Functional Characterization of Intracellular pH Regulators Responsible for Acid Extrusion in Human Radial Artery Smooth Muscle Cells

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Abstract

Intracellular pH (pH_i) is a critical factor influencing many important cellular functions. Acid extrusion carriers such as Na^+/H^+ exchanger (NHE), Na^+/HCO_3^- cotransporter (NBC) and monocarboxylate transporters (MCT) can be activated when cells are in an acidic condition ($pH_i < 7.1$). Human radial artery smooth muscle cells (HRASMCs) are an important conduit in coronary artery bypass graft surgery. However, such far, the pH_i regulators have not been characterized in HRASMCs. We therefore investigated the mechanism of pH_i recovery from intracellular acidosis and alkalosis, induced by NH_4Cl-prepulse and Na-acetate-prepulse, respectively, using intracellular 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein (BCECF)-fluorescence in HRASMCs. Cultured HRASMCs were derived from the segments of human radial artery that were obtained from patients undergoing bypass grafting. The resting pH_i is 7.22 ± 0.03 and 7.17 ± 0.02 for HEPES- (nominally HCO_3^--free) and CO_2/HCO_3^--buffered solution, respectively. In HEPES-buffered solution, a pH_i recovery from induced intracellular acidosis could be blocked completely by 30 μM HOE 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride) a specific NHE inhibitor, or by removing $[Na^+]_o$. In 3% CO_2/HCO_3^--buffered solution, HOE 694 slowed the pH_i recovery from the induced intracellular acidosis only, while adding together with DIDS (a specific NBC inhibitor) or removal of $[Na^+]_o$ entirely inhibited the acid extrusion. Moreover, α-cyano-4-hydroxycinnamate (CHC; a specific blocker of MCT) blocked the lactate-induced pH_i changes. In conclusion, we demonstrate, for the first time, those 3 different pH_i regulators responsible for acid extruding, *i.e.* NHE and NBC, and MCT, are functionally co-existed in cultured HRASMCs.

Key Words: BCECF, human radial artery smooth muscle cells (HRASMCs), intracellular pH (pH_i), monocarboxylate transporters (MCT), Na^+-H^+ exchanger (NHE), $Na^+-HCO_3^-$ cotransporter (NBC)

Introduction

Many important cellular functions are affected by a change of intracellular pH (pH_i). Mechanisms such as cell volume (27), permeability of ion channels (34), enzyme catalysts (33), cell differentiation, growth and apoptosis are sensitive to changes in pH_i (3, 23, 26, 50). For example, pH_i disturbances have recently

been claimed to be responsible for the irreversible endothelial dysfunction and development of hypertension, as well as vascular atherosclerosis in animal models (3, 4, 49). The pH_i in mammalian cells is kept within a narrow range (7.0-7.2) through the combined operation of active transmembrane transporters and passive intracellular buffering power (36). The membrane transporters can be divided into two main categories: acid extrusion carriers and acid loading carriers. Acid extrusion carriers such as a Na^+/H^+ exchanger (NHE) and Na^+/HCO_3^- cotransporter (NBC) can be activated when cells are in an acidic condition ($pH_i <$ 7.1) (3, 6, 39). On the contrary, when a cell is in an alkalized direction ($pH_i >$ 7.2), the acid loaders such as Cl^-/OH^- exchanger (CHE) and Cl^-/HCO_3^- exchanger (AE) will be triggered (51).

Net acid extrusion from VSMCs in rat and mice mesenteric small arteries is mediated by the Na^+/H^+-exchanger NHE1 (slc9a1) and the Na^+-HCO_3^--cotransporter NBCn1 (slc4a7) (3, 5-7). NHE mediates the electroneutral exchange of extracellular Na^+ for intracellular H^+ (1, 3, 25). pH_i recovery in HEPES-buffered media (HCO_3^--free condition) can be inhibited by the removal of extracellular Na^+ or by the addition of amiloride or Hoe 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride), a compound that inhibits NHE activity through its high affinity and selectivity (41). In our previous studies, following intracellular acidosis, in CO_2/HCO_3^- buffered Tyrode solution, Na^+-HCO_3^--dependent transport accompanies NHE to achieve acid-equivalent extrusion and, in the human ventricular myocyte, accounts for about 30-40% of the total acid efflux (39, 40). This symporter is largely 4,4-diisothiocyanatostilbene-2,2-disulphonic acid (DIDS) sensitive (56% to 91%), and it is amiloride- and HOE 694-resistant (3, 14, 39, 40, 46, 47). It is also inhibited by removal of external Na^+, whereas no inhibition of acid efflux following an intracellular acidosis has been found by removal of external K^+, decreasing external Ca^{2+} or, in mammalian cardiac cells, by removal of Cl^- (35).

Monocarboxylates such as pyruvate, lactate, and the ketone bodies (acetoacetate and b-hydroxybutyrate) play essential roles in carbohydrate, fat, and amino acid metabolism and must be rapidly transported across the plasma membrane of cells (45). Lactate, for example, is also a major respiratory fuel in cardiac and skeletal muscle under hypoxic conditions (9, 10, 18). Therefore, the transport of L-lactate across the transmembrane is of fundamental importance to most mammalian cells. The specific H^+-monocarboxylate symporter (MCT), a pH_i related carrier, has been identified in many different mammalian tissues, including mitochondrial cells, erythrocytes, hepatocytes, skeletal and cardiac muscle (19, 20, 28, 30, 32, 45). Moreover,

the drug, α–cyano-4-hydroxycinnamate (CHC), blocks pyruvate and lactate transport *via* this carrier by attenuating considerably the rapid fall of pH_i upon lactate-addition, and slows greatly its recovery upon lactate-removal (28, 29). The importance of MCTs is becoming increasingly evident as their extensive physiological and pathological roles are revealed (31). For example, it has been found that the up-regulation of muscle MCT1 expression in response to training exercise and MCT4 expression in response to hypoxia. The latter is mediated by hypoxia inducible factor 1α and often observed in tumor cells that rely almost entirely on glycolysis for their energy provision (31, 54, 55).

Atherosclerosis is major cause of cardiovascular disease, including the myocardial infraction (MI). Coronary artery bypass grafting is recognized to improve symptoms and prolong survival for MI (37, 43). Apart from the human greater saphenous vein, the human radial artery is the other major conduit of choice for the coronary arteries bypass grafts. The patency of radial artery is over 90% at 10 years, whereas the patency of saphenous vein grafts is 40~60% (24, 42). Na^+-H^+ exchangers have been reported to be implicated in vascular atherosclerosis and hypertension, further underlining the importance of pH_i regulation for normal cardiovascular function (2, 3, 5, 38). Therefore, more experimental evidence is required, however, to evaluate the cell biological effects of acid-base transport in vascular cells, especially in human tissues/cells. However, thus far there are not any related reports about active acid extruding transports in human radial artery smooth muscle cells (HRASMCs). Therefore, the aim of the present study is to use discarded conduits of human radial artery from surgery to derive the primary of HRASMCs for related experiments on pH_i regulation.

In conclusion, our present study, for the first time, has demonstrated that 3 different acid extruders, *i.e.* NHE, NBC and MCT, are functionally co-existed in HRASMCs.

Materials and Methods

Human Radial Artery Smooth Muscle Cells (HRASMCs)

With the approval of the Institutional Review Board of Tri-Service General Hospital, National Defense Medical Center (TSGHIRB No. 1-101-05-065) and with prior written informed consent of patients, HRASMCs were collected from surgically-leftover specimens of human radial arteries during heart transplant surgery at Tri-service General Hospital, Taipei, Taiwan. Primary HRASMCs were isolated by the explant technique which has been described in detail in Fletcher *et al*. (21) and cultured in HAM's F12K

medium containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM L-glutamate in a humidified incubator (at 37°C and 5% CO_2). The primary HRASMCs were used for experiments between 3 and 8 passages. The preparations were then perfused with oxygenated Tyrode solution, which was either 100% O_2 for nominally bicarbonate-free Tyrode solution or 5% CO_2/95% O_2 for bicarbonate-containing Tyrode solution, at 37°C, pH 7.40 ± 0.02 for experiments.

Immunocytochemistry

Cells were cultured on a 6-well plate (Macalaster Bicknell, New Haven, CT, USA) for 1-3 days. Cells were subsequently washed twice in phosphate-buffered saline (PBS). After washing, cells were fixed in 4% paraformaldehyde for 30 min at room temperature, then washed twice in PBS and blocked and permeabilized in PBS containing 0.3% triton and 5% normal goat serum for 60 min, and finally washed in PBS and incubated overnight with primary antibodies at 4°C. Cells were washed four times in PBS and labeled with secondary antibodies for 1 h in the dark. After labeling, cells were washed with PBS and incubated with DAPI (1 μg/ml) for 40 min. Cells were washed twice more and mounted onto slides with Gel/Mount (Biomedia Corp., Forest City, CA, USA). Images were acquired with an OLYMPUS 200M (Tokyo, Japan) microscope system.

Measurement and Calibration of the pH$_i$

Measurement of the pH$_i$ has been described in detail in our previous reports (39, 40). In brief, the pH$_i$ in the HRASMCS was measured using the pH-sensitive, dual excitation dual-emission fluorescent dye, 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein-acetoxymethyl (BCECF-AM) (Molecular Probes). The preparations were loaded with BCECF-AM (5 μM) by incubating them for 30 min at room temperature and exciting them alternately with 490 and 440 nm wavelength light. The BCECF fluorescence emission ratio of the 510 nm emission at 490 nm and 440 nm excitation (490/440) was calibrated using the K^+-nigericin method (39). Briefly, this method consisted of exposing a BCECF-loaded cell to the six nigericin calibration solutions (listed below in the Solution section) that clamps pH$_i$ to the value of pH$_o$ of the calibration solution. Fig. 1A showed the emission ratio changes seen on perfusing human artery smooth muscle cells with calibration solutions with different 6 pH values (5.5~9.5) in the presence of 10 μM nigericin. The emitted ratio 510 nm emission at 490 nm and 440 nm excitations (R; R = F_{490}/F_{440}) was increased as the pH value of superfusing solu-
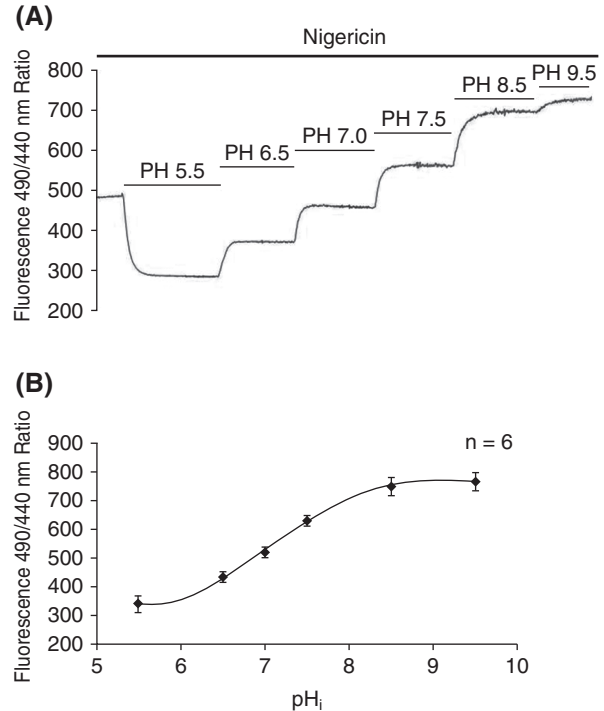

(A)

(B)

Fig. 1. *In situ* calibration of intracellular pH (pH$_i$) in human radial artery smooth muscle cells (HRASMCs). A and B: *In situ* pH$_i$ calibration curve in HRASMCs. A: The trace shows the BCECF fluorescence (510 nm emission at 440 nm and 490 nm excitations) in HRASMCs. (Please see *Materials and Methods* for details). B: The curve shows data pooled from 6 similar experiments shown in A.

tion was increased. R_{max} and R_{min} are, respectively, the maximum and minimum ratio values for the data curve. The fluorescence of BCECF at 490 nm to 440 nm is a function of pH$_i$ and the overall sampling rate in the experiment was 0.5 Hz for the recorded fluorescent ratio (490 nm/440 nm). Using the linear regression fit of the data (shown in the Fig. 1B) obtained from 6 calibration experiments similar to that shown in Fig. 1A, the mean apparent dissociation constant (pKa) at 37°C was found to be 7.17, very close to the value determined by our previous study of the human heart, as well as the value determined by other investigators (4, 39, 53). The following equation (11) was used to convert the fluorescent ratio in to pH$_i$:

$$pH_i = pK_a + \log [(R_{max} - R) / (R - R_{min})]$$

$$+ \log (F_{440min}/F_{440max})$$

where R is the ratio of the 510 nm fluorescence at 440 nm and 490 nm excitation, R_{max} and R_{min} are, respectively, the maximum and minimum ratio values from the data curve and the pK$_a$ (-log of dissociation con-

stant) is 7.17. F_{490min}/F_{440min} and F_{490max}/F_{440max} is the ratio of fluorescence measured at 440 nm of R_{min} and R_{max}, respectively.

Experimental Alteration of pH_i Weak Acid/Base Pre-Pulse Technique

NH_4Cl pre-pulse techniques were used in the present work to induce acute acid loading (4, 48). NH_4Cl pre-pulses were achieved with (~10 min) extracellular exposures to 20 mM NH_4Cl. Briefly, the mechanism of the NH_4Cl prepulse technique relies upon the characteristic of incomplete dissociation. Although both the charged and uncharged species of a weak base exist at the same time in solution, the uncharged species is lipid soluble and therefore able to permeate the lipid bi-layer of the cell membrane. In contrast, the charged species permeates relatively slowly, through various membrane protein routes. For example, the details of NH_4Cl prepulsing procedures, used in the present study, are given below. It can be explained in terms of four phases as shown in Fig. 3: rapid entry (see phase 1 in left part of Fig. 3A), slow recovery (see phase 2 in left part of Fig. 3A), rapid exit (see phase 3 in left part of Fig. 3A), and pH_i regulation (see phase 4 in left part of Fig. 3A) that the sudden acidosis activates pH_i regulatory proteins in the membrane, for instance Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter. Throughout the whole experiment, the change of pH_i induced by the tested drug was compared around the 3rd min after treating the drug, unless otherwise stated. The background fluorescence and auto-fluorescence were small (< 5%) and have been ignored.

Chemicals and Solutions

Standard HEPES-Buffered Tyrode Solution (air equilibrated) contained (mM): NaCl, 140; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$ 2.5; glucose, 11; HEPES, 20; pH adjusted to 7.4 with 4N NaOH. Unless otherwise stated, pH adjustments of all HEPES-buffered solutions were performed at 37°C (these adjustments included those where ionic-substitutions were made, see below). *Standard bicarbonate-buffered Tyrode solution* (equilibrated with 5%CO_2/23 mM HCO_3^-) was the same as above, except that the sodium chloride concentration was reduced to 117 mM, and 23 mM $NaHCO_3$ was added instead of the HEPES (pH 7.40 at 37°C).

Ion-Substituted Solutions: In a *Na^+-free, HEPES-buffered Tyrode solution*, NaCl was replaced with 140 mM N-methyl-D-glucamine (NMDG)-Cl, and the pH was adjusted to 7.4 with HCl. The *Na^+-free CO_2/HCO_3^--buffered Tyrode solution* was the same as the CO_2/HCO_3^--buffered Tyrode solution above, except that 117 mM NaCl and 23 mM HCO_3^- were replaced by 117 mM

NMDG and 23 mM NMDG, respectively, and pH was adjusted to 7.4 at 37°C with HCl under the condition of saturating with 5%CO_2/95%O_2. When 20 mM ammonium chloride was used, it was added directly as a solid to solution without osmotic compensation. DIDS and Hoe 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride) were added, as solids, to solutions shortly before use.

Nigericin Calibration Solutions contained (mM): KCl, 140; $MgCl_2$, 1; 10 μM nigericin; buffered with one of the following organic buffers: 20 mM 2-(N-morpholino) ethanesulphonic acid (MES, pH 5.5), 20 mM HEPES (pH 7.5) or 20 mM 3-(cyclohexylamino)-2-hydroxy-1-propane-sulphonic acid (CAPSO, pH 9.5), and were adjusted (37°C) to the correct pH with 4N NaOH.

Hoe 694 was kindly provided by Hoechst Aktiengesellshaft (Frankfurt, Germany). All other chemicals were from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Statistics

All data are expressed as the mean ± the standard error of the mean (SEM) for n preparations. Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) with Scheffe's posterior comparison. A *P* value smaller than 0.05 was regarded as significant.

Results

Success of Isolating HRASMCs from Tissue

Our present study successfully isolated HRASMCs from human artery tissue using the so-called explant method. The HRASMCs were significantly migrated out from artery tissue at the 7th day and 10th day in a time-dependent way, as shown in Fig. 2A and Fig. 2B, respectively. Also, immunocytochemistry technique was used in order to check out the purity of HRASMCs. In brief, HRASMCs were stained with α-SM-actin, the specific monoclonal antibody which serves as a smooth muscle differentiation marker (Fig.2 C; green color) and DAPI, the nuclei counterstained marker (Fig. 2D; blue color). The cell-pattern of Fig. 2E, merging from Fig. 2C from Fig. 2D, is nearly the same as that of Fig. 2C and Fig. 2D. This clearly indicates that the cells are HRASMCs. Therefore, we have successfully derived a single HRASMCS from tissue of human radial artery through explants technique.

Functional Existence of a Na^+-H^+ Exchanger (NHE)
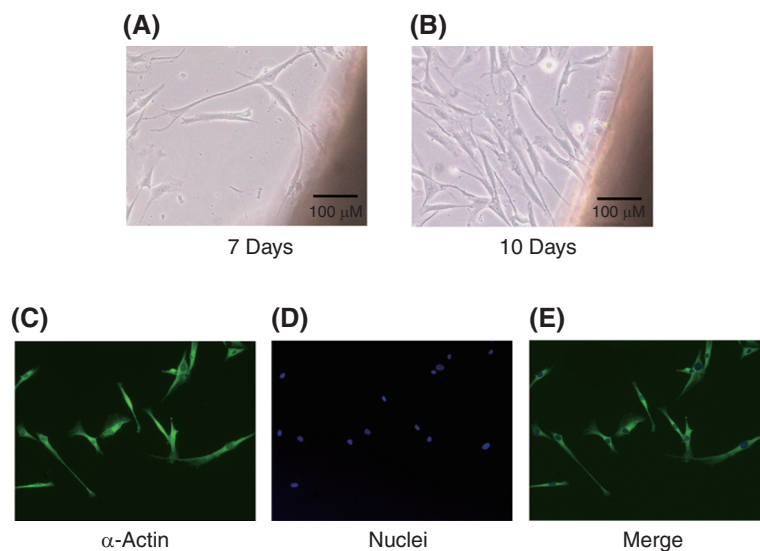
To examine whether an acid-extrusion mecha-

Fig. 2. The identification of human radial artery smooth muscle cells (HRASMCs). A and B: Phase-contrast micrographs of cultured HRASMCs (10×40), using explant technique. Cell cultured at the 7th day (A) and 10th days (B). The dark black area at the right bottom corner is the radial artery tissue. The bar below represents a length of 100 μm. C, D and E: Micrographs of immunohistochemistry of HRASMCs. C: HRASMCs stained for the anti-smooth muscle α actin (green). D: HRASMCs counterstained with DAPI for nuclei (blue). E: A merge micrograph that combines micrograph C and micrograph D (10×40).

nism exists in the HRASMCs, the experiments were first performed in HEPES-buffered superfusate (nominally free of CO_2/HCO_3^-). The steady-state pH_i value for the HRASMCs was found to be 7.22 ± 0.03 (n = 18) in HEPES-buffered solution. The steady-state pH_i value of HRASMCs is similar to 7.2, which is the value that was reported previously for mature mammalian cells of both animal and human models (36, 39).

As shown in the left part of Fig. 3A, the pH_i recovered completely from intracellular acidosis that was induced by using an NH_4Cl pre-pulse technique. This result indicated that there is a mechanism of acid extrusion in the HRASMCs. Removing extracellular Na^+ completely blocked the pH_i recovery from intracellular acidosis following the NH_4Cl prepulse, as shown in the middle part of Fig. 3A. The first and second columns of the histogram (Fig. 3B) shows the mean pH_i recovery slope (measured at $pH_i = 6.76 \pm 0.04$) before and after Na^+ removal for ten experiments that are similar to those whose results are shown in Fig. 3A. This clearly demonstrates that, under nominally CO_2/HCO_3^--free conditions, there is an Na^+-dependent, but CO_2/HCO_3^--independent, acid-extrusion mechanism involved in the pH_i recovery following induced intracellular acidosis in the HRASMCs. To further test if this Na^+-dependent acid extruder is the NHE, we added HOE 694, a specific NHE inhibitor, in the superfusate. As shown in the right part of Fig. 3A, HOE 694 (30 μM) entirely inhibited the pH_i recovery following the induced intracellular acidosis. The pH_i recovery rate (measured

at $pH_i = 6.79 \pm 0.03$) of seven similar experiments, like the result shown in Fig. 3C, were pooled in the first (before HOE 694 addition) and second columns (after HOE 694 addition) of Fig. 3D. Therefore, the present results provide clear pharmacological evidence that NHE functionally exists in HRASMCs.

Functional Existence of a Na^+-HCO_3^- Cotransporter (NBC)

The steady-state pH_i value for the human artery smooth muscle cells was found to be 7.17 ± 0.02 (n = 24) in CO_2/HCO_3^- buffered Tyrode solution. The steady-state pH_i value of HRASMCs is slightly lower than that in a HEPES-buffered Tyrode solution and the value was similar to that reported previously for mature mammalian cells of both animal and human models (36, 39).

The left part of the traces shown in Fig. 3A and 3C illustrate the pH_i recovery from an acid load induced in CO_2/HCO_3^- conditions. These conditions demonstrate the physical mechanism of acid extrusion. To test if the HCO_3^--dependent mechanism is also Na^+-dependent, Na^+ was removed from the solutions in the subsequent experiments; similar to what is shown in right panel of Fig. 4A. Removing Na^+ from the 5% CO_2/HCO_3^- Tyrode solution completely inhibited pH_i recovery following NH_4Cl-induced acidosis, as shown in the right part of Fig. 4A. The histogram in Fig. 4B shows the pH_i recovery rate, which was estimated at pH_i 6.77 ± 0.04 after averaging for eight experiments in HRASMCs. The present data suggests
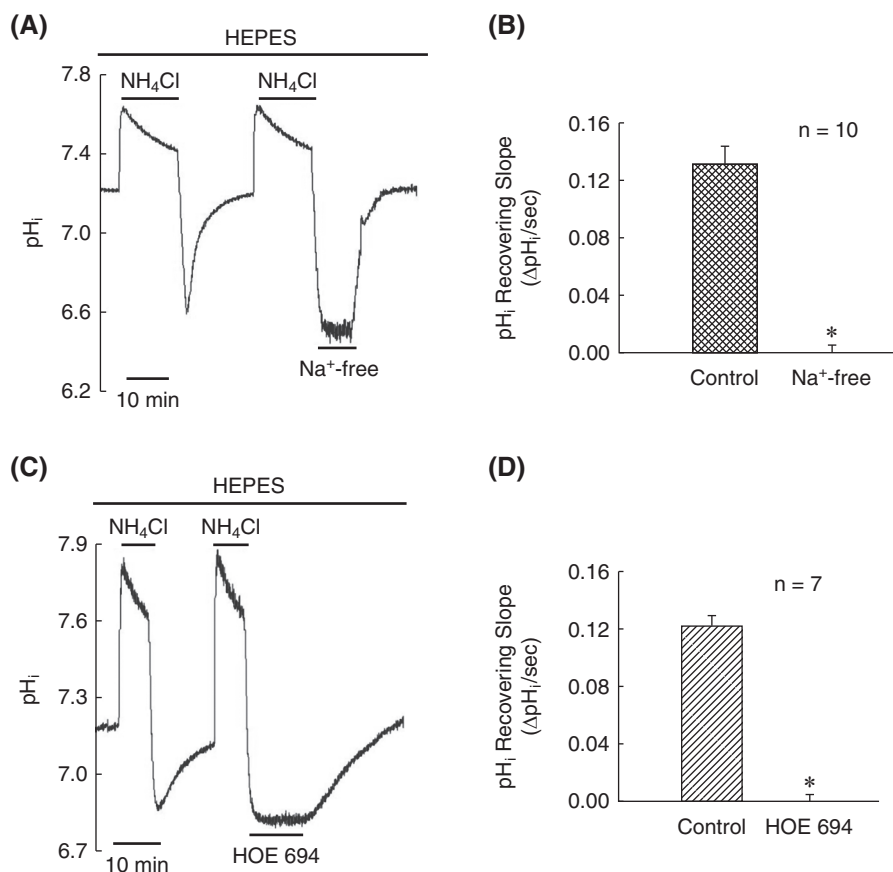
Fig. 3. Effect of Na$^+$-free and 30 μM HOE 694 on pH$_i$ recovery from induced acidosis (evidence of Na$^+$-H$^+$ exchanger) in HRASMCs superfused with HEPES-buffered Tyrode solution. A and C: Top bar shows buffer system used in the superfusate. The periods of application of NH$_4$Cl and tested drugs (Na$^+$-free solution, and 30 μM HOE 694, a NHE exchanger inhibitor, respectively) are indicated with bars above or below the trace. The left part of traces A and C show a typical recovery of pH$_i$-recovery from an intracellular acidosis induced by a 10 min NH$_4$Cl (20 mM) pre-pulse in HEPES-buffered Tyrode solution (pH$_o$ = 7.4, 37°C) in HRASMCs. For details of the mechanism of the pre-pulse technique, please see the *Materials and Methods* section. The right parts of traces A and C represents experiments showing the effect of Na$^+$-free and 30 μM HOE 694 on pH$_i$ recovery, respectively, in HRASMCs. B and D: Histograms, showing the pH$_i$ recovery slope of acid extrusion after NH$_4$Cl-induced intracellular acidosis averaged for 10 and 7 experiments (measured at pH$_i$ = 6.76 $\pm$ 0.04 and 6.79 $\pm$ 0.03, respectively) similar to those shown in A and C, respectively. *: *P* < 0.01 *vs.* control.

that this HCO$_3^-$-dependent acid-extrusion mechanism is also Na$^+$-dependent. To further test whether this pH$_i$ recovery HCO$_3^-$-dependent acid-extrusion mechanism is purely NHE that is found in HEPES-Tyrode superfusate above (*i.e.* Fig. 3), HOE 694 has been added. The pH$_i$ recovery was partially blocked, as expected, in the presence of HOE 694, as shown in the second part of Fig. 4C. The second column of the histogram in Fig. 4D shows the pH$_i$ recovery slope after acid loading for eight experiments (estimated at pH$_i$ 6.79 $\pm$ 0.03) in HRASMCs similar to the result shown in Fig. 4C. The significant difference between the first (control) and the second column (in presence of HOE 694 in a 5% CO$_2$/HCO$_3^-$ solution) indicated that, apart from NHE, there is another HCO$_3^-$-dependent acid-extrusion mechanism involved in the pH$_i$ recovery in 5% CO$_2$/HCO$_3^-$ Tyrode

solution is due to the NBC. In other words, we have provided, for the first time, functional evidence that both NHE and NBC play an important role in pH$_i$ regulation through acid extrusion in HRASMCs.

The Percentage of Contribution of NHE and NBC at Acid Condition

Although the percentage of contribution of NHE and NBC has been reported in animal cells and human cardiomyocytes (3, 36, 39), a corresponding report for the human smooth muscle cells have so far not been undertaken. In the latter part of Fig. 3C and 3E, acid extrusion with HOE 694 or DIDS alone represents the functional activity of NBC and NHE at the measuring pH$_i$, respectively. The right column of Fig. 4C and Fig. 5A, therefore, provide the activity percent-
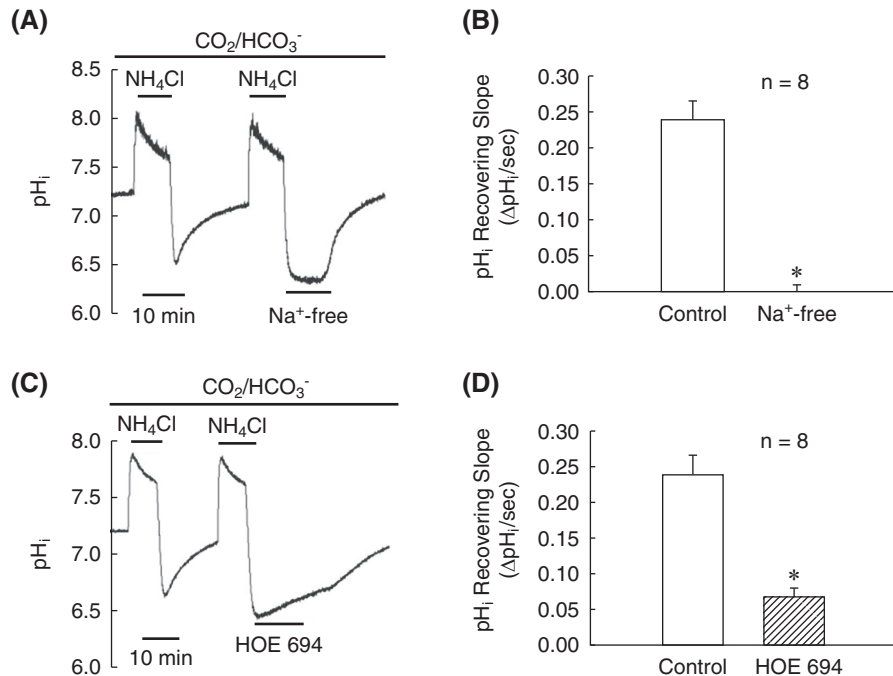
(A)

(B)

(C)

(D)

Fig. 4. Effect of Na^+-free and 30 μM HOE 694 on pH_i recovery from induced acidosis in HRASMCs superfused with 5% CO_2/HCO_3^- Tyrode solution. A and C: The top bar shows the buffer system used in the superfusate. The periods of application of NH_4Cl and tested drugs (Na^+-free solution and 30 μM HOE 694) are shown with bars above or below the trace. The left part of traces A and C shows a typical pH_i recovery from an intracellular acidosis induced by a 10 min NH_4Cl (20 mM) pre-pulse in 5% CO_2/HCO_3^- Tyrode solution (pH_o = 7.4, 37°C) in HRASMCs. For details of mechanism of the pre-pulse technique, please see the *Materials and Methods* section. The right part of traces A and C represents experiments showing the effect of Na^+-free solution and 30 μM HOE 694 (a NHE exchanger inhibitor) on pH_i recovery, respectively, in HRASMCs. B and D: Histograms, showing the pH_i recovery slope of acid extrusion after NH_4Cl-induced intracellular acidosis averaged for eight experiments similar to those shown in A and C, respectively. *: $P < 0.01$ *vs*. control.

age of acid extrusion through NBC and NHE, respectively. It can be seen clearly that the percentage of contribution of NBC was larger than that of NHE (70% and 30%, respectively) at the acidic condition, *i.e.* measuring pH_i around 6.75, in HRASMCs. Note that this is very different with that report in animal cells and human cardiomyocytes (see discussion for more details).

Functional Existence of a Monocarboxylate$^-$-H$^+$ Symporter (MCT)

Fig. 6 shows an experiment designed to demonstrate the effects on pH_i of activation of the transmembrane monocarboxylic acid carrier. The HRASMC was superfused with an isotonic solution of 100 mM sodium citrate (pH_o 7.40) at 25°C. In the steady state under these conditions (45), pH_i becomes somewhat alkaline, HCO_3^-- and Cl^--dependent acid equivalent carriers are inactivated, and because of the high pH_i and relatively low temperature, Na^+-H^+ exchange is virtually inactive (see below). Addition of extracellular Na L-lactate (10 mM) produces a monotonic and rapid fall of pH_i, which is reversed upon lactate

removal. These changes of pH_i are caused by transmembrane movement of lactic acid, much of which occurs *via* the monocarboxylic acid carrier (17, 28, 45, 56). The left part of Fig. 6 shows a typical lactate-induced fall and recovery of pH_i upon lactate addition and removal, respectively. The existence of MCT is confirmed as shown in the right part of Fig. 6A that CHC (alpha-cyano-4-hydroxycinnamate), an inhibitor of the carrier, attenuates considerably the rapid fall of pH_i upon lactate-addition (45). The histograms in Fig. 6B show the pH_i recovery rate, averaged for eight experiments, similar to that shown in Fig. 6A (estimated at pH_i 7.15 $\pm$ 0.07).

It has been demonstrated that the stilbene drug DIDS (0.4 mM) inhibits NBC effectively (3, 14, 39, 40, 46, 47); therefore, we further tested whether DIDS can inhibit this HCO_3^--dependent, but HOE 694-independent, acid-extrusion mechanism, in the HRASMCs. As shown in the right part of Fig. 5C, a combination of 30 μM HOE 694 and 0.4 mM DIDS entirely inhibited the pH_i recovery following induced intracellular acidosis with 5% CO_2/HCO_3^- Tyrode solution (right part of Fig. 5C), while the DIDS alone did not (the right part of Fig. 5A). The right histogram in Fig.
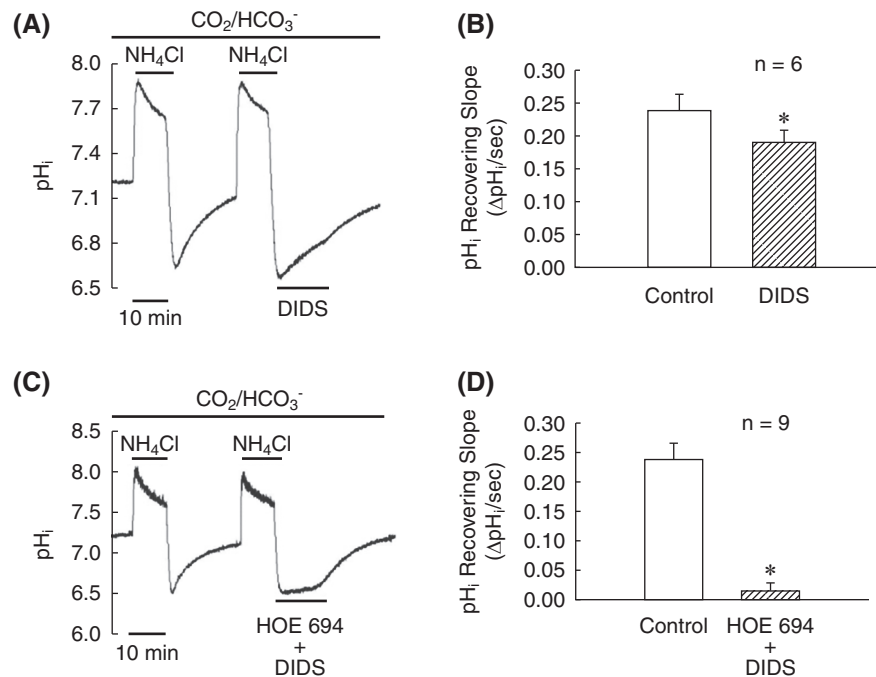
Fig. 5. Effect of 30 μM HOE 694 and 0.2 mM DIDS on pH_i recovery from induced acidosis in HRASMCs superfused with 5% CO_2/HCO_3^- Tyrode solution. A and C: The top bar shows the buffer system used in the superfusate. The periods of application of NH_4Cl and tested drugs (0.2 mM DIDS and HOE 694 pulse DIDS) are shown with bars above or below the trace. The left part of traces A and C shows a typical pH_i recovery from an intracellular acidosis induced by a 10 min NH_4Cl (20 mM) pre-pulse in 5% CO_2/HCO_3^- Tyrode solution (pH_o = 7.4, 37°C) in HRASMCs. For details of mechanism of the pre-pulse technique, please see the *Materials and Methods* section. The right part of traces A and C represents experiments showing the effect of 0.2 mM DIDS (a NBC exchanger inhibitor), and HOE 694 (a NHE exchanger inhibitor) plus DIDS on pH_i recovery, respectively, in HRASMCs. B and D: Histograms, showing the pH_i recovery slope of acid extrusion after NH_4Cl-induced intracellular acidosis averaged for six and nine experiments similar to those shown in A and C, respectively. *: $P < 0.01$ *vs.* control.
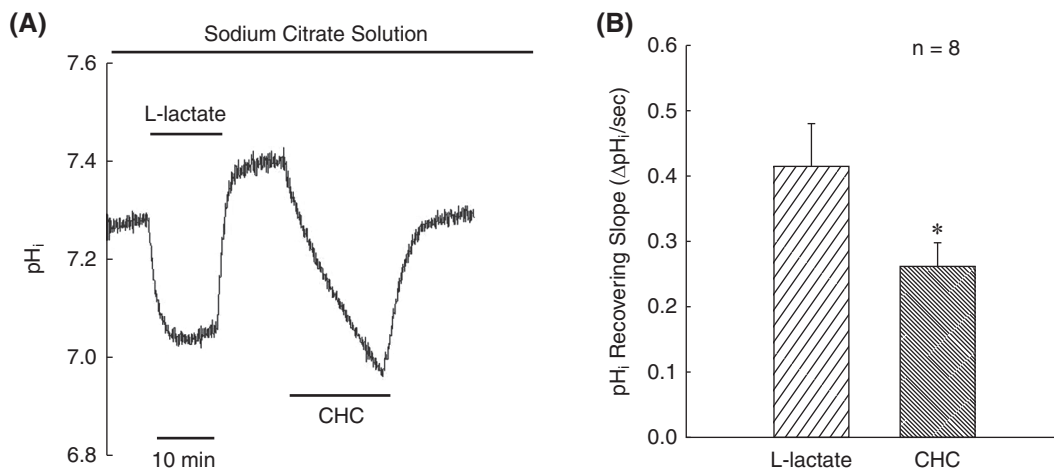

Fig. 6. Effect of cyano-hydroxycinnamate (CHC) on the L-lactate induced H^+-monocarboxylate symporter (MCT). A: The top bar shows the buffer system used in the superfusate. See text for details of the superfusate composition (essentially, an isotonic sodium citrate solution, nominally free of CO_2/HCO_3^-; pH_o 7.40; temperature 25°C). The periods of application of extracellular L-sodium lactate (10 mM) or cyano-hydroxycinnamate (CHC; 10 mM) are shown with bars above or below the trace. The left part of trace A shows a typical pH_i changes induced by addition or removal of L-lactate in sodium-citrate solution in HRASMCs. The right part of trace A represents experiment showing the effect of CHC on l-lactate induced pH_i changes in HRASMCs. B: Histograms, showing the change in pH_i recovery slope of acid loading after l-lactate addition averaged for 8 experiments similar to those shown in A (measured at pH_i = 7.15 ± 0.07), respectively. *: $P < 0.01$ *vs.* control.

5B and Fig. 5D show the pH_i recovery rate, averaged for several experiments, similar to that shown in Fig. 5A and Fig. 5C, respectively (estimated at pH_i 6.74 ± 0.03). The present data suggests that this Na^+- and HCO_3^--dependent acid-extrusion mechanism.

The present study has demonstrated for the first time, pharmacologically and physiologically, that NHE and NBC functionally co-exist for acid extrusion in HRASMCs. MCT has been identified as another transmembrane transporter to affect pH_i in HRASMCs.

Discussion

The Functional Evidence of Acid Extruding Regulators-NHE1 and NBC

Using the technique of microspectrofluorimetry, we have provided straight forward and convincing pharmacological evidence, for the first time, that NHE1 and another HCO_3^--dependent acid extruders, *i.e.* NBC, are functionally responsible for acid extrusion following induced acidosis in HRASMCs. NHE's activity was HCO_3^--independent and Na^+ dependent (the right part of Fig. 3A) (15, 39, 40). This conclusion was confirmed by the finding that the acid extruder could be entirely blocked by HOE 694 (the right part of Fig. 3C), a highly-specific NHE1 inhibitor (41). Among 9 different members of NHE, *i.e.* NHE1~9 (2), the NHE1 protein has been identified as an protein which ubiquitously expresses in different tissues, including heart and smooth muscle by molecular biology methods (22, 44). It has been shown that HOE 694 shows a high selectivity for cloned and expressed NHE1 that is two or more orders of magnitude higher than for the other isoforms, such as NHE 2 and 3 (16). Our present results revealed that the functioning NHE in the HRASMCs was also sensitive with low concentration of HOE 694 (30 μM) (Fig. 3C). Therefore, our study suggests that NHE isoform is purely NHE1, instead of NHE2 and NHE3. One might ask if the present data can exclude a significant presence of other members of NHE4~9 in HRASMCs. The answer would appear to be that it *can* be excluded, on the grounds that data available so far for NHE4,5 indicate that the acid extruder is essentially related insensitive to amiloride and HOE694, and that NHE6~9 only exists in membrane of intracellular organelles (2). Therefore, using pharmacological maneuvers, our present study has provided direct pharmacological evidence that the native NHE functioning during pH_i-regulation in the HRASMCs is the NHE1 isoform, instead of other members of NHE proteins.

Another category extruding mechanism whose activity was HCO_3^-- and Na^+-dependent (Fig. 4A) was NBC. This was supported by another result (Fig. 4C and Fig. 5A) in our present study, which showed that NBC was sensitive to DIDS, a NBC inhibitor, and insensitive to HOE 694 (15, 39, 41, 47). Indeed, our present study demonstrated functionally that there is a Na^+ and HCO_3^- dependent acid-extruding mechanism responsible for acid extrusion in the HRASMCs (Fig. 3). To know the exact stoichiometry between HCO_3^- and Na^+ (coupling ratio) of this carrier of NBC, *i.e.* isoforms, in the HRASMCs, further investigation of Western blot is required.

Potential Role of Inhibitors of NHE1 and NBCs in Clinic

In the HRASMCs, we found that the relative activity of NBC is much higher than that of NHE1 in the pH_i range of acidosis (< 6.8), as shown in the Fig. 4 and 5. In other words, NHE1 and NBC do not share the same percentage on acid extrusion during the conditions of server acidosis. Therefore, under conditions of intracellular acidosis such as ischaemia/reperfusion (*i.e.* pH_i < 6.9), the NBCs will be highly activated and responsible for nearly at least of 65 % of the acid extrusion in the HRASMCs. This result is slightly different to that derived from human atrium (39), and that of guinea pig ventricular myocytes (36) and, specially, animal vascular smooth cells (5-7). Both in rat or mouse vascular smooth cells, it has been found that NHE1 is predominantly active at lower pH_i values and plays a major role for acid extrusion during the conditions of severe intracellular acidification (5) whereas NBC is active at both low and near-physiological pH_i values (6, 7).

According to the finding above, we predicted that the alteration of activity of NBC will play a vital role on many patho-physiological conditions, such as ischaemia-reperfusion damage in HRASMCs, like that found by many other groups in other cell types (23, 26, 52, 53). Also, it is clear that NHE and NBC may involve an important approach for preventing some acute and chronic, pathological vascular illnesses in clinics, such as ischaemia-reperfusion induced damage that caused from rapid recovery of pH_i (8, 12, 13, 38, 57, 58). Moreover, our present study, therefore, can also implicate that a development of new and specific NBC inhibitor is another clue for preventing ischaemia/reperfusion-induced cardiovascular injury, apart from the knowing basic physiological mechanism of NBC.

Acknowledgments

supplying us with Hoe 694.

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