Direct modulation of basal and angiotensin II-stimulated aldosterone secretion by hydrogen ions

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Abstract

Disturbances in acid–base balance *in vivo* are associated with changes in plasma aldosterone concentration, and *in vitro* changes in extracellular pH (pH_o) influence the secretion of aldosterone by adrenocortical tissue or glomerulosa cells. There is considerable disparity, however, as to the direction of the effect. Furthermore, the mechanisms by which pH_o independently affects aldosterone secretion or interacts with other secretagogues are not defined. Thus, bovine glomerulosa cells maintained in primary monolayer culture were used to examine the direct effects of pH_o on cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) and aldosterone secretion under basal and angiotensin II (AngII)-stimulated conditions. pH_o was varied from 7.0 to 7.8 (corresponding inversely to changes in extracellular H^+ concentration from 16 nM to 100 nM). Whereas an elevation of pH_o from 7.4 to 7.8 had no consistent effect, reductions of pH_o from 7.4 to 7.2 or 7.0 caused proportionate increases in aldosterone secretion that were accompanied by increases in transmembrane Ca^{2+} fluxes and $[\text{Ca}^{2+}]_i$. These effects were abolished by removal of extracellular Ca^{2+} . A decrease in

pH_o from 7.4 to 7.0 also enhanced AngII-stimulated aldosterone secretion. This effect was more pronounced at low concentrations of AngII and was manifested as an increase in the magnitude of the secretory response with no effect on potency. In contrast to its effect on AngII-stimulated aldosterone secretion, a reduction of pH_o from 7.4 to 7.0 inhibited the Ca^{2+} signal elicited by low concentrations ($\leq 1 \times 10^{-10}$ M) of AngII, but did not affect the increase in $[\text{Ca}^{2+}]_i$ caused by a maximal concentration (1×10^{-8} M) of AngII. These data suggest that pH_o (i.e. H^+) has multiple effects on aldosterone secretion. It independently increases aldosterone secretion through a mechanism involving Ca^{2+} influx and an increase in $[\text{Ca}^{2+}]_i$. Also, it modulates the action of AngII by both decreasing the magnitude of the AngII-stimulated Ca^{2+} signal and increasing the sensitivity of a more distal site to intracellular Ca^{2+} . The latter action appears to be a more important determinant in the effects of pH_o on AngII-stimulated aldosterone secretion.

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Introduction

Plasma aldosterone concentration is known to change in response to alterations in acid–base balance. For example, aldosterone levels in plasma increased during diabetic ketoacidosis (Christlieb *et al.* 1975, Scott *et al.* 1978, Quigley *et al.* 1982), metabolic acidosis (Perez *et al.* 1977, 1979, 1980, Schambelan & Sebastian 1977) and acute respiratory acidosis (Raff & Roarty 1988). Conversely, during metabolic alkalosis (Kassirer *et al.* 1967, Julian *et al.* 1982), plasma aldosterone concentration decreased. Interpretation of these effects, however, is complicated by concurrent changes in plasma renin activity, plasma K^+ concentration and/or plasma adrenocorticotropin (ACTH) concentration – parameters that in and of themselves are important determinants of aldosterone secretion.

The possibility that alterations in extracellular pH (pH_o) directly affect the rate of aldosterone secretion has been examined under a variety of experimental conditions both *in vivo* (Jones *et al.* 1992, Yamauchi *et al.* 1997) and *in vitro* (Muller 1965, Chiu & Freer 1979, Gilchrist *et al.* 1983, Carroll *et al.* 1986, Radke *et al.* 1986a,b, Raff & Jankowski 1993). Although results of these studies generally support that premise, data concerning the specific nature of the effect are contradictory and interpretation of the results is complicated by a number of confounding variables. The possibility that indirect actions of H^+ (i.e. pH_o) contributed to its effects on aldosterone secretion by intact adrenal glands or adrenal sections can not be entirely excluded. In addition, there were considerable differences between the magnitudes of the changes in pH_o . Such differences may well contribute to disparities between the

apparent relationship between pH_o and aldosterone secretion. In some cases, the magnitudes of the changes in pH_o were such that the physiological relevance of the results is unclear. Also, differences in the means by which pH_o was altered may influence the rapidity and extent of compensation by the glomerulosa cells and, ultimately, aldosterone secretion.

The first objective of the present studies was to determine, using primary cultures of bovine glomerulosa cells as an experimental model, if changes in extracellular H^+ concentration ($[\text{H}^+]_o$) comparable to those that occur *in vivo* under physiological and pathophysiological conditions affect aldosterone secretion directly. The second objective was to determine if any direct steroidogenic effect of H^+ was associated with changes in cell Ca^{2+} . Changes in $[\text{H}^+]_o$ over the range of 16 to 100 nM (pH_o 7.8 to 7.0 respectively) were related to changes in transmembrane Ca^{2+} fluxes, cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) and aldosterone secretion. Finally, the ability of H^+ to modulate the Ca^{2+} signal and the aldosterone secretory response elicited by angiotensin II (AngII) was examined. The results indicate that H^+ has a direct action on glomerulosa cells to stimulate aldosterone secretion through a mechanism involving an increase in $[\text{Ca}^{2+}]_i$. H^+ also directly enhanced the secretory response to AngII, but this effect was dissociated from those of H^+ on AngII-stimulated increases in $[\text{Ca}^{2+}]_i$. Results of these investigations have been reported, in part, in an earlier communication (Robinson *et al.* 1990).

Materials and Methods

Cell culture

Glomerulosa cells were isolated from bovine adrenal glands and suspended in a modified Ham's F-12 medium containing horse serum (10% v/v) and Hepes (25 mM, pH 7.4 at 37 °C) buffer as previously described (Kramer 1988a). Cells were seeded to either Leighton tubes containing a fibronectin-coated (2 µg/cm²) 9 × 35 mm glass coverslip or fibronectin-coated cell culture dishes. Medium was changed 16–20 h after seeding and then at 48 h intervals. Cells achieved confluence within 5–7 days and were then maintained for an additional 24–96 h in serum-free Ham's F12/Hepes medium. Subsequent experiments were performed using Hanks' balanced salt solution (HBSS) modified to contain 0.2 mg/ml BSA, 4 mM KCl, 1.8 mM CaCl_2 , 4 mM NaHCO_3 and 10 mM Hepes buffer (pH 7.4 at 37 °C).

Ca^{2+} fluxes and $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was estimated from the ratio of fura2 fluorescences (emission 510 nm) when excited alternately at 340 and 380 nm (Grynkiewicz *et al.* 1985) after correction for cell

autofluorescences. Monolayers adhering to glass coverslips were incubated in the presence of 2 µM fura2 AM for 45–60 min. Then, fura2 fluorescences were monitored continuously as the cells were superfused with HBSS/Hepes buffer (pH 7.4 at 37 °C) at a rate of approximately 3 ml/min (Kramer 1991). The temperature of the superfusate within the cell chamber varied between 36 and 37.5 °C. The composition and/or pH of the superfusate was changed as indicated for specific experiments after steady-state basal data had been collected for at least 15 min. Alterations in pH_o over the range used in these experiments affected calibration parameters used in the calculation of $[\text{Ca}^{2+}]_i$ by less than 5%. These effects were ignored, since cytosolic pH (pH_i) changed over a narrower range (~ 7.05 – 7.30) than did pH_o . Calibration parameters were determined as described previously (Tsien *et al.* 1985).

To allow measurement of Ca^{2+} efflux, cells adhering to glass coverslips were incubated in the presence of 10 µCi [⁴⁵Ca]CaCl₂ for 2 h. In experiments in which ⁴⁵ Ca^{2+} efflux and $[\text{Ca}^{2+}]_i$ were measured simultaneously, 2 µM fura2 AM was also present during the second hour of incubation. Cells were then subjected to superfusion, and the superfusate was collected in 1 min fractions. The ⁴⁵ Ca^{2+} content of each fraction and the amount of ⁴⁵ Ca^{2+} remaining in the cells at the end of the experimental protocol were determined by liquid scintillation counting (Kramer 1988b). Calcium efflux during each 1 min interval is expressed as a percentage of the total ⁴⁵ Ca^{2+} contained within the cells at the beginning of that interval, i.e. as the Ca^{2+} efflux coefficient (Borle *et al.* 1982).

For the measurement of Ca^{2+} uptake, monolayers in 60 mm dishes or six-well clusters were first incubated in HBSS/Hepes buffer (pH 7.4 at 37 °C) for 60 min. Next, buffer was replaced with 2 ml HBSS/Hepes buffer of appropriate pH (7.8, 7.4 or 7.0) containing 2 µCi [⁴⁵Ca]CaCl₂. Accumulation of ⁴⁵ Ca^{2+} was terminated 10, 20, 30, 60 or 120 s thereafter, by the addition of an equal volume of cold 2 × stop solution (20 mM LaCl_3 /200 mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ pH 6.0). This solution was aspirated, and the cells were rinsed three times with 10 ml cold 1 × stop solution. Zero-time samples were prepared by adding 2 ml 2 × stop solution to the cells prior to addition of 2 ml HBSS/Hepes buffer containing [⁴⁵Ca]CaCl₂. Cells were lysed in 0.1 M nitric acid, and isotopic Ca^{2+} contents of the cell lysates were measured by liquid scintillation counting. Residual cell protein was rinsed with saline and then dissolved in 0.5 M NaOH for quantitation.

Aldosterone secretion

Cells previously grown to confluence in 48-well cluster dishes and maintained in serum-free Ham's F12 medium were incubated for 60 min in HBSS/Hepes buffer (pH 7.4 at 37 °C) containing 2 mg/ml BSA and then for an

additional 60 min in buffer containing 0.2 mg/ml BSA. In experiments in which the effects of H^+ alone were examined, cells were incubated for 120 min in fresh buffer that had been adjusted to pH 7.0, 7.2, 7.4 or 7.8. In experiments in which the interaction between H^+ and AngII was examined, cells were incubated first for 15 min in buffer of the indicated pH and then for 120 min after addition of AngII (1×10^{-12} to 1×10^{-8} M). Parallel incubations were ended at either 15 or 135 min. Media were collected, stored at -20°C and later used for the measurement of aldosterone. Cells were rinsed twice with saline, and total cell protein was measured against BSA standards using a bicinchoninic acid-based assay (Pierce, Rockford, IL, USA) after lysis of the cells in 0.5 M NaOH. Aldosterone contents of the incubation media were measured by direct RIA using ^{125}I -aldosterone and aldosterone antibody-coated tubes (Diagnostic Products, Los Angeles, CA, USA).

Specific reagents and buffers

Ham's F12 medium, HBSS, horse serum and antibiotics were obtained from Gibco (Grand Island, NY, USA), and other components of the media and buffers were from Sigma (St Louis, MO, USA). Dispase and collagenase were from Boehringer Mannheim (Indianapolis, IN, USA), and plastic culture dishes were from Co-Star (Cambridge, MA, USA). Fura2 and fura2 AM were obtained from Molecular Probes (Eugene, OR, USA). Isotopic Ca^{2+} was purchased from New England Nuclear (Boston, MA, USA), and AngII, [^5Val]AngII, was purchased from Bachem Inc. (Torrance, CA, USA).

Modified HBSS/Hepes buffers used for superfusion and static incubations were prepared as follows. A control buffer of pH 7.4 at 37°C was prepared that contained 4 mM KCl, 1 mM MgSO_4 , 1.8 mM CaCl_2 , 0.44 mM NaH_2PO_4 , 0.34 mM Na_2HPO_4 , 4 mM NaHCO_3 , 10 mM Hepes, 5.6 mM glucose, 0.2 mg/ml BSA, and a $[\text{Na}^+]$ from the addition of both NaOH and NaCl of 137 mM. The final $[\text{Na}^+]$ was approximately 142 mM, and the final osmolality was 278 ± 1 mosmol. Aliquots of the control buffer were removed, and the pH at 37°C was adjusted either upward (pH 7.8) by addition of NaOH or downward (pH 7.2 or 7.0) by addition of HCl. The osmolality of each of the resulting experimental buffers was 2–3 mosmol greater than the osmolality of the control buffer. No corrections for differences in osmolalities between control and experimental buffers were made. Final adjustments of pH of all buffers were made immediately prior to use. Specific additions to these buffers are noted for individual experiments.

Design and statistics

Temporal effects of pH_o or AngII on $[\text{Ca}^{2+}]_i$ were evaluated by comparing the average $[\text{Ca}^{2+}]_i$ measured

during the peak (4–5 min) or plateau (14–15 and 44–45 min) phase of the response and the average $[\text{Ca}^{2+}]_i$ measured during the minute immediately preceding the change in pH_o or the addition of agonist. Average $[\text{Ca}^{2+}]_i$ over equivalent periods was determined for control cells. In instances when the calcium signal was integrated to obtain an area-under-the-curve (AUC), the signal was first corrected for any slope in baseline $[\text{Ca}^{2+}]_i$. The effects of each experimental regimen were examined, for the most part, using individual monolayers from a single cell preparation. In cases where the effect of a given experimental regimen was examined using multiple monolayers from the same cell preparation, replicates were averaged to give a single value. Similarly, aldosterone secretion was assayed in triplicate using individual monolayers derived from a single cell preparation, replicates for each experimental condition representing monolayers from separate multiwell clusters. Results for each condition were averaged to give a single value, and the experiment repeated using independent cell preparations. Within an individual experiment (and, to the extent possible, between experiments), the times between the onset of superfusion (or incubation) with buffer of pH 7.4 and exposure of cells to buffer of another pH or agonist were constant between groups. All results are expressed as the mean $\pm$ s.e.m. with n representing the number of distinct cell preparations. Unless otherwise indicated, each experiment was repeated a minimum of four times. ANOVA followed by the Newman–Keul test for comparison of means was used to determine the effects of experimental treatments. A value of $P \leq 0.05$ was accepted as significant.

Results

Effects of changes in pH_o on $[\text{Ca}^{2+}]_i$

Reductions in pH_o from 7.4 (40 nM H^+) to 7.2 or 7.0 (63 or 100 nM H^+) caused graded increases in $[\text{Ca}^{2+}]_i$ that were characterized by an initial peak and a secondary plateau (Fig. 1A, Table 1). In response to a decrease in pH_o from 7.4 to 7.0, for example, $[\text{Ca}^{2+}]_i$ increased over 2-fold (254 ± 23 versus 113 ± 7 nM) within 4–5 min and then declined rapidly over the next 5–10 min and more slowly thereafter. Thirty minutes after the reduction in pH_o , $[\text{Ca}^{2+}]_i$ remained at a level greater than that of control cells (135 ± 7 vs 114 ± 8 nM). A smaller reduction in pH_o (i.e. from pH 7.4 to 7.2) caused proportionately smaller increases in $[\text{Ca}^{2+}]_i$ during both the peak and plateau phases of the Ca^{2+} signal. In contrast to the effects of a reduction in pH_o , an elevation in pH_o from 7.4 to 7.8 (corresponding to a decrease in $[\text{H}^+]_o$ from 40 to 16 nM) had no effect on $[\text{Ca}^{2+}]_i$ (Table 1).

A comparison of the Ca^{2+} signal elicited by H^+ with those elicited by K^+ and AngII is presented in Fig. 1B. Although the Ca^{2+} signal elicited by reduction of pH_o from 7.4 to 7.0, like the signal elicited by AngII

Figure 1 Cytosolic Ca^{2+} signal caused by reduction of pH_o . (A) $[Ca^{2+}]_i$ was monitored during superfusion of fura2-loaded glomerulosa cells with HBSS/Hepes buffer. Individual monolayers first were superfused for 15 min with buffer of pH 7.4. Then, pH_o was held at 7.4 or decreased to 7.2 or 7.0, and superfusion continued for an additional 30 min. Data presented are from a single experiment using individual coverslips for each pH_o . (B) Adherent fura2-loaded glomerulosa cells were superfused for 15 min with HBSS/Hepes buffer that was adjusted to pH 7.4 and contained 4 mM K^+ . Next, cells were exposed for an additional 15 min to superfusate that had been adjusted to pH 7.0 (H^+) or that contained 1×10^{-9} nM Ang II or 15 mM K^+ . Each line represents the average of two determinations using individual monolayers in a single experiment. The Ca^{2+} signal elicited by H^+ is representative of those noted in response to a similar reduction in pH_o during the course of the present studies, whereas the Ca^{2+} signals elicited by K^+ and Ang II are comparable to those noted in our previous studies using primary monolayer cultures of bovine glomerulosa cells.

($\geq 1 \times 10^{-9}$ M), exhibited an initial peak and secondary plateau, it was considerably smaller in magnitude than was the signal elicited by a maximal concentration of either AngII or K^+ .

The nature of the Ca^{2+} signal elicited by reductions in pH_o was independent of whether Hepes or bicarbonate was the primary extracellular buffer. A reduction of pH_o

from 7.4 to 7.0 during superfusion of cells with bicarbonate-buffered medium elicited both a peak (156 ± 18 nM) and plateau (22 ± 7 nM) in $[Ca^{2+}]_i$ that were comparable to the increases (155 ± 13 and 31 ± 4 nM respectively) elicited during superfusion of cells with Hepes-buffered medium ($n=4$).

Effects of changes in pH_o on Ca^{2+} fluxes

Alterations in pH_o caused changes in $^{45}Ca^{2+}$ efflux that generally paralleled their effects on $[Ca^{2+}]_i$ (Fig. 2A, Table 1). A decrease in pH of the superfusate from 7.4 to 7.2 increased Ca^{2+} efflux from a basal rate of $2.4 \pm 0.2\%/min$ to a maximal rate of $3.9 \pm 0.6\%/min$, whereas a reduction in superfusate pH from 7.4 to 7.0 increased the rate of Ca^{2+} efflux to a maximum of $5.6 \pm 0.8\%/min$. Rates of Ca^{2+} efflux subsequently declined during sustained reductions in pH_o . In cells superfused continuously with buffer of pH 7.0, the rate of Ca^{2+} efflux fell during a 20 min period to a level about 1.4-fold greater than the rate of Ca^{2+} efflux from control cells, whereas the rate of Ca^{2+} efflux from cells superfused continuously with medium of pH 7.2 returned to near control levels. The rate of Ca^{2+} efflux was not affected by an elevation in the pH of the superfusate from 7.4 to 7.8.

A reduction of pH_o from 7.4 to 7.0 enhanced the initial rate of Ca^{2+} influx from 15.5 ± 3.0 to 143.2 ± 20.3 c.p.m./s per mg protein (Fig. 2B), whereas an elevation of pH_o to 7.8 had no effect. Within 40–60 s, however, the rate of Ca^{2+} accumulation in cells exposed to pH_o 7.0 paralleled that of control cells.

Effects of changes in pH_o on aldosterone secretion

The rate of aldosterone secretion by cells incubated in medium of pH 7.4 (40 nM H^+) was 23.3 ± 1.4 pmol/2 h per mg protein ($n=8$). An elevation of pH_o from 7.4 to 7.8 (16 nM H^+) did not affect the rate of aldosterone secretion (21.9 ± 1.1). In contrast, reductions in pH_o caused increases in the rate of aldosterone secretion that correlated closely with H^+ -stimulated increases in $[Ca^{2+}]_i$ (Fig. 3). Aldosterone secretion increased approximately 1.5-fold (34.7 ± 2.8) in response to a reduction of pH_o from 7.4 to 7.2 (63 nM H^+) and approximately 2-fold (46.82 ± 3.6) in response to a reduction of pH_o to 7.0 (100 nM H^+). These rates of aldosterone secretion elicited by reductions in pH_o compare to maximal rates of aldosterone secretion elicited by AngII (1×10^{-8} M) and K^+ (15 mM) of 261.6 ± 8.0 and 194.7 ± 12.7 pmol/2 h per mg protein respectively.

Role of extracellular Ca^{2+} in the actions of H^+

In the presence of extracellular Ca^{2+} , there is a close correlation between H^+ -induced increases in $[Ca^{2+}]_i$ and aldosterone secretion (Fig. 3). In the absence of

Table 1 Summary of time-dependent changes in $[Ca^{2+}]_i$ and transmembrane Ca^{2+} fluxes caused by alterations in pH_o . For the measurement of $[Ca^{2+}]_i$ and Ca^{2+} efflux, cells previously loaded with fura2 and $^{45}Ca^{2+}$ were superfused for an initial 15 min control period with buffer of pH 7.4 and then for a 25–30 min experimental period during which the pH of the superfusate was 7.0, 7.2, 7.4 or 7.8. Data for each experimental group represent the differences between values measured at the end of the control period and those measured at the indicated times after the start of the experimental period. Changes in $[Ca^{2+}]_i$ and Ca^{2+} efflux during the experimental period are indicated also by AUC (nM•min or percent•min, as appropriate). Values for $[Ca^{2+}]_i$ represent the mean $\pm$ S.E.M. of six to eight independent determinations, whereas values for $^{45}Ca^{2+}$ efflux represent the mean $\pm$ S.E.M. of four determinations. Initial rates of Ca^{2+} influx were calculated from the accumulation of $^{45}Ca^{2+}$ in cells incubated in buffer of the indicated pH_o and are expressed as the mean $\pm$ S.E.M. of five experiments

pH_o	$\Delta[Ca^{2+}]_i$ (nM)				$\Delta^{45}Ca^{2+}$ efflux (%)			$^{45}Ca^{2+}$ influx (c.p.m./s/mg protein)
	4–5 min	15 min	30 min	AUC	6 min	25 min	AUC	
7.0	141 $\pm$ 20*	42 $\pm$ 3*	27 $\pm$ 2*	1572 $\pm$ 171*	2.8 $\pm$ 0.3*	0.8 $\pm$ 0.4*	31.6 $\pm$ 3.7*	126.5 $\pm$ 20.6*
7.2	65 $\pm$ 6*	23 $\pm$ 5*	16 $\pm$ 3*	907 $\pm$ 141*	1.5 $\pm$ 0.4*	0.2 $\pm$ 0.3	11.3 $\pm$ 1.3*	nd
7.4	1 $\pm$ 2	0 $\pm$ 1	1 $\pm$ 1	0 $\pm$ 5	–0.1 $\pm$ 0.2	–0.2 $\pm$ 0.2	–2.1 $\pm$ 2.1	11.8 $\pm$ 1.9
7.8	–1 $\pm$ 1	2 $\pm$ 1	1 $\pm$ 2	–3 $\pm$ 3	–0.3 $\pm$ 0.2	–0.3 $\pm$ 0.3	–6.0 $\pm$ 2.8	11.0 $\pm$ 1.5

nd, not determined; * $P < 0.05$ vs pH_o 7.4.

extracellular Ca^{2+} (i.e. Ca^{2+} -free buffer containing 0.05 mM EGTA), increases in $[Ca^{2+}]_i$ and Ca^{2+} efflux caused by reduction of pH_o from 7.4 to 7.0 were abolished (Table 2). Removal of extracellular Ca^{2+} also abolished the increase in aldosterone secretion caused by acidification (pH_o 7.0) of the extracellular medium (Table 2). In contrast, rates of aldosterone secretion by cells incubated under control (pH_o 7.4; 26.6 $\pm$ 1.2 vs 27.1 $\pm$ 1.5 pmol/2 h per mg protein) or alkalotic (pH_o 7.8; 25.4 $\pm$ 1.6 vs 23.4 $\pm$ 1.2 pmol/2 h per mg protein) conditions in the absence of extracellular Ca^{2+} were similar to rates of secretion in the presence of extracellular Ca^{2+} .

Effects of pH_o on AngII-stimulated increases in $[Ca^{2+}]_i$ and aldosterone secretion

The ability of AngII to increase $[Ca^{2+}]_i$ was compared in control (pH_o 7.4) cells and cells exposed to acidotic (pH_o 7.0) conditions for 15 min. At the time of addition of AngII, initial transients in $[Ca^{2+}]_i$ caused by reduction of pH_o had subsided, and $[Ca^{2+}]_i$ had achieved a new steady-state (Fig. 4). The results of a representative experiment in which pH_o was decreased from 7.4 to 7.0 prior to stimulation with AngII are presented in Fig. 4A and B, and a summary of the results of four to six independent experiments is presented in Fig. 4C.

Prior acidification of the extracellular medium (i.e. reduction of pH_o from 7.4 to 7.0), attenuated the increase in $[Ca^{2+}]_i$ caused by 1×10^{-10} M AngII, as evidenced by decreases in the rate-of-rise, the final magnitude and AUC of the Ca^{2+} signal. In contrast, the Ca^{2+} signal caused by 1×10^{-8} M AngII was not affected. At the lower concentration of AngII, the combined integrated Ca^{2+} signal (i.e. AUC) for H^+ (pH_o 7.0) plus AngII approximated the integrated signal for AngII alone at pH 7.4 (Fig. 4C). At 1×10^{-8} M AngII, in contrast, the combined integrated

Ca^{2+} signal for H^+ plus AngII was greater than that for AngII alone.

AngII-stimulated aldosterone secretion was not affected by an increase of pH_o from 7.4 to 7.8, but it was augmented by a reduction of pH_o to 7.0 (Fig. 5). The enhanced aldosterone secretion caused by extracellular acidification was manifested as an increase in the magnitude of the response rather than by an increase in the sensitivity to AngII; the EC_{50} of AngII at both pH_o 7.4 and 7.0 was approximately 7×10^{-10} M. Nonetheless, when the increase in aldosterone secretion caused by AngII (1×10^{-11} , 1×10^{-10} and 1×10^{-8} M) is examined as a function of the integrated AngII-stimulated Ca^{2+} signal (Fig. 6), it is apparent that a reduction of pH_o from 7.4 to 7.0 increased the sensitivity and gain of a more distal component(s) of the signaling cascade or the steroidogenic pathway to Ca^{2+} . For comparison, the increase in aldosterone secretion caused by both H^+ and AngII is also presented as a function of the sum of their integrated Ca^{2+} signals. As expected, the total steroidogenic response under acidotic conditions, as is the response under control conditions (pH_o 7.4), is directly related to the magnitude of the Ca^{2+} signal.

Discussion

The premise that H^+ (i.e. pH_o) has a modulatory role in the control of aldosterone secretion arose from correlations between plasma aldosterone concentration and acid-base status (Christleib *et al.* 1975, Kassirer *et al.* 1976, Perez *et al.* 1977, 1979, 1980, Schambelan & Sebastian 1977, Scott *et al.* 1978, Raff & Roarty 1988, Julian *et al.* 1982). The premise developed further in light of the ability of alterations in pH_o to affect aldosterone secretion *in vitro* (Muller 1965, Chiu & Freer 1979, Gilchrist *et al.* 1983,

Figure 2 Effects of changes in pH_o on transmembrane Ca^{2+} fluxes. Calcium efflux (A) was monitored simultaneously with $[\text{Ca}^{2+}]_i$ (see Fig. 1A) during superfusion of adherent glomerulosa cells previously loaded with fura2 and $^{45}\text{Ca}^{2+}$. Individual monolayers first were superfused with buffer of pH 7.4 for 15 min and then for an additional 30 min with buffer of the indicated pH. Data are presented as the mean $\pm$ S.E.M. of four experiments. For the measurement of Ca^{2+} influx (B), cells were incubated initially in HBSS/Hepes buffer of pH 7.4 for 60 min and then in fresh buffer of pH 7.4, pH 7.0 or pH 7.8 containing $2 \mu\text{Ci } ^{45}\text{Ca}^{2+}$. Intracellular accumulation of $^{45}\text{Ca}^{2+}$ was terminated at the indicated times by the addition of an equal volume of cold $\text{LaCl}_3/\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ (20 mM/200 mM, pH 6.0) solution. Values are expressed as the mean $\pm$ S.E.M. of five experiments.

Carroll *et al.* 1986, Radke *et al.* 1986a,b). Results of the present studies extend these previous observations and demonstrate that H^+ acts directly on glomerulosa cells to increase aldosterone secretion. Importantly, the effect of H^+ to increase aldosterone secretion was apparent over a range of pH_o *in vitro* that is compatible with the range of plasma pH evidenced *in vivo* under physiological and pathophysiological conditions (Singer & Hastings 1948).

Reductions of pH_o from 7.4 to 7.2 or 7.0 resulted in proportionate increases in the basal rate of aldosterone secretion. Given the action of aldosterone to promote Na^+ reabsorption by the collecting tubule and the reciprocal relationship between Na^+ reabsorption and H^+ excretion, an increase in aldosterone secretion resulting from a direct action of H^+ on glomerulosa cells might elicit a compensatory increase in proton excretion that contributes to the restoration of acid-base status during periods of acidosis. If such a mechanism operates *in vivo*, the present data suggest that it operates predominantly, if not solely, in response to an acid load. This seems incongruous with the conservation of H^+ that might be achieved secondary to a decrease in Na^+ reabsorption during alkalosis. However, independent effects of H^+ on aldosterone secretion may be less important under such circumstances than are effects resulting from its interaction with other agonists. In this context, metabolic alkalosis induced by infusion of NaHCO_3 in humans was associated with a fall in serum aldosterone without an accompanying change in serum K^+ , ACTH or renin activity (Jones *et al.* 1992, Yamauchi *et al.* 1997). Also, AngII- and K^+ -stimulated aldosterone secretion by perfused canine adrenal glands were attenuated by increases in pH_o comparable to those of the present study (Radke *et al.* 1986a,b).

A modulatory role for pH_o in the regulation of aldosterone secretion is reinforced by comparison of the cytosolic Ca^{2+} and steroidogenic responses elicited by H^+ with those elicited by AngII or K^+ . Whereas AngII (1×10^{-8} M) and K^+ (15 mM) caused approximately 10-fold increases in aldosterone secretion, H^+ (100 nM; pH 7.0) increased aldosterone secretion only about 2-fold over the same period. Also, the final increase in $[\text{Ca}^{2+}]_i$ caused by 100 nM H^+ was about one-tenth the increase caused by AngII or K^+ . Finally, the ability of H^+ to modulate increases in $[\text{Ca}^{2+}]_i$ and aldosterone secretion by cultured glomerulosa cells caused by a low concentration (e.g. 1×10^{-11} M) of AngII is not apparent at a maximal concentration (1×10^{-8} M) of AngII.

Comparisons of the data obtained in the present studies with data obtained over a similar range of pH_o in previous studies suggests an increased complexity to the actions of H^+ as well as the regulation of aldosterone secretion. For instance, Raff & Jankowski (1993) using bovine glomerulosa cells within 24–48 h of isolation failed to observe a change in basal aldosterone secretion in response to a reduction of pH_o from about 7.2 to 7.0–6.8. Gilchrist *et al.* (1983) using freshly isolated rat glomerulosa cells reported that basal aldosterone secretion was inhibited by both a reduction in pH_o from 7.4 to 7.1–6.8 and an elevation of pH_o to 7.7. Furthermore, Radke *et al.* (1986a,b) reported that basal aldosterone secretion by isolated perfused canine adrenal glands did not change in response to a decrease in pH_o from 7.4 to approximately 7.2, but increased in response to an elevation of pH_o to approximately 7.8.

Figure 3 Relationships between pH_o, aldosterone secretion and [Ca²⁺]_i. Aldosterone secretion was allowed to proceed for 2 h during static incubations of glomerulosa cells in HBSS/Hepes buffer adjusted to pH 7.0, 7.2, 7.4 or 7.8. Values represent the mean ± S.E.M. obtained from six to eight experiments. In each experiment, triplicate monolayers were incubated under each condition, and replicates averaged. Changes in [Ca²⁺]_i caused by alterations in pH_o from 7.4 were determined in four of the same cell preparations in which aldosterone secretion was measured. The Ca²⁺ response was integrated over the initial 15 min of the experimental period (see Fig. 1 for details) and is presented as an AUC. Values are presented as mean ± S.E.M.

Some discrepancies between results of these and the present studies may be attributed to alterations in extracellular Na⁺ concentration [Na⁺]_o, osmolality or initial pH_o; reasons for others are not readily explained. Although buffers as well as the means used to alter pH_o differed between studies, Radke *et al.* (1986a, b) concluded

that the effects observed using perfused adrenal glands resulted from H⁺ *per se*. Similarly, in cultured bovine glomerulosa cells, H⁺- and AngII-induced changes in pH_i, [Ca²⁺]_i and aldosterone secretion are qualitatively similar in Hepes- and bicarbonate-buffered media (Kramer 1991, R E Kramer, unpublished observations). Possibly, some

Table 2 Relationship of extracellular Ca²⁺ to H⁺-stimulated Ca²⁺ signals and aldosterone secretion. The effects of a reduction in pH_o from 7.4 to 7.0 on [Ca²⁺]_i, Ca²⁺ efflux and aldosterone secretion were compared in the presence (+Ca²⁺, 1.8 mM CaCl₂) and absence (−Ca²⁺; nominal Ca²⁺+0.05 mM EGTA) of extracellular Ca²⁺. Measurements of [Ca²⁺]_i and Ca²⁺ efflux were made simultaneously. After being loaded with both fura2 and ⁴⁵Ca²⁺, one set of cells (+Ca²⁺) was superfused with HBSS/Hepes buffer (pH 7.4) containing 1.8 mM CaCl₂ for 20 min and then with buffer of pH 7.0 for 15 min. A second set (−Ca²⁺) was superfused sequentially with HBSS/Hepes buffer (pH 7.4, 1.8 mM CaCl₂) for 15 min, nominal Ca²⁺/EGTA buffer of pH 7.4 for 5 min and, finally, nominal Ca²⁺/EGTA buffer of pH 7.0 for 15 min. Peak (4–5 min) and plateau (15 min) values for [Ca²⁺]_i and ⁴⁵Ca²⁺ efflux are in nM and percent respectively. In addition, changes in cytosolic [Ca²⁺]_i and Ca²⁺ efflux during the final 15 min experimental period were integrated and are presented as AUC (nM*min and percent*min respectively). Aldosterone secretion was assayed during static incubations using cell preparations different from those used to measure changes in cell Ca²⁺. Rates of aldosterone secretion by control cells (Hepes buffer, pH_o 7.4) were 26.4 ± 1.5 and 29.6 ± 2.2 pmol/2 h per mg protein respectively, in the presence and absence of extracellular Ca²⁺. All values represent the change elicited by reduction of pH_o relative to control (pH_o 7.4) and are expressed as the mean ± S.E.M. derived from four experiments

Group	Δ[Ca ²⁺] _i (nM or nM*min)			Δ ⁴⁵ Ca ²⁺ efflux (% or %*min)			Δ Aldosterone (pmol/2 h/mg protein)
	4–5 min	15 min	AUC	4–5 min	15 min	AUC	
+Ca ²⁺	139 ± 20	58 ± 9	1213 ± 74	3.1 ± 0.3	1.1 ± 0.3	16.4 ± 2.0	20.2 ± 2.1
−Ca ²⁺	−1 ± 1	1 ± 1*	−1 ± 4*	−0.2 ± 0.2*	−0.3 ± 0.2*	−4.2 ± 3.1*	−1.6 ± 1.4*

*P<0.05 vs +Ca²⁺.

Figure 4 Changes in AngII-stimulated Ca^{2+} signals caused by reduction of pH_o . After being superfused with HBSS/Hepes buffer of pH_o 7.4 for 15 min, adherent fura2-loaded glomerulosa cells were superfused for an additional 30 min with buffer of pH_o 7.4 or 7.0. AngII was added to the superfusate at 30 min. Representative changes in $[\text{Ca}^{2+}]_i$ caused by reduction of pH_o and by AngII (1×10^{-8} M or 1×10^{-10} M) at pH_o 7.4 and 7.0 are reported in (A) and (B), whereas mean $\pm$ S.E.M. peak, plateau and AUC for AngII-stimulated Ca^{2+} signals from five to eight experiments are reported in (C). Peak values were measured 2–3 (1×10^{-8} M) or 4–5 (1×10^{-10} M) min after addition of AngII, whereas plateau values were determined after 15 min. AUC for AngII-stimulated Ca^{2+} signals was calculated by integrating $[\text{Ca}^{2+}]_i$ from the time of addition of AngII to the end of the experimental period (i.e. from 30 to 45 min). The combined integrated Ca^{2+} signal caused by H^+ and AngII was calculated from the time of reduction of pH_o (i.e. 15 min). * $P < 0.05$ versus the AngII response at pH_o 7.4.

differences in aldosterone secretion observed following alterations in pH_o result from the presence of factors in the intact adrenal gland or the whole animal that modulate the response to H^+ . These factors, or their effects on glomerulosa cells, may be lost during the course of primary culture. Likewise, a higher 'basal' activity of freshly isolated cells may make subtle changes in aldosterone secretion caused by alterations in pH_o more readily apparent than they would be in cells maintained in long-term

culture. Subtle differences in aldosterone secretion also may be more apparent during perfusion than during static incubations. And, possibly the response of glomerulosa cells to changes in pH_o differs between species. Nonetheless, changes in aldosterone secretion by cultured bovine glomerulosa cells in response to reductions in pH_o noted here are in accord with those noted in humans in response to reductions in plasma pH in the absence of apparent changes in other potentially confounding variables (Jones *et al.* 1992). Moreover, cultured bovine glomerulosa cells respond to other physiological variables in a manner expected of the adrenal gland *in vivo* (Schneider & Kramer 1986, Kramer 1988a, Wang *et al.* 1992). In any event, the present data as well as much of the data from previous studies indicate that H^+ has a direct action on glomerulosa cells to increase aldosterone secretion.

The present data indicate that the mechanism by which H^+ increases aldosterone secretion is Ca^{2+} -dependent. Increases in aldosterone secretion associated with reductions in pH_o were accompanied by proportionate increases in $[\text{Ca}^{2+}]_i$ and Ca^{2+} efflux. The rate of Ca^{2+} influx was also increased upon initial reduction of pH_o , though this effect was not sustained. Nonetheless, influx of extracellular Ca^{2+} appears to be critical to the action of H^+ . Increases in $[\text{Ca}^{2+}]_i$, Ca^{2+} efflux and aldosterone secretion elicited by a reduction of pH_o in the presence of extracellular Ca^{2+} were abolished in the absence of extracellular Ca^{2+} . The participation of Ca^{2+} in the actions of H^+ is consistent with the importance of the divalent cation in the regulation of aldosterone secretion.

Although the present data indicate an important role for Ca^{2+} in the actions of H^+ , other ions may contribute to the overall steroidogenic response. Certainly, Na^+ and H^+ fluxes change concurrently with those of Ca^{2+} , and alterations in the intracellular concentrations of Na^+ and H^+ may affect aldosterone synthesis through mechanisms that are not directly related to $[\text{Ca}^{2+}]_i$ or Ca^{2+} flux across the plasma membrane (Jefcoate & Boyd 1971, Matsuoka *et al.* 1984, Rossier *et al.* 1987). A decrease in pH_i in glomerulosa cells occurs in response to extracellular acidification, and alterations of pH_i have been implicated in the stimulation of aldosterone secretion by AngII and K^+ (Horiuchi *et al.* 1989, Conlin *et al.* 1990, 1993). Similarly, intracellular Na^+ concentration ($[\text{Na}^+]_i$) increases in response to AngII (Van der Bent *et al.* 1993), and changes in $[\text{Na}^+]_i$ arising from increases in $\text{Na}^+/\text{Ca}^{2+}$ - and Na^+/H^+ -exchange may contribute to the steroidogenic response to H^+ . A resulting depolarization of the plasma membrane may affect $[\text{Ca}^{2+}]_i$ through activation of voltage-dependent Ca^{2+} channels. These and other possibilities are supported by the ability of pH to influence membrane potential, ion conductances, inositol 1,4,5-trisphosphate (IP_3)- and Ca^{2+} -induced Ca^{2+} release, and diacylglycerol-induced association of protein kinase C with plasma membrane in other cell types (Albuquerque & Leffler 1998, Hulme & Orchard 1998, Nishino *et al.* 1998,

Figure 5 Effects of pH_o on AngII-stimulated aldosterone secretion. Aldosterone secretion by adherent glomerulosa cells was measured under static conditions at pH_o 7.8, 7.4 or 7.0 in the absence or presence of AngII (1×10^{-12} to 1×10^{-7} M). The insert depicts aldosterone secretion as a function of pH_o in the absence of AngII. For each experiment, mean secretory rates of control (i.e. pH_o only) cells were subtracted from the secretory rate of AngII-treated cells incubated at the same pH_o . Data represent the mean $\pm$ S.E.M. of four independent experiments; in each, rates measured in triplicate cultures were averaged to give a single value. * $P < 0.05$ vs the same AngII concentration.

Peng *et al.* 1998, Wu & Fry 1998, Kourie 1999). Interrelationships between Ca^{2+} , H^+ and Na^+ fluxes and cytosolic concentrations provide potential means through which pH_o may independently affect aldosterone secretion. Such interactions may also allow pH_o to modulate the actions of AngII and K^+ as well as other factors, notably extracellular osmolality (Schneider & Kramer 1986, Wang *et al.* 1992) and $[\text{Na}^+]_o$ (Saruta *et al.* 1972, Lobo *et al.* 1978), that influence aldosterone secretion through Ca^{2+} -dependent mechanisms.

Indeed, results of the present studies confirm previous reports (Chiu & Freer 1978, Radke *et al.* 1986b, Raff & Jankowski 1993) that reductions in pH_o increased AngII-stimulated aldosterone secretion. Some differences, however, in the specific effects of H^+ are apparent. For example, aldosterone secretion by perfused dog adrenals was directly related to $[\text{H}^+]_o$ over a range of approximately 16 to 70 nM (pH_o 7.8 to 7.16) (Radke *et al.* 1986b), whereas aldosterone secretion by cultured bovine glomerulosa cells was not significantly affected at a $[\text{H}^+]_o$ of less than 40 nM (pH_o 7.4). In rabbit glomerulosa cells, a reduction of pH_o from 7.4 to 6.5 increased both the sensitivity to AngII and the magnitude of the secretory

response (Chiu & Freer 1978). In the present studies, only the secretory response to AngII was increased, albeit by a smaller reduction of pH_o (from 7.4 to 7.0).

The mechanisms by which H^+ modulates AngII-stimulated aldosterone secretion are presently unclear, but appear to differ from the means by which it independently increases basal aldosterone secretion. Increased association of AngII with its receptor (Carroll *et al.* 1986) can not account for the increase in AngII-stimulated aldosterone secretion caused by H^+ reported here. To the contrary, transduction between AngII receptor binding and generation of a Ca^{2+} signal was attenuated by H^+ . This effect may be partially explained by decreased binding of IP_3 in adrenocortical microsomes and, consequently, decreased Ca^{2+} mobilization (Guillemette & Segui 1988, Rossier *et al.* 1989). It may be rationalized also by the fact that H^+ independently elicited a Ca^{2+} signal. If it did so through a mechanism common to the action of AngII, the magnitude of the AngII-stimulated Ca^{2+} -signal would be correspondingly decreased. In keeping with a modulatory role for H^+ , attenuation of the AngII-stimulated Ca^{2+} -signal would be greater at lower concentrations of the peptide than it would be at higher concentrations. This expectation is

Figure 6 Effect of extracellular acidification on the relationship between AngII-stimulated Ca^{2+} and secretory responses. Increases in aldosterone secretion and $[\text{Ca}^{2+}]_i$ caused by AngII (1×10^{-11} , 1×10^{-10} and 1×10^{-8} M) were measured in parallel incubations in medium of pH_o 7.4 or 7.0. In each instance, responses to AngII were corrected for basal (pH_o 7.4 or 7.0 without AngII) values and were concentration-dependent. The Ca^{2+} signal was integrated and is expressed as an AUC. Also, the combined increase in aldosterone secretion caused by H^+ plus AngII is plotted as a function of the combined integrated Ca^{2+} response. Data represent the mean $\pm$ S.E.M. for four independent experiments.

consistent with data presented in Fig. 4 as well as an effect of H^+ to decrease binding of IP_3 in adrenocortical microsomes while not affecting maximal IP_3 -stimulated Ca^{2+} release over the range of pH used in the present studies (Guillemette & Segui 1988). The effect of H^+ to enhance AngII-stimulated aldosterone secretion in the presence of a reduced AngII-stimulated Ca^{2+} signal indicates an action of H^+ distal to Ca^{2+} mobilization. Given the obligatory role for Ca^{2+} in AngII-stimulated aldosterone secretion, the present data also suggest that this late action(s) results in a greater Ca^{2+} sensitivity of a more distal component of the AngII-dependent signaling cascade. In this respect, the transient Ca^{2+} signal (or another intracellular signal) elicited by H^+ may partially substitute for the initial 'trigger' Ca^{2+} mobilized by AngII (Barrett *et al.* 1989) and contribute to the gain of AngII-stimulated aldosterone secretion. Alternatively, H^+ may enhance the activation of adenylyl cyclase by AngII (Baukal *et al.* 1994, Python *et al.* 1995). Stimulation through separate, but convergent, Ca^{2+} - and cAMP-dependent pathways could account for the potentiating effect of H^+ on aldosterone secretion at low concentration of AngII. The present data, however, do not directly address these possibilities.

Although H^+ increased both basal and AngII-stimulated aldosterone secretion, the site in the steroidogenic pathway

at which it acts remains to be identified. It would be expected that an effect of H^+ to increase aldosterone secretion would be manifested at the level of cholesterol side-chain cleavage or conversion of corticosterone to aldosterone. These reactions limit aldosterone biosynthesis and are subject to regulation by AngII and other primary agonists (McKenna *et al.* 1978, Aguilera *et al.* 1980, Kramer *et al.* 1980). An increase in the activity of either enzyme, or both enzymes, in response to H^+ would reasonably explain the increases in aldosterone secretion caused by reduction of pH_o . Further, an effect of H^+ to increase the transfer of cholesterol to mitochondria, with or without a concomitant effect to increase its translocation from the outer to the inner membrane, could account for the greater Ca^{2+} sensitivity of AngII-stimulated aldosterone secretion. Jefcoate & Boyd (1971) presented evidence that H^+ directly stimulated cholesterol side-chain cleavage in isolated bovine adrenocortical mitochondria, and Radke *et al.* (1986b) suggested that H^+ increased the conversion of corticosterone to aldosterone by perfused canine adrenals. However, neither the rate of pregnenolone production from endogenous cholesterol nor the rate of aldosterone production from exogenous corticosterone was affected by reduction of pH_o from 7.2 to 6.8 (Raff & Jankowski 1993).

In summary, the present data indicate that H^+ has direct actions on glomerulosa cells to increase $[Ca^{2+}]_i$ and promote aldosterone secretion. In light of these actions, pH_o participates in the regulation of aldosterone secretion. These actions of H^+ contribute to changes in plasma aldosterone concentration associated with disturbances in acid–base balance by changing the basal rate of aldosterone secretion as well as by modulating the responses to other secretagogues, notably AngII. Furthermore, in light of results of these and previous studies, it seems reasonable to predict that the actions of H^+ are themselves subject to modulation; the direction and magnitude of the response being determined by the concentration of primary agonists as well as other factors that modulate aldosterone secretion.

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