis. We hypothesized that colonic crypts, recently demonstrated to absorb fluid, would respond rapidly to aldosterone.

Cytoplasmic pH changes in crypts loaded with a pH-sensitive, fluorescent dye (BCECF) were recorded with confocal laser imaging. An intracellular alkalization of colonic crypts was observed within one minute of aldosterone application that was inhibited by ethylisopropylamiloride or the absence of extracellular sodium, yet unaffected by inhibitors of protein synthesis. The genesis of this rapid and distinct steroid action involves a signal transduction pathway that involves G proteins, protein kinase C, and prostaglandins.

We have identified, by real-time imaging, a nongenomic upregulation of sodium-hydrogen exchange in colonic crypts by aldosterone that occurs independent of the traditional receptor. This distinct, rapid onset effect of aldosterone on epithelial ion transport has major implications for our understanding of fluid and electrolyte homeostasis in health and disease.

Key words: Steroid — Sodium-hydrogen exchange — Prostaglandins — Protein kinase C

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Introduction

The traditional mode of action for steroid hormones involves binding to an intracellular receptor (Class 1 receptor) and translocation of the hormone-receptor complex to the genome. The initiation of hormone action, via subsequent modification of deoxyribonucleic acid (DNA) transcription, messenger ribonucleic acid (mRNA) translation and protein synthesis, occurs following a lag of sixty minutes [43, 45]. These transcriptional changes are too slow to account for some of the effects that occur within minutes of hormone application. Studies of human mononuclear lymphocytes have shown effects of aldosterone within minutes [12]. Similarly rapid aldosterone responses have been observed in frog skin epithelium [51], rat cortical collecting tubule [20], and colonic mucosa [16, 30]. The term "nongenomic" has been proposed for these fast aldosterone responses since they are neither affected by inhibitors of DNA transcription and mRNA translation nor inhibited by competitive antagonists of the Class 1 aldosterone receptor. The rapid effects of aldosterone have been attributed to a functionally distinct binding site (possibly on the cell membrane) termed the Class 2 receptor [19]. A recent demonstration of increased erythrocyte sodium-hydrogen exchange activity in patients with primary hyperaldosteronism provides further evidence for nongenomic effects of aldosterone [27].

The natriferic steroid hormone aldosterone plays a central role in the homeostatic regulation of extracellular fluid volume and stimulates colonic electrolyte transport [57]. Mammalian colon is a major target for aldosterone and the level of mineralocorticoid receptor gene expression is higher in the distal colon than in other target tissues including the kidney [18]. In rat distal colon, aldosterone causes a switch from an electroneutral NaCl

absorption to stimulated electrogenic Na^+ absorption by inducing apical amiloride-sensitive Na^+ channels and enhancing basolateral Na^+/K^+/ATPase pump activity [4]. In parallel with this aldosterone-induced alteration of Na^+ transport, net K^+ absorption in the rat distal colon is converted to net K^+ secretion by aldosterone, which reflects, at least in part, the induction of apical K^+ channels [49]. These effector mechanisms involve binding of aldosterone to intracellular, type I mineralocorticoid receptors and the stimulation of genomic events.

Transepithelial electrogenic sodium transport is dependent upon the permeability of apical membrane Na^+ channels and the activity of Na^+/K^+ ATPase pumps on the basolateral membrane. In tight epithelia, aldosterone increases both the permeability of the apical membrane to Na^+ and the activity of the basolateral membrane Na^+/K^+-ATPase [21, 38]. To maintain the favorable electrogenic gradient for sodium absorption a recycling of potassium ions through basolateral membrane channels must occur [38, 51]. It has been proposed that a reduction in intracellular H^+ ion concentration (increase in pH_i) by an upregulation of sodium-proton exchange (Na^+/H^+ exchange) is responsible for activation of the potassium channels [30, 38, 51]. Many of the ion transport pathways affected by aldosterone, including membrane ion channels, display sensitivity to fluctuations in intracellular H^+ ion concentrations (pH_i) [38, 54]. This provides a plausible hypothesis for rapidly responsive signaling by aldosterone via an alteration of pH_i for facilitation of electrolyte transport.

We examined this hypothesis by observing the effect of acute aldosterone exposure on colonic crypt cells. By imaging crypt cells loaded with a pH-sensitive, fluorescent dye real-time observations of pH_i changes in response to aldosterone at physiological doses was possible. We found that the fluorescent emission intensity of BCECF-loaded colonic cells increased within one minute of aldosterone application. The aldosterone-induced cytoplasmic alkalization was abrogated by ethylisopropylamiloride or the absence of extracellular sodium, and was unaffected by the nominal absence of bicarbonate from the perfusate or an inhibition of anion exchange. The response of the cytoplasmic pH was neither reduced by inhibitors of DNA transcription and mRNA translation, nor the classical receptor antagonist spironolactone. We believe that this indicates an upregulation of a membrane Na^+/H^+ exchange by aldosterone that (i) is too rapid to be mediated by a genomic mechanism; (ii) occurs independent of the traditional receptor; and (iii) is transduced by a signaling pathway independent of protein synthesis.

Materials and Methods

Chemicals

BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein-acetoxy-methylester) was obtained from Molecular Probes (Portland, OR). All other drugs and chemicals were purchased from Sigma Chemicals.

Tissue Preparation

Male Sprague-Dawley rats (100–180 grams) that had been fed on a normal diet were euthanized with ether. The distal colon was immediately harvested and exposed to a calcium chelation solution (composition in mM: NaCl 96, KCl 1.5, HEPES/Tris 10, NaEDTA 27, Sorbitol 45, Sucrose 28) for 30 min at room temperature. A pellet of isolated crypts was formed by centrifugation at 200 revolutions per min for 1 min and was resuspended in a Hepes buffered Ringers solution (HBRS) at pH 7.4 (composition in mM: NaCl 140, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, Na-HEPES 6.0, Glucose 11.1). Where indicated, the bathing solution sodium concentration was reduced to 0 mM by replacement of NaCl with N-Methyl-*d*-Glucamine (NMDG) (composition in mM: NaCl 0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 32.2, Glucose 10.5, NMDG 120). Experiments under conditions of bicarbonate (HCO_3^-) were performed at pH 7.4 in a 5% CO_2-bubbled, HCO_3^--buffered solution (composition in mM: $NaHCO_3^-$ 22, NaCl 125, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 11.1). A total of 40 distal colonic segments from 40 animals were included in this study.

Fluorescent Dye Loading

Isolated crypts bathed in HBRS were attached to 24 × 40 mm coverslips (VWR Scientific) coated with Cell-Tak® in perfusion chambers. Crypt cells were subsequently incubated in HBRS containing 3 μmol/L 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluoroscein-acetoxymethyl ester (BCECF-AM) at room temperature for 30 min. Cytoplasmic esterase hydrolysis of the acetomethoxy-group results in conversion of the dye, to the pH-sensitive, fluorescent form of the dye BCECF within the cell [41]. Following dye-loading, residual BCECF-AM in the crypt bathing solution was removed by perfusing the attached crypts with fresh HBRS for 5 min prior to the start of the experiment.

Imaging

Dye-loaded crypts were imaged with a laser scanning confocal microscope (inverted LSM 410, Carl Zeiss, Thornwood, NY) using a 40 × 1.4 oil immersion lens with infinity corrected optics. Dye molecules were excited with an argon laser at a wavelength of 488 nM and emission was recorded in the wavelength range of 515–565 nM (single excitation/single emission). Experiments were performed at room temperature to maximize crypt viability and minimize dye leakage. Greater than 95% of all crypts in this study demonstrated a stable basal cellular fluorescent emission intensity with structural integrity and metabolic viability. The system was calibrated in vitro using a H^+/K^+ exchange ionophore (nigericin at a concentration of 1 μg/ml) solubilized in buffered solutions of varying pH as described previously [33].

Data Acquisition and Analysis

Experimental images were recorded to the hard drive of the Zeiss Confocal workstation. Fluorescent emission intensity was quantified in terms of the pixel intensity recorded from a defined area (250 μM^2). Recordings were acquired from three separate cells from each of the base, middle and upper portions of the crypts (total of nine recordings per crypt) in each image. Results were processed using a statistical software package (Statview) and a Student's *t*-test was employed for

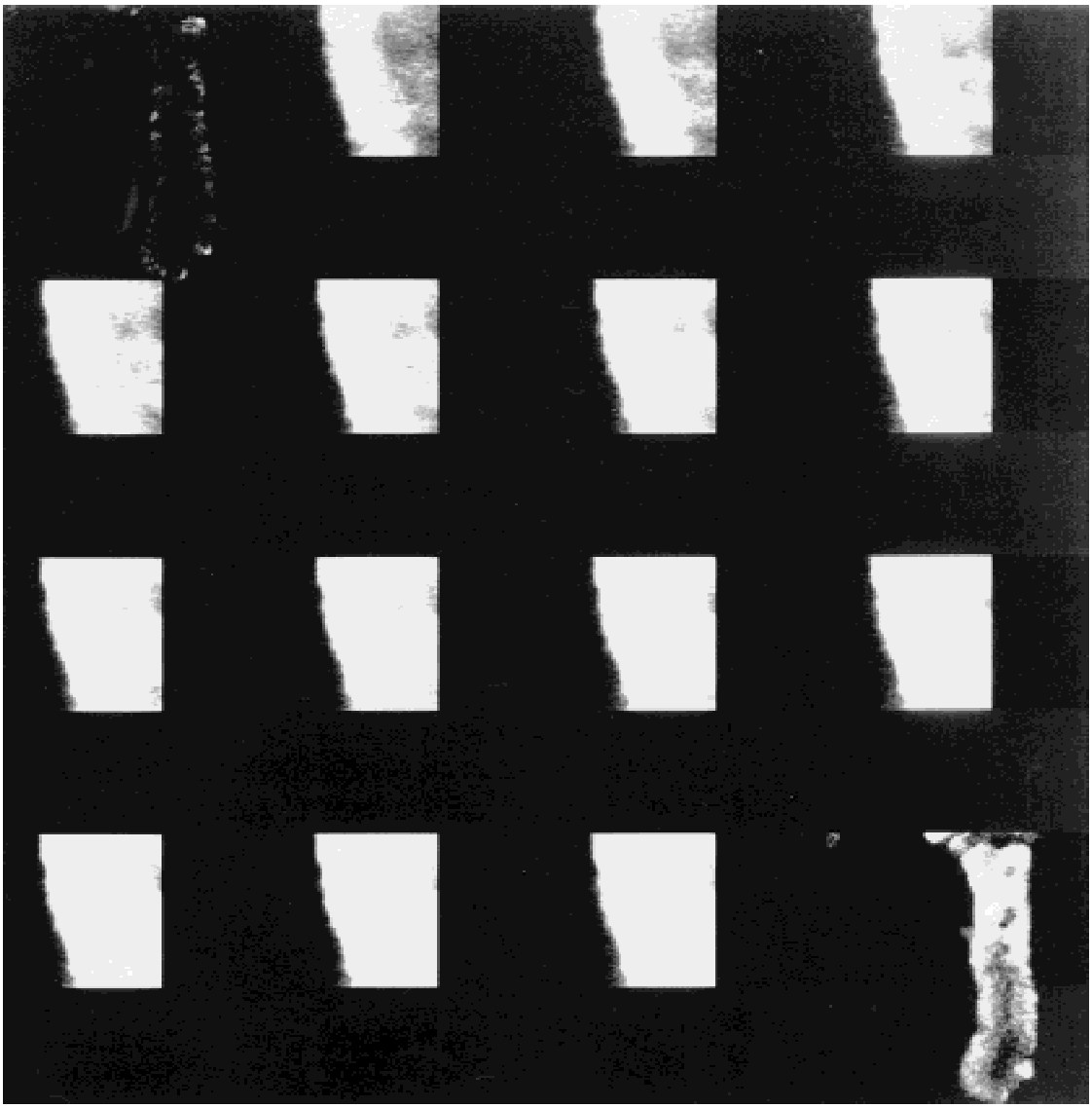

Fig. 1. This composite of confocal laser microscopic images from a representative experiment illustrate the rapid effect of aldosterone (0.1 nM) on colonic crypt pH. The first image is of the BCECF-loaded crypt at the beginning of the experiment. The subsequent zoom images (from left to right) are taken at the basolateral membrane of two cells on the left side of the lumen in the upper third of the crypt. The first image is prior to aldosterone exposure and each image is taken at 15-sec intervals thereafter. Aldosterone increases cellular fluorescent intensity (alkalization) within 1 min. The final image demonstrates that the entire crypt responds to aldosterone.

analysis, where $P < 0.05$ was considered significant. Results are expressed as percentage change of basal fluorescent intensity (% F.I.) presented as mean ± SEM, indicating the dose of aldosterone in each case. The experimental number is indicated in each case where n = number of cell recordings × number of crypts × number of animals.

Results

BCECF-loaded crypts displayed a steady basal intensity that corresponded, using the nigericin technique for calibration, with a cytoplasmic pH of 7.11 ± 0.08. An increase in cellular fluorescent emission intensity was observed within one minute of aldosterone exposure (% F.I. at one minute = 144.7 ± 8.29%; n = 270; 0.1 nM aldosterone) throughout the longitudinal axis of crypts (Fig. 1). A stable plateau was reached within 13.8 ± 0.62 min (% F.I. = 223.6 ± 26.5%; n = 270; 0.1 nM aldosterone) (Fig. 2). This represented an absolute change of 0.17 ± 0.38 pH units (mean ± SD) when calibrated with nigericin. A dose-dependence over a range of hormone concentrations from 0.005 to 100 nM was demonstrated with an EC_{50} of 0.8 nM (Fig. 3). There was a slight heterogeneity to the degree of alkalization observed along the crypt axis, in that the maximal response of the middle and upper one-third of crypts was greater than that of the basal third. However, comparisons of inter-

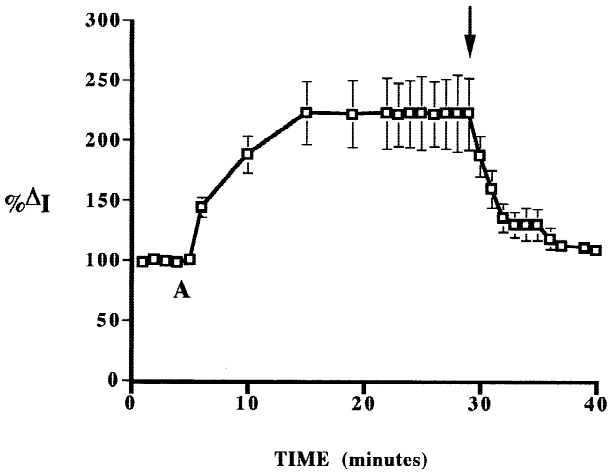

Fig. 2. Graphical representation of experimental recordings of changes in fluorescent intensity, expressed as a percentage of basal (% F.I.), from a BCECF-loaded crypt exposed to 0.1 nM aldosterone. Time points represent percentage of basal intensity in mean ± SEM. A stable fluorescent intensity is observed under basal conditions. Perfusion of aldosterone (marked point A) rapidly increases the fluorescent intensity (indicating a cytoplasmic alkalization) to a higher plateau. This is inhibited by the introduction of 200 μM amiloride into the perfusate (marked with an arrow) indicating a dependence of this aldosterone effect on sodium-hydrogen exchange.

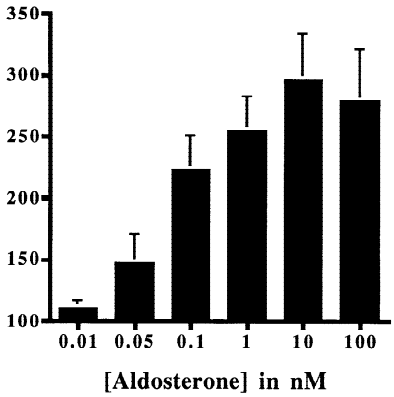

Fig. 3. The dose-response characteristics of the rapid effect of aldosterone on cytoplasmic pH in isolated colonic crypts. The alkalization (increase in intensity) of crypt cells exposed to aldosterone is dose dependent with an EC_{50} of 0.8 nM. The range of doses tested (5–100 nM) indicate responses within the physiological range (0.08–0.2 nM) of plasma aldosterone under normal circumstances (*see* text). Demonstrable effects of aldosterone at higher doses is not surprising because the plasma levels exceed 1 nM under conditions of primary or secondary hyperaldosteronism. Data indicate maximal change in intensity, expressed as a percentage of basal (% F.I.), in mean ± SEM for $n = 108$ in each case.

segmental variations failed to achieve statistical significance.

The aldosterone-induced alkalization occurred in the presence (HCO_3^- buffered Ringers solution) or nominal absence (CO_2-free HEPES buffered Ringers solution) of bicarbonate (% F.I. = 220.1 ± 29.3% *vs.* 228.7 ± 24.1%, respectively; $n = 72$ at 0.1 nM aldosterone for each; $P > 0.4$). In separate experiments anion exchange was inhibited with 200 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) without adversely influencing the change in pH_i (Fig. 2). However, the increase in pH_i was inhibited with 200 μM amiloride (% F.I. = 133.7 ± 10.7%; $P < 0.01$, $n = 198$; 0.1 nM aldosterone) and further with 1 mM amiloride (% F.I. = 109.3 ± 3.6%; $P < 0.01$, $n = 72$; 0.1 nM aldosterone) (Fig. 2). Since amiloride is an inhibitor of both Na^+/H^+ exchange and sodium channels (the latter effect may depolarize the cell and effect transport function indirectly), 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), an analogue with a higher specificity for Na^+/H^+ exchange [52], was employed in separate experiments to inhibit the response to aldosterone. EIPA effectively abrogated aldosterone-induced changes in intracellular pH at doses of 10 μM (% F.I. = 122.5 ± 6.9%; $P < 0.01$, $n = 124$; 0.1 nM aldosterone) and 50 μM (% F.I. = 107.8 ± 4.7%; $P < 0.01$, $n = 124$; 0.1 nM aldosterone). When crypts were bathed in a sodium-free solution (sodium replaced with N-methyl-*d*-glucamine$^+$), a reversible cytoplasmic acidification was observed as a result of a reversal in the direction of Na^+/H^+ exchange and a limitation of sodium-dependent H^+ extrusion [2]. Under these experimental conditions, no significant alkalization of crypt cells occurred in response to aldosterone (Fig. 4). These experiments demonstrate that Na^+/H^+ exchange mediates the rapid alkalization of the crypt cells in response to aldosterone.

The traditional, protein synthesis-mediated mechanism of aldosterone action is inhibited by competitive antagonists of the mineralocorticoid receptor (e.g., spironolactone and canrenone). Spironolactone did not antagonize the effect of aldosterone on pH_i in this study (% F.I. = 224.3 ± 15.2%; $P > 0.5$; $n = 108$; 0.1 nM aldosterone) even at a dose 10,000 times that of aldosterone (1 μM). Abrogation of genomic influences, by inhibiting DNA transcription and mRNA translation with actinomycin D (50 μg/ml) and cycloheximide (20 μg/ml) respectively, did not influence the effect of aldosterone on pH_i (Fig. 5). This indicates that the action of aldosterone on crypt pH_i is independent of classical aldosterone (or mineralocorticoid) receptors and is mediated by a signal transduction mechanism other than protein synthesis. We examined the effect of synthetic steroids on crypt cell pH_i to evaluate the specificity of the aldosterone response. Neither dexamethasone (500 nM–1 μM) nor hydrocortisone (500 nM–1 μM), two synthetic steroids that exhibit mineralocorticoid and glucocorticoid responses in vivo, caused a change in the intracellular pH over 40 min ($n = 108$; $P > 0.2$). This is further evidence of a distinct signaling pathway specific to aldosterone that is mediated independent of mineralocorticoid receptor ligation. Therefore, we went on to elucidate some of

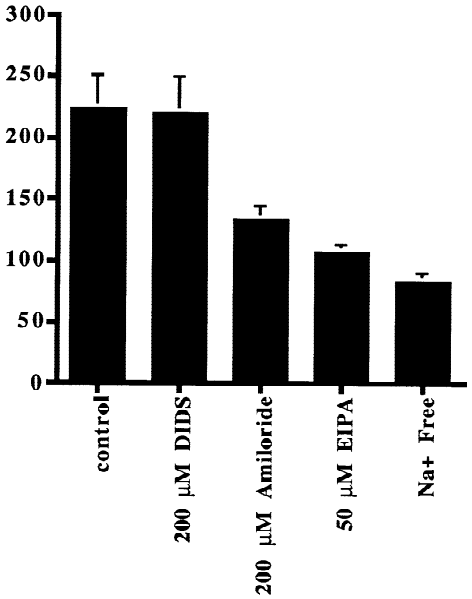

Fig. 4. The rapid effect of aldosterone on crypt cytoplasmic pH is dependent on sodium-hydrogen exchange. Aldosterone rapidly increased fluorescent intensity (alkalization) in controls bathed in a HEPES-buffered Ringers solution ($n = 144$). Pretreatment of crypts with 200 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of anion exchange, did not significantly influence the effect of aldosterone ($n = 108$; $P > 0.5$). However, inhibiting sodium-hydrogen exchange with amiloride (200 μM; $n = 198$), ethylisopropylamiloride (EIPA) (50 μM; $n = 124$), or bathing crypts in a sodium-free (Na^+ Free) perfusate (sodium replaced with N-Methyl-*d*-Glucamine) ($n = 144$) significantly reduced subsequent aldosterone effects on cellular pH (all $P < 0.01$). Data indicate maximal change in intensity, expressed as a percentage of basal (% F.I.), in mean ± SEM for 0.1 nM aldosterone in each case.

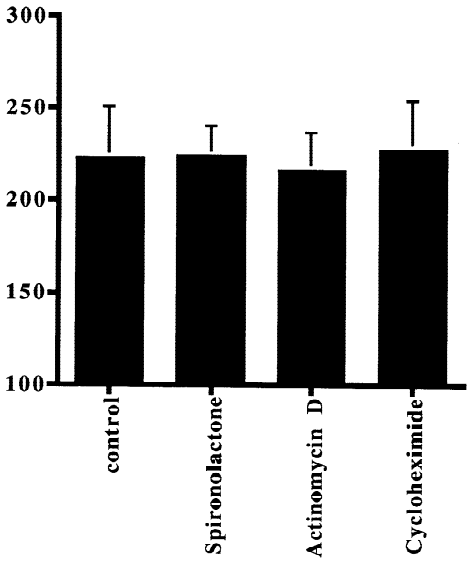

Fig. 5. The rapid effect of aldosterone on crypt cell pH is independent of protein synthesis. Pretreatment of crypts with 1 μM spironolactone, an antagonist of the traditional, protein synthesis-mediated receptor, did not inhibit the rapid alkalization of crypt cells in response to aldosterone ($n = 108$; $P > 0.5$). Neither did abrogation of *de novo* protein synthesis with either 50 μg/ml actinomycin D ($n = 108$; $P > 0.5$), or 20 μg/ml cycloheximide ($n = 108$; $P > 0.5$) significantly influence the fast action of aldosterone on cytoplasmic pH. Data indicate maximal change in intensity, expressed as a percentage of basal (% F.I.), in mean ± SEM for 0.1 nM aldosterone in each case.

the signal transduction pathways in the mediation of this fast aldosterone action.

Na-H exchange isoforms may be differentially regulated by protein kinases, in that protein kinase C (PKC) can be stimulatory or inhibitory depending on the isoform and tissue type [36]. Similarly, elevation of cellular adenosine 3′,5′-cyclic monophosphate (cAMP) inhibits NHE-1 via protein kinase A (PKA) yet stimulates the trout β-NHE [36]. Inhibition of PKC with 1 μM chelerythrine chloride (Fig. 6) reduced the cellular alkalization induced by aldosterone (% F.I. = 124.2 ± 2.7%; $P < 0.01$; $n = 72$; 0.1 nM aldosterone). A cytosolic alkalization could be achieved by directly stimulating PKC with 1 μM phorbol 12-myristate-13-acetate (a phorbol ester) (% F.I. maximum = 234.5 ± 47.2%; $P < 0.01$; $n = 135$). Phorbol ester-induced alkalization was similarly inhibited by 200 μM amiloride (% F.I. = 149.8 ± 15.2%; $P < 0.01$), superfusing a sodium-free Ringer's solution (% F.I. = 91.0 ± 12.5%; $P < 0.01$) or pretreatment with chelerythrine chloride (% F.I. = 137.6 ± 16.4%; $P < 0.01$). Generating cAMP via direct stimulation of adenylate cyclase acidified crypts (% F.I. = 82.7 ± 8.5%; $P < 0.01$; $n = 72$; 10 μM forskolin) and inhibited subsequent activation of Na-H exchange by aldosterone (% F.I. = 84.9 ± 10.2; $P > 0.2$; $n = 72$; 0.1 nM aldosterone). Similarly, forskolin treatment resulted in an acidification of the cytoplasm (beneath initial basal levels) following aldosterone-induced alkalization (% F.I. = 89.2 ± 4.7%; $P < 0.01$; $n = 72$; 0.1 nM aldosterone). This may simply reflect a differential magnitude of stimulus to the protein kinases, or indicate that PKA is the dominant regulator of Na-H exchange activity. The latter seems more plausible in light of the recent demonstration that colonic crypts constitutively absorb fluid, in a sodium-dependent fashion, yet secrete in response to a cell permeant analogue of cAMP, or via its generation by native neurohumoral stimulus [47]. These experiments indicate that the rapid onset effect of aldosterone on Na-H exchange is PKC-mediated. Moreover, they confirm that PKC and PKA have opposing effects on Na-H exchange activity, which has important implications for the regulation of crypt fluid and electrolyte transport.

We examined the role of phospholipase activity, arachidonic acid, prostaglandins and leukotrienes in the rapid aldosterone action on cellular pH in colonic crypts. The known stimulatory effect of arachidonic acid on PKC [31] may directly account for increased Na^+/H^+

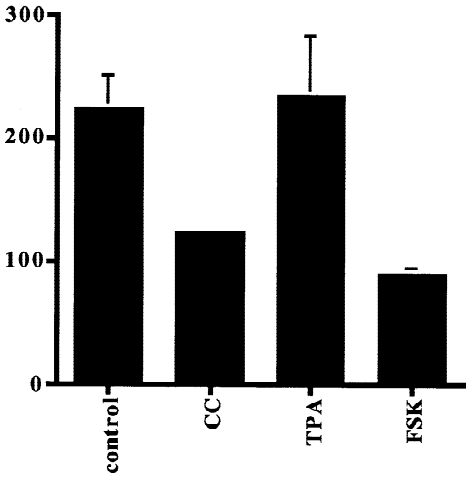

Fig. 6. Rapid aldosterone-induced alkalization of crypt cells is protein kinase C-dependent. Inhibition of protein kinase C with 1 μM chelerythrine chloride (CC) reduced subsequent cytoplasmic pH responses to aldosterone ($n = 72$; $P < 0.01$). In keeping with a proposed protein kinase C-mediated activation of sodium-hydrogen exchange by aldosterone, direct stimulation of protein kinase C with 1 μM phorbol-12-myristate-13-acetate (TPA) also increased fluorescent intensity (cellular alkalization) in separate experiments ($n = 135$; $P < 0.01$). However, increasing cellular adenosine 3′,5′-cyclic monophosphate levels by stimulation of adenylate cyclase with 10 μM forskolin acidified the crypt cells ($n = 72$; $P < 0.01$) and inhibited subsequent aldosterone-induced alkalization ($n = 72$; $P > 0.5$). Data indicate maximal change in intensity, expressed as a percentage of basal (% F.I.), in mean ± SEM for 0.1 nM aldosterone in each case.

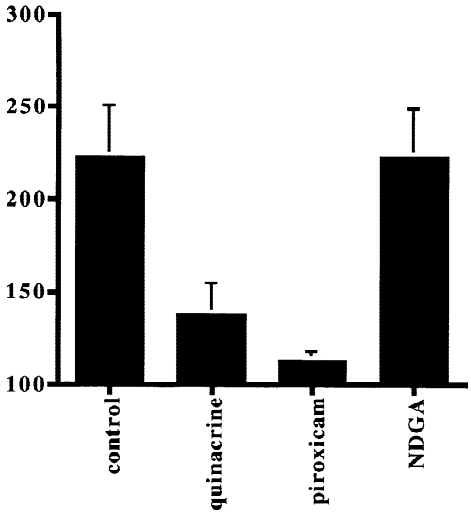

Fig. 7. The role of arachidonic acid metabolites in the rapid effect of aldosterone on cytoplasmic pH. Pretreatment of crypts with blockers of phospholipase (50 μM quinacrine; $n = 108$; $P < 0.01$) or cyclooxygenase (10 μM piroxicam; $n = 108$; $P < 0.01$) inhibited the rapid effects of aldosterone on intracellular pH. However, prior exposure to 100 μM nordihydroguaiararetic acid (NDGA), a lipoxygenase inhibitor, did not significantly affect rapidly responsive aldosterone-induced alkalization in separate experiments (increase in intensity). Therefore, there appears to be arachidonic acid metabolism through the cyclooxygenase pathway (prostaglandins), rather than lipoxygenase direction (leukotrienes), in rapid aldosterone effects. Data indicate maximal change in intensity, expressed as a percentage of basal (% F.I.), in mean ± SEM for 0.1 nM aldosterone in each case.

exchangers. Prostaglandins have been demonstrated to regulate renal Na^+/H^+ exchange activity [46] while leukotrienes are involved in cellular mechanisms which may employ Na^+/H^+ exchangers [14]. Inhibition of phospholipase A_2 with 50 μM quinacrine reduced aldosterone-induced alkalization (% F.I. = 137.9 ± 16.8%; $P < 0.01$; $n = 108$; 0.1 nM aldosterone). Blockade of prostaglandin or leukotriene synthesis was achieved by incubating the isolated colonic crypts with inhibitors of cyclooxygenase (10 μM piroxicam) or lipoxygenase (100 μM nordihydroguaiararetic acid), respectively. Inhibition of cyclooxygenase (% F.I. = 113.3 ± 4.41%; $P < 0.01$; $n = 108$; 0.1 nM aldosterone), but not lipoxygenase (% F.I. = 223.2 ± 25.1%; $P > 0.5$; $n = 72$; 0.1 nM aldosterone), reduced the cytosolic pH response to subsequent aldosterone exposure. These results, summarized in Fig. 7, suggest that synthesis of prostaglandins, and not leukotrienes, are necessary to the transduction of the rapid aldosterone action on colonic crypts.

Heterotrimeric guanosine triphosphate-binding proteins (G proteins) regulate a variety of processes involved in electrolyte transport, including Na^+/H^+ exchange activity [9, 14]. Pertussis toxin-sensitive regulatory mechanisms of epithelial Na^+ transport have been identified [10]. Moreover, the α_{i-3} G protein subunit interacts, both directly and indirectly with phospholipase A_2, with membrane transport function [10, 29]. Therefore, since inhibiting phospholipase A_2 and arachadonic acid metabolites blocked this rapid aldosterone effect on Na^+/H^+ exchange we postulated that G proteins would be regulators of the signal transduction. Pretreatment of crypts with 1 μg/ml pertussis toxin inhibited subsequent aldosterone-induced increases in pH_i (% F.I. = 130.7 ± 12.6%; $P < 0.01$; $n = 144$; 0.1 nM aldosterone). However, crypts incubated with 10 μg/ml cholera toxin prior to aldosterone exposure responded in a similar manner to controls (% F.I. = 213.8 ± 31.4%; $n = 108$, $P > 0.4$; 0.1 nM aldosterone). This is evidence of a regulatory role for G proteins (possibly G_i) in the rapid effects of aldosterone on colonic crypts. However, this does not necessitate that a membrane-bound, G-protein coupled receptor exists for aldosterone in crypt cells. Rather, it may reflect an inhibition of protein packaging and sorting of vesicles to the membrane [15, 36].

Discussion

There have been reports of the existence of membrane receptors for steroid hormones in several nonepithelial tissues [8, 24, 42]. Whether or not there is a membrane

ligand(s) for steroids that is structurally and functionally distinct from the traditional receptor is ill-defined. Some of these investigators have demonstrated a distinct binding coefficient for a proposed membrane receptor but definitive structural studies have not been performed to date.

However, many tissues respond to steroid hormones in a rapid, nongenomic fashion (independent of protein synthesis) which may indicate a separate pathway, if not a different receptor coupling, for this class of hormone. For example, nongenomic actions of testosterone in ventricular myocardium [26], progesterone in spermatozoa and oocytes [6], estrogens in glomerulosa cells [34], and vitamin D in colonic epithelial cells [53] have been demonstrated. Rapid onset effects of aldosterone on second messengers such as cytosolic calcium and protein kinase C are known to occur [12, 16]. A rapid intracellular alkalization in response to treatment with aldosterone has been reported in other epithelia [22, 38]. These actions are either (i) too fast to be transduced by protein synthesis, (ii) refractory to receptor antagonism with spironolactone, or (iii) unaffected by inhibitors of DNA transcription and mRNA translation. This has led to a hypothesis that there is a second signaling pathway for aldosterone which may involve membrane events (if not a membrane receptor domain) distinct from the traditional, protein synthesis-based mode of action.

Until recently, the accepted dictum had been that colonic absorptive and secretory functions were segregated to the surface epithelial cells and crypt cells, respectively. It has been proposed, however, based on theoretical considerations and indirect evidence, that crypts were involved in colonic fluid absorption [35]. Direct measurement of net fluid absorption by isolated, perfused crypts from rat distal colon was recently described [47]. This has led to a focus on the crypt cells as targets for effects of aldosterone [16]. In this study, we examined cytoplasmic pH responses to acute aldosterone exposure using confocal laser scanning microscopy of BCECF-loaded colonic crypts. BCECF is ideal for examining intracellular pH because the pK is within physiological pH ranges and the dye is excluded by organelles, ensuring true cytoplasmic pH recordings [41]. Thus it has been used previously both to identify, and study, Na^+/H^+ exchange in mammalian colon [23, 25]. Confocal microscopy confers distinct advantages over conventional fluorescence microscopy because true cellular fluorescence is recorded through the elimination out-of-focus signals.

The basal pH_i measured in the colonic crypts in our study was 7.11 ± 0.08 which approximates the resting pH_i found in enterocytes [24.]. We observed a cytoplasmic alkalization (increase in fluorescent intensity) within one minute of aldosterone exposure to isolated colonic crypts. The rapid change in fluorescent intensity induced by aldosterone in crypt cells corresponds with an increase of 0.17 ± 0.38 pH units (mean ± SD at 0.1 nM aldosterone), in keeping with that observed in other tissues [22, 38]. A dose dependence was observed over a range of 0.005–100 nM with an EC_{50} of 0.8 nM. Therefore, this effect occurs within the normal physiological range of aldosterone which varies between approximately 0.08 nmol/l and 0.2 nmol/l in both human [1] and rat plasma [32]. A tissue response to doses in excess of these values is not surprising since, in primary hyperaldosteronism the plasma concentration may be greater than 0.6 nmol/l [27] and under conditions of low salt diet (secondary hyperaldosteronism), plasma aldosterone levels may exceed 1 nmol/l.

This aldosterone-induced alkalization occurred in both the presence or nominal absence of bicarbonate in the system. This rapid aldosterone action (increase in pH_i) was reduced by inhibiting Na^+/H^+ exchange both with amiloride and through removal of sodium from the perfusate. Therefore, we propose that an increase in Na^+/H^+ exchange activity is responsible for the change in pH. This is supported by the observed aldosterone-induced increase of cytoplasmic pH by activation of amiloride-sensitive Na^+/H^+ exchange in frog skin [22].

In-vivo perfusion experiments and in-vitro transport studies have demonstrated that Na^+ absorption by the rat distal colon (as in some other mammals) is an electroneutral process mediated by parallel apical membrane Na^+/H^+ and Cl^-/HCO_3^- exchange [5]. *In-situ* perfusion experiments have shown that net Na^+ absorption is inhibited by millimolar concentrations of amiloride, consistent with the dependence on colonic Na^+/H^+ exchange [28]. Studies on apical membrane vesicles have confirmed the presence of Na^+/H^+ exchange in both surface epithelial cells [5] and crypt cells [44] of rat distal colon. Na^+/H^+ exchange is ubiquitous in vertebrate cell membranes where it exchanges external sodium for intracellular H^+ ions with a fixed stoichiometry of 1:1 [2].

To date, at least four distinct membrane proteins have been identified as Na^+/H^+ exchange isoforms [50]. The ubiquitous NHE-1 isoform is localized to the basolateral membrane of epithelial cells, regulates intracellular pH and is referred to as the "housekeeper NHE" [23, 50]. NHE-3 has been found on the apical membrane rat intestine (including colon) but, while present in surface epithelial cells, it is not localized in crypt cells [7]. The message for both NHE-2 and NHE-4 has been identified in colon [13, 39] but their exact location within the surface cell-crypt axis remains to be elucidated. A chloride-dependent Na-H exchange has been recently reported in rat colonic mucosa [44]. It is unlikely to be either the NHE-1 or NHE-3 isoform because it is situated on the apical membrane of crypt cells and is relatively insensitive to amiloride (in contrast to the chloride-independent Na-H exchange on the apical and basolateral membrane

of surface cells) [40]. However, we believe that the Na-H exchange involved in the present study is the NHE-1 isoform because it is located in rat colonic crypts, is blocked by ethylisopropylamiloride, and is sensitive to protein kinase C (both directly and via aldosterone) [50].

The classical genomic mechanism of action for aldosterone involves binding of the hormone to a cytoplasmic receptor, migration of the hormone-receptor complex to the nucleus, and subsequent production of aldosterone-induced proteins (AIPs) via DNA synthesis and messenger ribonucleic acid (mRNA) translation [43]. Aldosterone-induced proteins are thought to cause alterations in sodium reabsorption at the Na^+ channel and Na^+/K^+ ATPase pump steps. The classical receptor has been cloned and does not distinguish between aldosterone and hydrocortisone [3]. The classical receptor mediates the genomic effects of aldosterone which are characterized by sensitivity to inhibitors of transcription and translation (cycloheximide and actinomycin D) and a latency of onset (greater than 60 min). The aldosterone action on pH_i in our study was specific for aldosterone, was not mediated via genomic events or protein synthesis, and occurred independent of the traditional receptor antagonist. Therefore, we investigated the role of alternative signal transduction mediators in the generation of this rapid aldosterone effect.

A rapid effect may be explained by aldosterone-induced carboxymethylation [56] of the membrane protein that forms the Na^+/H^+ exchange or by phosphorylation of the exchanger. There is considerable evidence for the latter mechanism. Protein kinase C (PKC) regulates Na^+/H^+ exchange via phosphorylation [48]. This mechanism is partly responsible for a nongenomic increase in Na^+/H^+ exchange activity by aldosterone in mature red blood cells [27] and a rapid activation of PKC by aldosterone in mucosal epithelium of distal rat colon was recently reported [16]. The steroid hormone vitamin D in its active form, 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), displays both delayed genomic and rapid, PKC-mediated, nongenomic actions [37, 53]. In this study, we examined the role of PKC in the mediation of the aldosterone action on pH_i in colonic crypt cells. We found that blocking PKC with a specific inhibitor, chelerythrine chloride, abrogated the cytoplasmic pH increase in response to aldosterone. It was possible to mimic this alkalization by direct stimulation of protein kinases with a phorbol ester. The effect of PMA was demonstrated to be due to direct stimulation of PKC since it was similarly inhibited with chelerythrine (*data not shown*). Increasing cellular cAMP by activation of adenylate cyclase with forskolin had a profound inhibitory influence on the cytoplasmic alkalization induced by either aldosterone or phorbol ester. This underlines the complex, interactive regulatory influences of protein kinase A and protein kinase C on Na^+/H^+ exchange [11, 55] and demonstrates that PKC activation is required for rapid aldosterone action on cell pH.

We employed a pharmacological approach in order to examine a putative role for arachidonic acid metabolites in the rapid aldosterone action on cellular pH in colonic crypts. Prostaglandins have been demonstrated to regulate renal Na^+/H^+ exchange activity, e.g., subclass F2∝ [46], while leukotrienes are involved in regulatory volume mechanisms which may employ Na^+/H^+ exchangers [14]. Inhibition of phospholipase A2 with quinacrine reduced aldosterone-induced alkalization. Blockade of prostaglandin or leukotriene synthesis was achieved by incubating the isolated colonic crypts with inhibitors of cyclooxygenase (piroxicam) or lipoxygenase (nordihydroguaiararetic acid), respectively. Inhibition of cyclooxygenase, but not lipoxygenase, reduced the cytosolic pH response to subsequent aldosterone exposure. We propose that prostaglandins, and not leukotrienes, are involved in the signal transduction of the rapid, alternative pathway for aldosterone action in colonic crypt cells.

Heterotrimeric guanosine triphosphate-binding proteins (G proteins) play a central role in coupling membrane receptors to effector mechanisms within the cell. G proteins have been identified as regulators of Na^+/H^+ exchange activity in a variety of cells and by a diverse range of stimuli [14, 17]. In this study, we found that the effect of aldosterone on Na^+/H^+ exchange activity was abrogated by pertussis toxin. However, Cholera toxin had no adverse influence on the rapid aldosterone action. This underlines the role of membrane-associated signaling events in the transduction of this rapid steroid hormone action on ion transport. Previous studies have demonstrate guanosine nucleotide-dependent activation of cation channels [9] and pertussis toxin-sensitive regulatory mechanisms of epithelial Na^+ transport [10]. The α_{i-3} G protein subunit plays a pivotal role in this regulation and has been demonstrated to interact both directly with membrane channels, or indirectly with phospholipase A2 [10, 29]. Furthermore, a role for G proteins in vesicular and protein traffic has been determined, with the subunits α_{i-2} and α_{i-3} implicated in basolateral membrane events [15, 36]. If the G protein subunits α_{i-2} or α_{i-3} are involved in the rapid activation of Na^+/H^+ exchange by aldosterone in colonic crypt cells then they may similarly upregulate or shuttle Na^+ channels and/or the Na^+/K^+ ATPase pumps. Ongoing research will determine whether aldosterone can rapidly alter net transepithelial fluid and electrolyte transport and/or cellular Na^+ concentrations.

Theories as to the physiological impact of this nongenomic aldosterone action are speculative but it is tempting to propose that it represents a mechanism for 'fine-tuning' cellular conditions for enhanced transepithelial sodium absorption. Additionally, it may prime

the cell for induction of the transport functions of synthesized proteins that characterize the latent, genomic action. Some evidence suggests that active regulation of pH_i is crucial for processing aldosterone-induced proteins [20, 28, 43, 56]. Therefore, the cellular role of a rapid upregulation of membrane Na–H exchange may be to facilitate the slower, nuclear events that lead to aldosterone-induced protein synthesis while providing an immediate tissue response in the interim.

In conclusion, aldosterone induces a cytoplasmic alkalization of colonic crypt cells within minutes of exposure by upregulating Na^+/H^+ exchange activity. This demonstrates a mode of action, in a classical aldosterone target tissue, that occurs independent of the traditional receptor and protein synthesis. Protein kinase C stimulation and prostaglandin synthesis are essential steps in the genesis of this rapidly responsive signal transduction. This is particularly relevant to our understanding of intestinal function in light of the recent demonstration that sodium transport and fluid absorption are constitutive functions of colonic crypts [47]. Further investigation is required in order to define this fast aldosterone action and its role in colonic absorptive function, and to determine if a receptor for aldosterone exists on crypt cell membranes.

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