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EVIDENCE FOR A NA^+/H^+ ANTIPORT IN STOMACH SMOOTH MUSCLE CELLS

Trevor J. Finkle and A. K. Grover

Department of Neurosciences,
McMaster University Health Sciences Centre,
Hamilton, Ontario, Canada. L8N 3Z5

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SUMMARY: Single smooth muscle cells were isolated from circular muscle of the canine gastric corpus by collagenase incubation. Cytoplasmic pH (pH_i) of these cells was measured fluorometrically using the trapped dye 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein. Cells were examined for their Na^+/H^+ exchange activity after intracellular acidification. Cells acid-loaded by propionate exposure, the NH_4^+ prepulse technique or suspension in a Na^+-depleted medium regained almost normal pH_i upon exposure to a Na^+ medium. The Na^+-dependent alkalinization was amiloride sensitive. As well, addition of amiloride to cells suspended in a Na^+ medium caused a concurrent decrease in pH_i. The study indicates that a Na^+/H^+ antiport is present in these smooth muscle cells. © 1988 Academic Press, Inc.

The amiloride sensitive cellular membrane transport system responsible for the exchange of extracellular Na^+ for intracellular H^+ has been described for many cell types (1-5). It may be involved in regulation of cell volume (6,7) and intracellular pH (pH_i) (1,3). Recent reports indicate this antiport is present in cultured vascular smooth muscle cells of the rat (8,9). Sodium influx in cultured vascular smooth muscle cells, by this pathway, can be activated by the vasoconstrictor peptide angiotensin II (10-12) and by the mitogenic agent PDGF (11,13). Thus, the Na^+/H^+ antiport may be very important in both excitation-response

Abbreviations: BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxy-fluorescein; pHi, intracellular pH; NMG^+, N-methyl-d-glucamine.

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coupling and cellular proliferation in vascular smooth muscle. Exposure of gastric smooth muscle to low pH is known to cause an increase in baseline tension, a reduction in amplitude and frequency of spontaneous contractions and impairment of relaxation (14). In pathological conditions a damage of the mucosal barrier may result in a low Na^+ concentration and a high pH in the interstitial tissue bathing the gastric smooth muscle cells (15,16). Little is known, however, about Na^+/H^+ exchange and its involvement in intracellular pH regulation in gastrointestinal or other nonvascular smooth muscle. In this present study, freshly isolated contractile smooth muscle cells of the dog corpus were examined for Na^+/H^+ activity. To demonstrate Na^+ influx by this transport system, cytoplasmic pH was manipulated.

Materials and Methods

Chemicals: BCECF-AM was obtained from HSC Research Development Corporation (Toronto, Ontario). Vitamins and amino acids contained in Hepes-buffered vitamin solution were purchased in concentrated form from Gibco (Grand Island, New York). Sodium pyruvate was from Boehringer Mannheim (West Germany). All other reagents were purchased from Sigma.

Solutions: Hepes-buffered vitamin solution contained (in mM) 124 NaCl, 4.9 KCl, 3.1 NaH_2PO_4 H_2O, 6.15 sodium glutamate, 6.15 sodium fumarate, 6.15 sodium pyruvate, 14.2 glucose, 23.5 Hepes, 1.8 $CaCl_2$, 1.2 $MgSO_4$, and 2% (vol/vol) amino acid mixture, 1% (vol/vol) vitamin solution, 0.1% (wt/vol) fatty acid free bovine serum albumin and 0.01% (wt/vol) trypsin inhibitor. The solution was adjusted to a pH of 7.4 with NaOH. The osmolarity was 297 mosmol.

The NaCl-medium contained (in mM) 135 NaCl, 5.8 KCl, 2.2 KH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgCl_2$ $6H_2O$, 25.0 Hepes and 12.0 glucose with 0.1% (wt/vol) fatty acid free bovine serum albumin. The medium was adjusted to a pH of 7.4 with Tris. The osmolarity was 297 mosmol. In NMG^+-medium and $choline^+$-medium, NaCl was isosmotically replaced with NMGCl and choline chloride.

Cell isolation: Isolated smooth muscle cells were prepared using modifications of the techniques previously outlined by Collins and Gardner (17) and Collins and Crankshaw (18). Briefly, stomachs were removed from sodium pentobarbitol-anesthetized dogs and the circular muscle dissected from the gastric corpus. The muscle was sliced into thin sheets using a tissue slicer, cut into smaller pieces and filtered through 508 um nylon mesh. During this procedure, the tissue was maintained in Hepes-

buffered vitamin solution. The tissue was then incubated in a Dubnoff shaking water bath (20 oscillations/minute) for two successive 25 minute periods at 31°C in Hepes-buffered vitamin solution containing 0.1 % (wt/vol) collagenase (Sigma Type II) and 0.1% (wt/vol) soybean trypsin inhibitor. The digested tissue was then filtered through 200 um nylon mesh, washed and placed in a minimal volume of Na^+-medium. The tissue was left in Na^+-medium for 1 hour in a shaking water bath at 37°C. During this time the cells dispersed. The cell suspension and tissue was subsequently filtered once again and the remaining suspension of cells kept in a water bath at 37°C. This isolation method usually yielded 12-15 ml of cell suspension at a concentration of 2-5 x 10^5 cells/ml. Throughout cell isolation and incubation the solutions were perfused with 100% O_2.

For fluorometric studies, cells were incubated for 30 min. at 37°C in 2-3 ug BCECF per ml of cell suspension. Excess dye was then removed by centrifugation at 200g for 5 min. and the pellet rinsed to remove any remaining dye. The cells were resuspended in a fresh volume of NaCl-medium to a final concentration of 1-2 x 10^5 cells/ml.

Intracellular pH measurements were performed using a Spex Fluorolog fluorescence spectrometer at 37°C while stirring. An excitation wavelength of 500 nm and an emission wavelength of 530 nm were used. Intracellular pH was calculated by calibrating fluorescence against pH after cell disruption with 0.1 % (final) Triton X-100. A red shift correction factor was determined independently by the nigericin technique (1,19) and applied to measurements obtained with Triton X-100. All experiments were repeated at least 4 times.

Results and Discussion

Na^+/H^+ exchange activity can be monitored by inducing intracellular acidification and then observing the recovery of pH_i in a physiological solution with or without Na^+. Dog stomach smooth muscle cells were acid-loaded by introduction of propionate or preincubation with NH_4Cl (20,21).

Smooth muscle cells suspended in NMG^+-medium exhibited a rapid intracellular acidification upon addition of NMG-propionate (Fig. 1) as expected (20). Intracellular pH dereased to approximately 6.75 in the prescence of 10 mM NMG-propionate. Following the decline in pH_i there was a slow recovery phase which was unaffected by the addition of KCl or NMGCl. In contrast, a rapid return to normal pH_i (7.1-7.2) could be induced by NaCl (Fig. 1a). This alkalinization could largely be abolished by amiloride (Fig. 1b) which is known to inhibit Na^+/H^+ exchange (22).

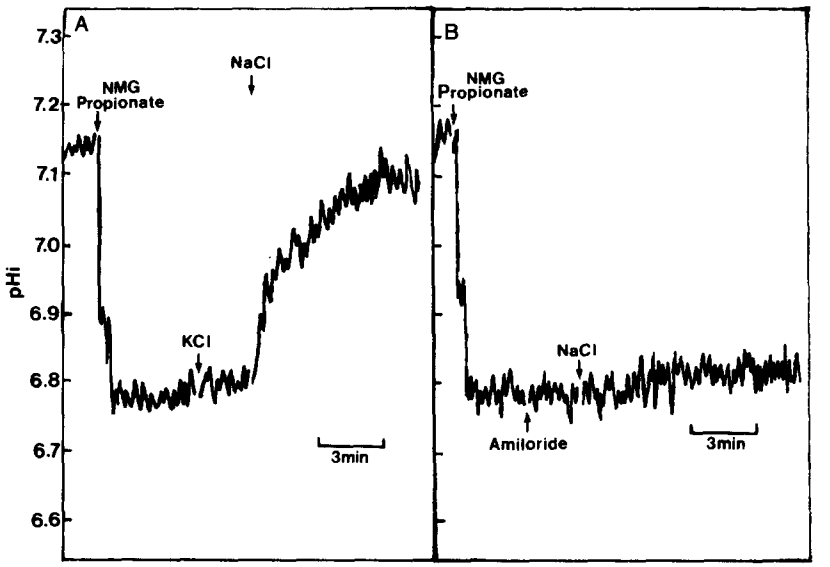

Fig. 1. Intracellular pH (pH_i) measurements in propionic acid-loaded smooth muscle cells. 1-2 x 10^5 cells/ml were suspended in 135mM NMG^+-medium. Where indicated 25mM (final) NMG-propionate was added. After maximum intracellular acidification was achieved either KCl and NaCl were added to a concentration of 50mM respectively (A) or 1mM amiloride (final) and 50mM (final) NaCl were added (B).

The Na^+-dependent recovery of pH_i after an acid load could also be demonstrated using the NH_4^+ prepulse technique. Smooth muscle cells resuspended in Na^+-medium after removal of NH_4Cl exhibited a rapid recovery in pH_i that was not induced in either NMG^+ (Fig. 2A) or choline$^+$ substituted (not shown) Na^+-medium. The alkalinization in Na^+-medium could, again be prevented by amiloride (Fig 2B). Results from these acid-loading experiments indicate that sodium influx via a Na^+_o/H^+_i antiport may be activated by intracellular acidification. This has been demonstrated in cultured vascular smooth muscle cells using the ionophore nigercin (8,9).

To further elucidate the role extracellular Na^+ has in regulation of pH_i, circular smooth muscle cells were suspended in an NMG^+ or a choline$^+$ medium. Exposure to a Na^+-depleted medium resulted in a slow decrease in pH_i (Fig 3A) over time. Addition of NaCl quickly restored normal pH_i. In the prescence of

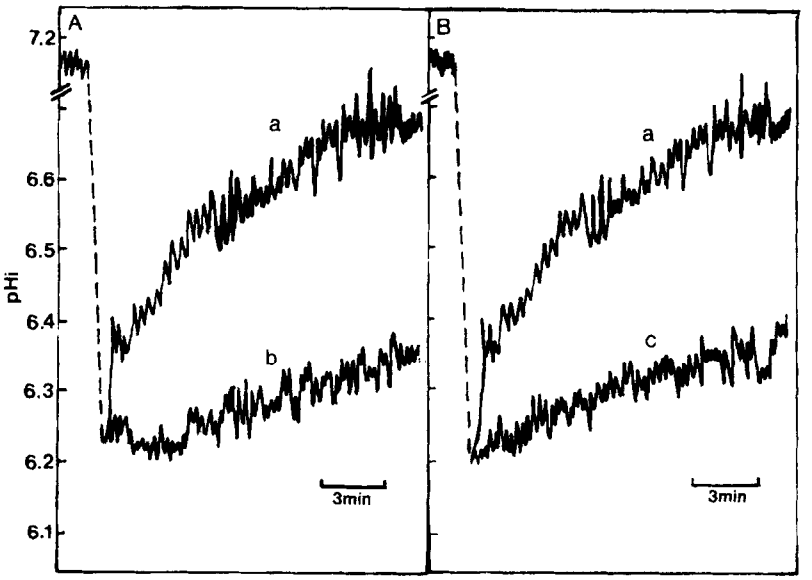

Fig. 2. Intracellular pH (pH_i) measurements in smooth muscle cells after preincubation with NH_4Cl. 1-2x10⁵ cells/ml suspended in Na^+-medium were briefly centrifuged and resuspended in Na^+-medium containing 50mM NH_4Cl (85mM NaCl) for 5 min. at room temperature. Cells were then recentrifuged and resuspended in 135mM NaCl medium (curve a), 135mM NMG chloride medium (curve b), 135mM choline chloride medium (curve b) or 135mM NaCl medium with 1mM (final) amiloride added (curve c). Dotted line represents the time during which the cells were incubated with NH_4Cl.

amiloride, NaCl had no affect and pH_i declined to a minimum value of appproximately 6.9. Based on this experiment it would appear that extracellular Na^+ was necessary for maintenance of normal pH_i. Since amiloride prevented recovery of pHi after additon of NaCl, Na^+-influx seemed to be mediated by a Na^+/H^+ antiport. To support this statement another experiment was performed in

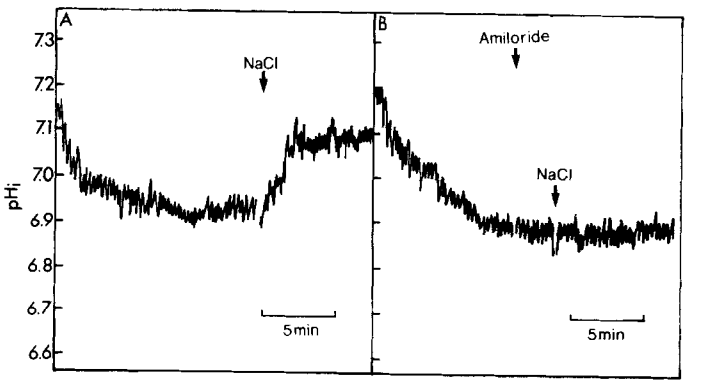

Fig. 3. Dependence of pHi on Na^+/H^+ antiport activity. 1-2x10⁵ cells/ml were suspended in 135mM NMG chloride medium or 135mM choline chloride medium. Where indicated NaCl was added to a final concentration of 50mM (A) or 1mM (final) amiloride and 50mM (final) NaCl were added (B).

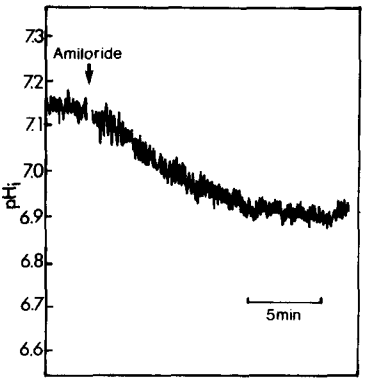

Fig. 4. Effect of amiloride on Na^+/H^+ activity on cells in Na^+-medium. $1-2x10^5$ cells/ml were suspended in 135mM NaCl medium. Where indicated 1mM (final) amiloride was added.

which amiloride was introduced to cells suspended in a normal Na^+-medium. Amiloride caused an initial rapid decrease in pH_i (not shown). This was in part due to the quenching of BCECF by amiloride. Amiloride is permeable to cells in its neutral form and has been shown to quench 6-carboxyfluorescein in cardiac muscle (23). Intracellular pH values had to be adjusted to compensate for this amiloride effect in experiments where amiloride was added during continuous scanning. After adjustment, the effect of amiloride was to cause a slow decrease in pHi to a final value of approximately 6.9 (Fig. 4). The degree to which pH_i decreased was dependent on the concentration of amiloride present (not shown). This was to be expected if extracellular Na^+ was being transported through a Na^+/H^+ antiport.

These experiments suggest that the Na^+/H^+ antiport may be active under normal physiological conditions. Thus by this mechanism, Na^+ might continually enter, while protons produced during normal metabolic activity are extruded. This is in agreement with studies on cultured smooth muscle cells (8) which showed that addition of ethylisopropyl amiloride, a specific inhibitor of Na^+/H^+ exchange, resulted in a slow decline in pH_i.

Results from these series of experiments strongly suggest the prescence of a Na^+/H^+ antiport that is responsible for main-

tenance of normal pH_i and is activated upon intracellular acidification in circular smooth muscle of the dog gastric corpus. This exchanger may be involved in maintaining pH_i during acidosis encountered under certain pathological conditions.

Acknowledgements

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