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Nongenomic Effects of Aldosterone on Intracellular pH in Vascular Smooth Muscle Cells

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The aim of the present study was to investigate rapid effects of aldosterone and other steroids on intracellular pH of vascular smooth muscle cells and to compare these effects with those of peptide hormones. After addition of 100 nmol/L aldosterone, initial acidification is followed by significant alkalinisation occurring within two minutes, while 1 μmol/L hydrocortison does not affect intracellular pH. The initial response to 100 nmol/L angiotensin II is similar; however, subsequent alkalinization is not seen for this agonist. PDGF induces an initial acidification followed by a minor recovery so that cells remain acidified for eight minutes. Both pH recovery after angiotensin II and alkalinization after aldosterone were blocked in sodium-free medium. These results demonstrate rapid effects of aldosterone on intracellular pH in vascular smooth muscle cells, which include final alkalinization not seen after angiotensin II or PDGF.

Recently, rapid in vitro effects of aldosterone on sodium, potassium and calcium concentrations and cell volume of human mononuclear leukocytes (HML; 1–3), and on the activity of the sodium-proton-exchanger of the cell membrane in HML and vascular smooth muscle cells (VSMC; 4–6) have been demonstrated. Not being compatible with the involvement of the classical type-I-mineralocorticoid receptors, these nongenomic effects rather suggested the existence of distinct receptors which were subsequently described in plasma membranes from HML and pig kidney and liver (7–9). The phosphoinositide pathway, protein kinase C and free intracellular calcium appear to be involved in intracellular signalling in HML and VSMC (4, 10–12). In the present paper, rapid effects of aldosterone on intracellular pH were investigated in cultured VSMC by single cell imaging of BCECF fluorescence and compared with effects of angiotensin II and PDGF.

MATERIALS AND METHODS

Aldosterone and nigericin were from Fluka (Buchs, Switzerland); hydrocortisone was from Sigma (St. Louis, MO) and angiotensin II and PDGF-BB were from Calbiochem (Bad Soden, Germany). HOE694 was a gift from Hoechst AG (Frankfurt/Main, Germany), 2′-7′-bis (carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) purchased from Molecular Probes, Inc. (Eugene, OR). The other reagents were from Merck (Darmstadt, FRG, analytical grade).

Culture of vascular smooth muscle cells. The investigation complies with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). VSMC were prepared enzymatically from rat (Sprague-Dawley) thoracic aortae, as described earlier (13, 14). Cells were grown in Waymouth M752/Ham F12 medium supplemented with 10% FCS after enzymatic digestion. Early passage cells (passages 1–4) grown on cover slips were used 48–96 h after seeding, medium was changed 24 hours after seeding on cover slips then every 48 hours. Before the experiments, VSMC were growth arrested by serum deprivation for 24 hours. The depriving medium was identical to growth medium except that FCS had been replaced by 0.1% bovine serum albumin (BSA).

Measurement of intracellular pH. Imaging of free intracellular pH was performed in single cells which were washed 3× with 2 mL physiological salt solution (PSS; 135 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4) to remove serum and loaded with 4 μmol/L BCECF from a 2 mmol/L stock

Abbreviations: BCECF, 2′–7′-bis (carboxyethyl)-5(6)-carboxyfluorescein; HML, human mononuclear leukocytes; PDGF, platelet-derived growth factor; VMSC, vascular smooth muscle cells.

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solution in dimethyl sulfoxide for 30 minutes at 37°C. At the end of the loading period, cells were washed with PSS-buffer (3 × 2mL) and used within one hour. They were placed in a thermostatically controlled ring chamber (37°C; Life Science Resources Ltd., Cambridge, UK). Drugs were added in volumes of 100–200 μL to a final volume of 0.3–1 mL PSS-buffer. For experiments in Na+ free PSS, NaCl was replaced by equimolar amounts of choline chloride.

Cell imaging of pH was performed using a dual wavelength imaging system (Till Photonics GmbH, Gräfelfing, Germany) attached to a Zeiss Axiovert 35 (Zeiss, Hanau, Germany) inverted fluorescence microscope with a Fluor 40/1.30 oil immersion objective. The imaging camera was a AE2000 system from General Scanning GmbH (Planegg, Germany). Excitation wavelengths were 500 and 440 nm, and emitted light 540 nm collected via a dichroic mirror at 510 nm. Integration time was 0.10 s at excitation wavelengths of 500 and 440 nm, and a time increment of 6 s. Autofluorescence was determined in unloaded cells and subtracted from each reading before calculation of basal pH. The measurements of intracellular pH in VSMC were performed according to the method of Thomas et al. (15). The calibration curve was obtained by permeabilizing the cells with 7 μg/mL nigericin in PSS containing 140 mmol/L KCl instead of NaCl adjusted to pH 6.8, 7.0, 7.4 and 7.6. Steroids, peptides and HOE694 at the concentrations employed were checked for autofluorescence which was insignificant for the conditions mentioned above. All readings were checked for stability of baseline for 2 min. At times indicated, agonists were added from stock solutions (10 mmol/L) in ethanol. pH was analysed on serial images in three regions of interest (ROI) in the nucleus, in the perinuclear region of the cell, and one near the plasmalemma. Statistical comparisons were done by the nonparametric Mann-Whitney U-test. A $p < 0.05$ was considered statistically significant. Means ± SE are given.

RESULTS

Resting intracellular pH in VSMC was 7.17 ± 0.016 (n = 20). In Figure 1, typical traces of intracellular pH are shown in response to the addition of 100 nmol/L aldosterone, 100 nmol/L angiotensin II or 100 ng/mL PDGF. As can be seen, shapes of all responses are different: after aldosterone, a transient minor acidification is followed by a continued alkalinization reaching a new plateau about 0.1 pH unit above the initial baseline within 6–8 min. Angiotensin II induces acidification which recedes to initial values within ten minutes. PDGF induces acidification without subsequent regression to initial values. These different types of responses have also been analysed numerically; results are shown in Table 1. It is obvious, that only for aldosterone a significant alkalinization is finally seen. The table also shows that the latency for a deflection of the pH curve was shortest for angiotensin II with effects of aldosterone and PDGF being delayed by two-fold.

In the presence of the specific inhibitor of the sodium-proton-exchanger, HOE694, alkalinization is absent regardless of the agonist used (Figure 2; n = 7–12). HOE694 induces a slight baseline shift towards lower pH. In sodium-free medium, acidification, but no alkalinization is seen in response to the agonists (not shown).

The glucocorticoid hydrocortisone is inactive at a concentration of 100 nmol/L; at 1 μmol/L, acidification, but no alkalinization is seen (Figure 3; n = 6–8). There were no obvious differences in the time course or extent of pH changes with regard to ROI's in different areas of the cells. The effect of aldosterone on pH in ROI's is exemplified in Figure 4, but there were no obvious

TABLE 1
Changes of pH in VSMC by Different Stimuli

	ΔpH vs. baseline at pH minimum	ΔpH vs. baseline at the end of the experiment	latency [s] of pH deflection	n
aldosterone	-0.044^{xx}	$+0.093^{xx}$	28.5	12
[100 nmol/L]	±0.010	±0.025	±3.39	
angiotensin II	-0.032^{xx}	$+0.018^{aa}$	12.00^{a}	14
[100 nmol/L]	±0.004	±0.005	±1.26	
PDGF	$-0.146^{xx,aa}$	$-0.127^{xx,aa}$	30.00	5
[100 ng/ml]	±0.017	±0.036	±2.68	

xx: $p < 0.01$ vs. baseline.
a, aa: $p < 0.05/0.01$ vs. aldosterone.

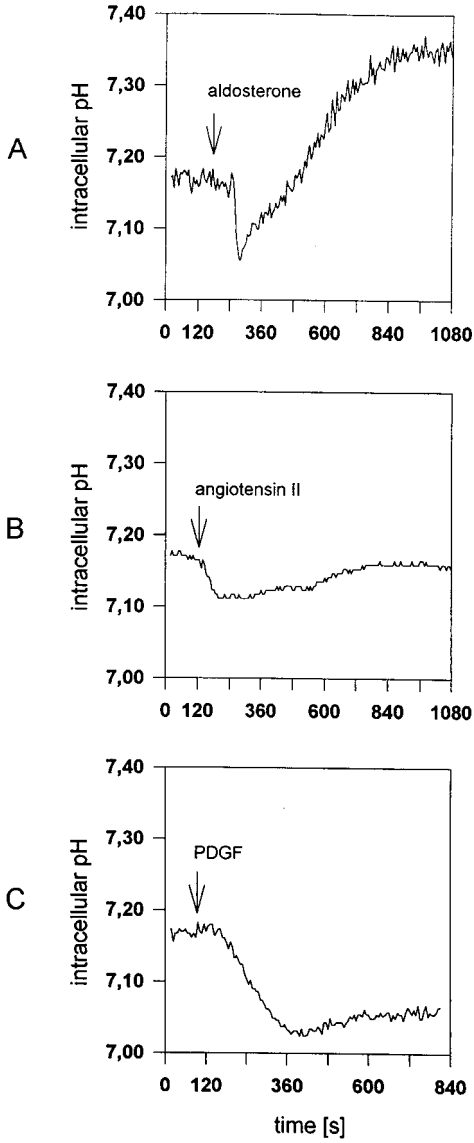

FIG. 1. Representative traces of intracellular pH as determined by BCECF fluorescence in single vascular smooth muscle cells in response to various stimuli. At times indicated, 100 nmol/L aldosterone (A), 100 nmol/L angiotensin II (B) or 100 ng/mL PDGF (C) were added.

differences in the localization of pH changes induced by angiotensin II and PDGF as well (not shown).

DISCUSSION

The main finding of this paper is the demonstration of a rapid effect of 100 nmol/L aldosterone, but not cortisol on intracellular pH in VSMC. Compared with the effects of angiotensin and PDGF, aldosterone was the only agonist which induced a persistent alkalinization after an initial acidification of the cell. Alkalinization most likely reflects the increased activity of the sodium-proton-exchanger as it is blocked in sodium- free medium (16) and by the specific inhibitor HOE694 (17). It should be mentioned that the experiments were performed in bicarbonate-free buffer thus ex-

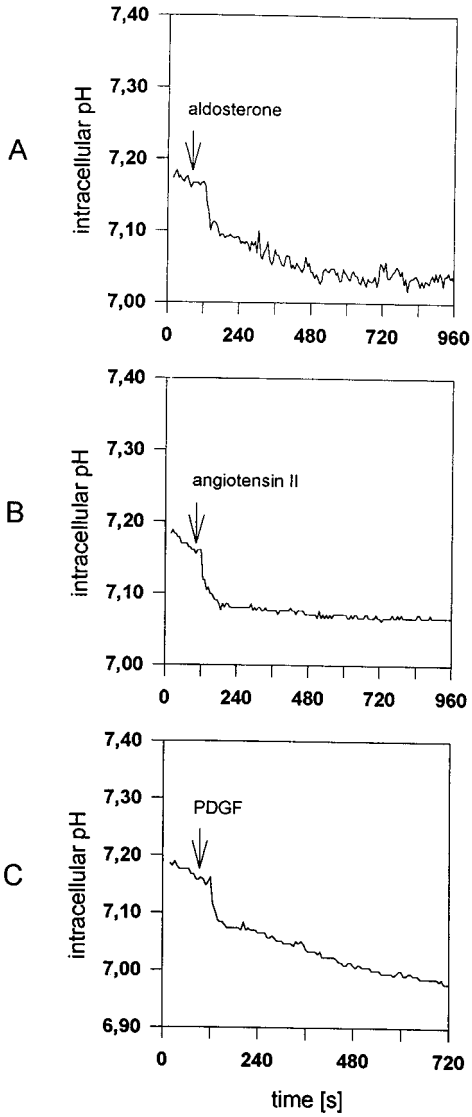

FIG. 2. Agonist effects on intracellular pH in normal PSS containing 10 mol/L HOE694 determined by BCECF fluorescence in single vascular smooth muscle cells. Traces are shown for 100 nmol/L aldosterone (A), 100 nmol/L angiotensin II (B) or 100 ng/mL PDGF (C) added at times indicated.

cluding Cl^-/HCO_3^- exchange which might have obscured the effect. The results reported here are in perfect agreement with previous studies on rapid aldosterone effects both in HML and VSMC. A significant stimulation of the sodium-proton-exchanger was found in these cells as early as 2–4 minutes after application of aldosterone at an EC_{50} of $\sim$0.1 nmol/L with hydrocortisone being inactive at 1000-10.000 fold higher concentrations. The specificity of rapid aldosterone effects is a landmark feature of this novel pathway of nongenomic mineralocorticoid action and was found here as well (18).

The interpretation of the data presented would be compatible with the assumption, that all agonists employed here primarily lead to an increased proton liberation which is reflected by the initial acidification of the cell as shown earlier (19). In turn, activation of the sodium-proton-

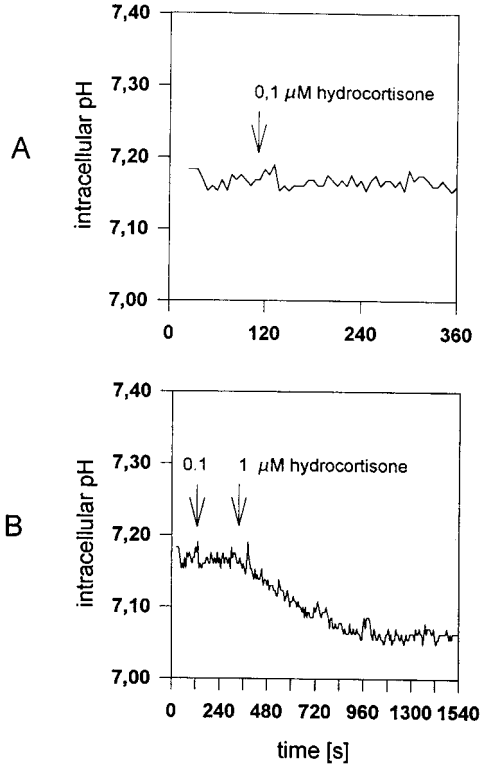

FIG. 3. Representative traces of intracellular pH as determined by BCECF fluorescence after the addition of 0.1 μmol/L (A) and 0.1 μmol/L followed by 1 μmol/L (B) hydrocortisone.

exchanger tends to counteract this increased proton load resulting in complete or partial regression of pH-shifts towards base-line. This activation could just reflect a passive increase of activity at lower pH; however, this assumption alone is not sufficient to fully explain the effect of aldosterone which after an initial acidification tends to further alkalinize the cell above baseline values.

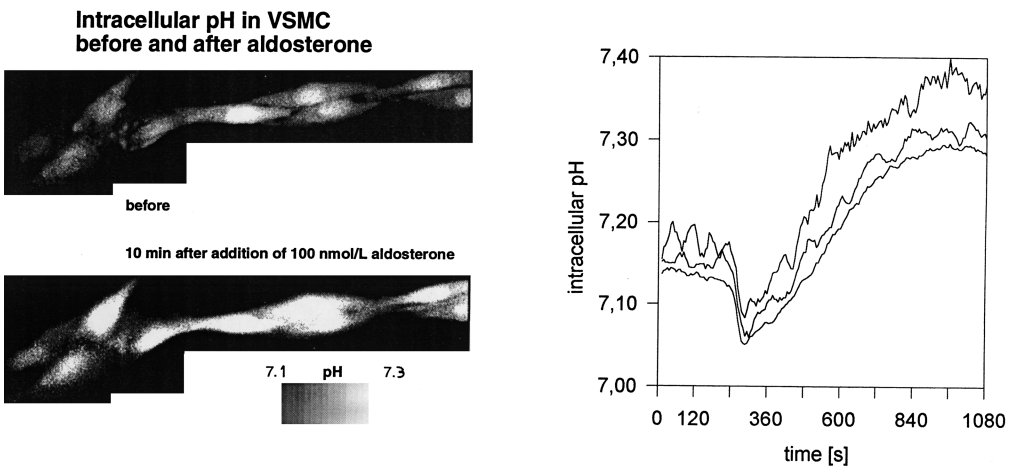

FIG. 4. Images of intracellular pH (left) and time courses of pH in three different regions of interest in the nucleus, in the perinuclear space and in the subplasmalemmal space (right) are shown.

Excessive alkalinization of vascular smooth muscle cells in response to angiotensin II has been shown for cells from spontaneously hypertensive rats and for suspended VSMC from Wistar Kyoto rats whereas in coverslip cells from Wistar Kyoto rats, as reported here, no excessive alkalinization was seen in response to angiotensin II (20, 21). This shows that under certain circumstances angiotensin II might also act as a direct stimulator of the sodium-proton-exchanger. It is yet not clear which biochemical features of cells from hypertensive rats or of trypsinized cells are important in this regard, but differential influences of pathological states (hypertensive rats) or trypsinization on signalling pathways may be assumed. Unlike those former results on free intracellular calcium in VSMC (22), there was no obvious difference in the changes of pH for different cellular compartments.

In conclusion, these data on acute changes of intracellular pH in VSCMM by aldosterone extend earlier observations of nongenomic mineralocorticoid effects on intracellular second-messengers and the sodium-proton-exchanger. Further analysis of the pH response to different stimuli should target the assessment of the relative importance of contributing signalling pathways and metabolic alterations.

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REFERENCES

1. Wehling, M., Armanini, D., Strasser, T., and Weber, C. (1987) *Am. J. Physiol.* **252,** E505–E508.
2. Wehling, M., Käsmayr, J., and Theisen, K. (1990) *Cell Calcium* **11,** 565–571.
3. Wehling, M., Kuhls, S., and Armanini, D. (1989) *Am. J. Physiol.* **257,** E170–E174.
4. Christ, M., Douwes, K., Eisen, C., Bechtner, G., Theisen, K., and Wehling, M. (1995) *Hypertension* **25,** 117–123.
5. Wehling, M., Käsmayr, J., and Theisen, K. (1989) *Biochem. Biophys. Res. Com.* **164,** 961–967.
6. Wehling, M., Käsmayr, J., and Theisen, K. (1991) *Am. J. Physiol.* **260,** E719–E726.
7. Christ, M., Sippel, K., Eisen, C., and Wehling, M. (1994) *Mol. Cell. Endocrinol.* **99,** R31–R34.
8. Wehling, M., Christ, M., and Theisen, K. (1992) *Am. J. Physiol.* **263,** E974–E979.
9. Meyer, C., Christ, M., and Wehling, M. (1995) *Eur. J. Biochem.* **229,** 736–740.
10. Christ, M., Eisen, C., Aktas, J., Theisen, K., and Wehling, M. (1993) *J. Clin. Endocrinol. Metab.* **77,** 1452–1457.
11. Christ, M., Meyer, C., Sippel, K., and Wehling, M. (1995) *Biochem. Biophys. Res. Com.* **213,** 123–129.
12. Wehling, M., Neylon, C. B., Fullerton, M., Bobik, A., and Funder, J. W. (1995) *Circ. Res.* **76,** 973–979.
13. Brown, M. L., and Deykin, D. (1991) *Arteriosclerosis Thromb.* **11,** 167–173.
14. Little, P. J., Cragoe, E. J., and Bobik, A. (1986) *Am. J. Physiol.* **251,** C707–C712.
15. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* **18,** 2210–2218.
16. Berk, B. C., Brock, T. A., Gimbrone, M. A. Jr., and Alexander, R. W. (1987) *J. Biol. Chem.* **262,** 5065–5072.
17. Woll, E., Ritter, M., Offner, F., Lang, H. J., Schölkens, B., Hussinger, D., and Lang, F. (1993) *Eur. J. Pharmacol.* **246,** 269–273.
18. Wehling, M. (1994) *Trends Endocrinol. Metab.* **5,** 347–353.
19. McConnell, H. M., Owicki, J. C., Parce, J. W., Miller, D. L., Baxter, G. T., Wada, H. G., and Pitchford, S. (1992) *Science* **257,** 1906–1912.
20. Berk, B. C., Aronow, M. S., Brock, T. A., Cragoe Jr, E., Gimbrone Jr, M. A., and Alexander, R. W. (1987) *J. Biol. Chem.* **262,** 5057–5064.
21. Berk, B. C., Vallega, G., Muslin, A. J., Gordon, H. M., Canessa, M., and Alexander, R. W. (1989) *J. Clin. Invest.* **83,** 822–829.
22. Wehling, M., Ulsenheimer, A., Schneider, M., Neylon, C., and Christ, M. (1994) *Biochem. Biophys. Res. Com.* **204,** 475–481.