Endothelin-induced changes in intracellular pH and Ca^{2+} in coronary smooth muscle: role of $\text{Na}^+\text{-H}^+$ exchange

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The relationship between endothelin-1 (ET-1)-induced stimulation of $\text{Na}^+\text{-H}^+$ exchange and intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) was examined in primary cultures of porcine coronary artery smooth muscle cells. Intracellular pH (pH_i) and $[\text{Ca}^{2+}]_i$ were measured using 2,7-bis-carboxyethyl-5(6)-carboxyfluorescein and the acetoxymethyl ester of fura-2 respectively. In HCO_3^- -free buffer ($\text{pH} = 7.4$), ET-1 (0.1–50 nM) induced a sustained, dose-dependent increase in pH_i . ET-1 (10 nM) increased pH_i from 6.83 ± 0.01 to 6.93 ± 0.02 ($P < 0.01$). The alkalization was blocked by the $\text{Na}^+\text{-H}^+$ exchange inhibitor, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA, 3 μM) or by substitution of Na^+ with *N*-methylglucamine or choline chloride ($P < 0.05$). Recovery of pH_i in response to acidification, induced by washout of a 20 mM NH_4Cl prepulse, was $> 90\%$ inhibited by EIPA (3 μM), confirming the presence of an ET-1-responsive $\text{Na}^+\text{-H}^+$ exchanger. Coronary smooth muscle cells responded to ET-1 with a dose-

dependent, biphasic increase in $[\text{Ca}^{2+}]_i$ which was not inhibited by manipulations (EIPA pretreatment or Na^+ -free media) shown to block the $\text{Na}^+\text{-H}^+$ exchanger. The ET-1-mediated alkalization was not inhibited by removal of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$). However, complete blockade of the ET-1-mediated $[\text{Ca}^{2+}]_i$ response using the intracellular Ca^{2+} -chelator, [bis-(2-amino-5-methylphenoxy)ethane-*NNN'*-tetraacetic acid tetraacetoxymethyl ester] (MAPTAM), in $[\text{Ca}^{2+}]_o$ -free media, demonstrated that an increment in $[\text{Ca}^{2+}]_i$ is required for activation of the $\text{Na}^+\text{-H}^+$ exchanger by ET-1. The ET-1-induced rise in $[\text{Ca}^{2+}]_i$ was not associated with a rise in pH_i in the presence of $\text{CO}_2/\text{HCO}_3^-$. We conclude that: (1) activation of $\text{Na}^+\text{-H}^+$ exchange by ET-1 requires an increment in $[\text{Ca}^{2+}]_i$; and (2) ET-1 stimulates EIPA-sensitive $\text{Na}^+\text{-H}^+$ exchange, but this stimulation does not modulate ET-1-induced changes in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

The mechanism by which endothelin-1 (ET-1) promotes mobilization of intracellular free calcium ($[\text{Ca}^{2+}]_i$) and contraction in vascular smooth muscle (VSM) is not completely understood [1]. Results of several studies, including our own, suggest that ET-1-mediated stimulus-response coupling in VSM involves multiple Ca^{2+} -influx pathways as well as changes in the levels of other intracellular cations [1–3]. The relationship between changes in intracellular concentrations of Ca^{2+} , H^+ and Na^+ in the mechanism of ET-1 action, however, remains unclear.

Raising intracellular pH (pH_i) in the absence of an agonist has been shown to raise $[\text{Ca}^{2+}]_i$ in VSM cells and increase contractility of isolated arteries [4–7]. Stimulation of plasmalemmal $\text{Na}^+\text{-H}^+$ exchange could lead to increases in $[\text{Ca}^{2+}]_i$, stemming either from intracellular alkalization or increases in cytosolic Na^+ [7–10]. Several groups have shown that ET-1 can induce alkalization in VSM cells or coronary myocytes by stimulation of $\text{Na}^+\text{-H}^+$ exchange [9, 11, 12]. Similarly, a variety of other $[\text{Ca}^{2+}]_i$ -mobilizing agonists stimulate this activity in VSM [13–15]. An association between activation of $\text{Na}^+\text{-H}^+$ exchange and increases in $[\text{Ca}^{2+}]_i$ has been described in a number of cell systems, including VSM, and may contribute to contraction of VSM [9, 16–18]. However, in part because of the use of inhibitors of questionable specificity, controversy exists regarding the available data [16, 19–21]. The potential link between ET-1-mediated stimulation of the $\text{Na}^+\text{-H}^+$ exchanger and the transduction pathways leading to increased $[\text{Ca}^{2+}]_i$ in VSM has not been adequately examined. We, therefore, tested the hypothesis that activation of $\text{Na}^+\text{-H}^+$ exchange is

integral to the mechanism by which ET-1 increases $[\text{Ca}^{2+}]_i$ in primary cultures of coronary VSM cells. To probe further the relationship between $[\text{Ca}^{2+}]_i$ and $\text{Na}^+\text{-H}^+$ exchange in response to ET-1, we also assessed the Ca^{2+} -dependence of the ET-1-mediated activation of $\text{Na}^+\text{-H}^+$ exchange. These studies were performed using a nominally HCO_3^- -free buffer system in order to isolate $\text{Na}^+\text{-H}^+$ exchange activity from HCO_3^- -dependent pH_i regulatory systems.

Most studies demonstrating ET-1-induced cytosolic alkalization in VSM cells were performed in the nominal absence of HCO_3^- [9, 11]. In the presence of a $\text{CO}_2/\text{HCO}_3^-$ -containing buffer, however, agonists may stimulate HCO_3^- -dependent transporters in addition to $\text{Na}^+\text{-H}^+$ exchange activity. The combined effect often is that cytosolic alkalization is not observed [15, 22, 23]. In rat ventricular cells, however, ET-1 has been reported to induce alkalization ascribed to stimulation of $\text{Na}^+\text{-H}^+$ exchange in the presence of $\text{CO}_2/\text{HCO}_3^-$ [12]. In view of the controversy concerning the ability of ET-1 or other agonists to induce alkalization via $\text{Na}^+\text{-H}^+$ exchange in the presence of $\text{CO}_2/\text{HCO}_3^-$ [12, 23, 24], we also assessed the pH_i response to ET-1 using this buffer system.

METHODS

Primary cell culture

Pig hearts were obtained from a local slaughterhouse and transported in ice-cold, sterile, calcium-free Hanks' salts solution containing penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and

Abbreviations used: ET-1, endothelin-1; $[\text{Ca}^{2+}]_i$, intracellular free calcium; pH_i , intracellular pH; BCECF, 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein; fura 2 AM, acetoxymethyl ester of fura 2; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; Hepes-PSS, Hepes-buffered physiologic salts solution; MAPTAM, [bis-(2-amino-5-methylphenoxy)ethane-*NNN'*-tetraacetic acid tetraacetoxymethyl ester]; DMEM/F-12, Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham); DMSO, dimethyl sulphoxide; VSM, vascular smooth muscle.

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fungizone (2.5 $\mu\text{g/ml}$; Gibco Laboratories, Grand Island, NY). For each culture preparation, the left anterior descending coronary artery was dissected from ten hearts and thoroughly cleaned of fat and connective tissue. Each artery was cut longitudinally to expose the intima, which was then rubbed with a cotton swab to remove the endothelial cell layer. The vessels were cut into 2 mm² segments, the segments pooled, and incubated with shaking at 37 °C for 2 h in collagenase solution. Collagenase solution was 20 mM Hepes-buffered Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham) (DMEM/F-12 medium; Gibco) containing penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$) and ascorbate (50 $\mu\text{g/ml}$; Sigma Chemical Co., St. Louis, MO, U.S.A.) and collagenase (CLS2, 300 U/ml; Worthington Biochemical Corp., Freehold, NJ, U.S.A.). The tissue pieces were then triturated using a 25 ml sterile pipette, the enzyme solution removed and replaced with fresh collagenase solution and the tissues incubated for an additional 4 h. The solution containing the tissue was filtered through a 500-mesh nylon screen to separate the dispersed cells from small clumps of adventitia and other debris. The dispersed cells were then pelleted and resuspended in Hepes-buffered DMEM/F-12 medium containing 10% fetal bovine serum (Gibco), 50 $\mu\text{g/ml}$ ascorbate and penicillin (100 U/ml)/streptomycin (100 $\mu\text{g/ml}$). Cells were plated at high density onto circular glass coverslips (No.1, 25 mm; Bellco Glass Inc., Vineland, NJ, U.S.A.) in 6-well chambers and placed in a humidified tissue-culture incubator maintained at 37 °C and equilibrated with 5% CO₂/filtered room-air. Culture media were changed every 48 h. After reaching confluence (7–10 days post-plating) and at 24–48 h before use, the cells were transferred to the same culture medium, except serum was omitted and 5 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin and 5 ng/ml sodium selenite were added (Sigma). Cell type was verified by morphology, by positive immunofluorescent staining for smooth muscle-specific α -actin (Sigma) and for desmin (Sigma) and by negative immunofluorescent staining for von Willebrand factor (Accurate Chemical and Scientific Corp., Westbury, NY, U.S.A.), and for 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labelled acetylated low-density lipoprotein uptake (Biomedical Technologies, Inc., Stoughton, MA, U.S.A.), using cultured porcine endothelial cells as a positive control.

Measurement of pH_i

Intracellular pH was quantified by computerized fluorescence imaging using the H⁺-sensitive fluorophore, 2,7-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF). Coverslips of confluent coronary VSM cells were loaded with the permeable acetoxymethyl derivative, BCECF AM (Molecular Probes Inc., Eugene, OR, U.S.A.) at 5 μM final concentration by incubation for 30 min at 25 °C in Hepes-buffered physiologic salts solution (Hepes-PSS). Coverslips were then mounted in a Sykes–Moore chamber (Bellco Glass, Inc.) and placed in a thermostatically controlled heat-exchanger mounted on a Zeiss IM35 fluorescence microscope equipped with a 40 $\times$ quartz objective. Unless otherwise noted, monolayers were perfused with prewarmed 33–35 °C Hepes-PSS at a rate of 1 ml/min for 15 min before, and during, experimentation. Image-1 fluorescence software (Universal Imaging Corp., West Chester, PA, U.S.A.) was used for data collection from the output of an SIT camera (Hamamatsu Photonic Sys. Corp., Bridgewater, NJ, U.S.A.) mounted on the microscope. Cells were exposed to excitation light (xenon) at two alternating wavelengths using a computer-driven filter wheel; 495 nm and 440 nm excitation filters were used (Omega Optical, Brattleboro, VT, U.S.A.). Emission signals were passed through a 520 nm

long-pass filter (Omega Optical). Individual cytoplasmic regions of 15–35 cells were monitored per coverslip. After automatic subtraction of background fluorescence (obtained from a region of the coverslip devoid of cells), data were expressed as the average ratio of the emission signals following excitation at 495 and 440 nm respectively. A fluorescence ratio–pH_i calibration curve was generated at the end of each experiment by determining fluorescence ratios at pH_i 7.4, 7.0, and 6.6, where pH_i was set using the nigericin/high-K⁺ technique [25]. By acting as a H⁺-K⁺ ionophore, nigericin (5 $\mu\text{g/ml}$; Sigma) equilibrates pH_i with pH_o when [K⁺]_o is adjusted to equal [K⁺]_i. Fluorescence ratios obtained were analysed by linear regression analysis and the equation thus derived used to calculate experimental pH_i values.

Measurement of [Ca²⁺]_i

Cells were loaded with 10 mM fura 2 AM [1 mM stock in dimethyl sulphoxide (DMSO); Molecular Probes] plus 0.1% Pluronic detergent (Molecular Probes) in Hepes-PSS for 60 min at 25 °C, followed by a 30 min wash to allow for ester hydrolysis. Coverslips of VSM cells were mounted on the microscope and perfused as described above. Alternating excitation wavelengths of 340 and 380 nm were used and the emission signal filtered through a 470 nm long-pass filter (Omega Optical). Results are expressed as the average ratio of the fluorescence emission following excitation at the two wavelengths.

Calibration of [Ca²⁺]_i was performed in duplicate by an *ex vivo* multipoint calibration method using serial dilutions of Ca²⁺-free and high-Ca²⁺ (10 mM) buffers containing 10 mM Mops, 10 mM EGTA and 100 mM KCl [26]. Estimates of free Ca²⁺ were predicted by the method of Fabiato [26] at pH 6.85 and 33 °C. A linear regression of free Ca²⁺ versus fluorescence ratio ($r = 0.997$) predicted a free [Ca²⁺]_i concentration of 600 nM to be equivalent to a 340 nm/380 nm emission ratio of 1.0. In view of the assumptions and potential artifacts associated with [Ca²⁺]_i calibrations [27], the data presented in the Figures are expressed as ratio emission values.

Solutions and reagents

Hepes-PSS had the following composition (mM): NaCl (137); KCl (5.4); CaCl₂ (1.25); KH₂PO₄ (0.44); Na₂HPO₄ (0.34); MgSO₄ (0.8); glucose (5.5); Hepes (20.0). HCO₃⁻-PSS contained (mM): NaCl (113); KCl (4.7); CaCl₂ (1.6); KH₂PO₄ (1.18); MgSO₄ (1.17); NaHCO₃ (24); glucose (5.5). For selected experiments requiring HCO₃⁻, monolayers were perfused with HCO₃⁻-PSS in the absence of Hepes. In this case, buffer storage flasks were vigorously gassed with 5% CO₂/room air, and gas-impermeable tubing was used for perfusion of all solutions. In addition, the surface of the Sykes–Moore chamber was equilibrated with 5% CO₂/room air, directed from a small conical dome covering the chamber so as to maintain the solution pH in the chamber at 7.4.

Nigericin calibration solution was nominally Na⁺-free Hepes-PSS containing 5 $\mu\text{g/ml}$ nigericin and 144 mM KCl instead of NaCl. All solutions for ammonia prepulse experiments were made by isosmotic replacement of NaCl with NH₄Cl. Nominally Na⁺-free Hepes-PSS was made by isosmotic replacement of NaCl with choline chloride or *N*-methyl-D-glucamine chloride; in addition, Na₂HPO₄ was substituted by K₂HPO₄ and KCl was reduced appropriately. Nominally Ca²⁺-free Hepes-PSS was made without CaCl₂ and supplemented with 0.5 mM EGTA.

ET-1 was obtained from Peptides International, Inc. (Louisville, KY, U.S.A.). Histamine, choline chloride and *N*-methyl-D-glucamine chloride were from Sigma. [Bis-(2-amino-5-methylphenoxy)ethane-*NNN'*-tetraacetic acid tetraacetoxymethyl

ester] (MAPTAM) was from Calbiochem, La Jolla, CA., U.S.A., and was stored in DMSO (250 mM stock) at -20°C until use. 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) was the gift of Dr. H.-J. Lang, Hoechst Pharmaceuticals, Frankfurt, Germany.

Experimental design

Each experiment was performed on primary cultures of cells pooled from the epicardial arteries of ten porcine hearts. Unless otherwise indicated, figures are represented as the mean ($\pm$ S.E.M.) pH_i or $[\text{Ca}^{2+}]_i$ responses of coronary VSM cells from a representative coverslip, with the number of cells monitored indicated. Experiments examining pH_i and $[\text{Ca}^{2+}]_i$ inter-relationships, used coverslips treated identically and from the same culture plating. Typically, $>90\%$ of cells in a given field demonstrated pH_i or $[\text{Ca}^{2+}]_i$ responses to 10 nM ET-1; less responsive monolayers were excluded from the data set. The results of each experiment were verified on at least three coverslips from at least two different primary cell cultures. Statistical significance was determined by Student's *t*-test or by Newman-Keuls multiple range test for multiple comparisons. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of ET-1 on $\text{Na}^+\text{-H}^+$ exchange in coronary VSM cells

Figure 1(a) shows a representative time-course of the effect of 10 nM ET-1 on pH_i of coronary VSM cells perfused with a nominally HCO_3^- -free buffer system (Hepes-PSS) to isolate the exchanger from HCO_3^- -dependent pH_i regulatory systems. ET-1 induced a sustained cytosolic alkalization, which was observed within 1 min of exposure to the agonist. Baseline pH_i averaged 6.83 ± 0.01 and increased to 6.93 ± 0.02 after 5 min exposure to 10 nM ET-1 ($P < 0.01$). In parallel experiments, pH_i did not rise over the same time-period in the absence of the agonist (results not shown). Changes in pH_i produced by ET-1 (range 0.1–50 nM ET-1) were dose-dependent (Figure 2).

The initial, baseline pH_i observed was slightly lower than resting pH_i values reported for murine fibroblasts (6.88 ± 0.01) [28], for the smooth muscle-like cell line BC3H-1 (6.89 ± 0.01) [29] and for canine femoral artery VSM cells (7.10 ± 0.03) [15]. Whether these variations in resting pH_i are attributable to differences among cell type or differences in experimental conditions cannot be readily determined.

In the absence of ambient $\text{CO}_2/\text{HCO}_3^-$, some investigators have noted a biphasic pH_i response to ET-1 or other agonists in VSM, consisting of a transient acidification followed by an $\text{Na}^+\text{-H}^+$ exchange-mediated, sustained alkalization [11,30]. The initial acidification may involve activation of the plasmalemmal $\text{Ca}^{2+}\text{-ATPase}$, resulting in H^+ influx [30]. Our coronary VSM cells responded to ET-1 without detectable initial acidification. Our results are similar to other reports in which VSM cells alkalinized in response to agonists without initial acidification [9,15,23]. Whether the absence of initial acidification is due to differences in cell type or experimental conditions is presently unknown.

To examine the role of the $\text{Na}^+\text{-H}^+$ exchanger in ET-1-induced alkalization responses to ET-1, experiments were performed in the presence and absence of the $\text{Na}^+\text{-H}^+$ exchange inhibitor, EIPA. As illustrated in Figure 1(b), 3 μM EIPA was sufficient to block the alkalization response to 10 nM ET-1 (ΔpH_i (10 nM ET-1 alone) = 0.10 ± 0.01 versus ΔpH_i (10 nM ET-1 + 3 μM EIPA) = 0.006 ± 0.01 ; $n = 5$ experiments; $P < 0.05$). Similar inhibition of ET-1-induced alkalization was produced by 10, 35 and 100 μM EIPA, whereas 0.3 μM EIPA produced a partial

Figure 1 Effect of ET-1 on pH_i in primary cultures of porcine coronary VSM

Cells were perfused with Hepes-PSS. pH_i was determined as described in the Materials and methods section. (a) A representative pH_i -time-course before and during addition of 10 nM ET-1 to the perfusate. Values represent mean responses $\pm$ S.E.M. ($n = 20$ cells). (b) Effect of pretreatment with 3 μM EIPA on pH_i -time-course response to 10 nM ET-1. EIPA was included in the perfusate and ET-1 was added after stabilization of baseline fluorescence. Values represent mean responses $\pm$ S.E.M. ($n = 21$ cells).

inhibition, visualized as a delay in the time-course of the response (results not shown). As exemplified by Figure 1(b), 3–100 μM EIPA alone also induced significant baseline cytosolic acidification. This observation implies that $\text{Na}^+\text{-H}^+$ exchange contributes to basal pH_i regulation by extruding acid in Hepes-PSS perfused cells.

To assess more directly the presence of $\text{Na}^+\text{-H}^+$ exchange in cultured coronary VSM cells, we examined the recovery of pH_i after acidification induced by a standard procedure involving washout of a 4–5 min pulse of 20 mM NH_4Cl in Hepes-PSS [10]. Washout of NH_4Cl from the media caused a rapid fall of pH_i to values (6.5–6.6) lower than the initial baseline. This was followed by prompt recovery of pH_i to initial baseline values (recovery rate = 0.22 ± 0.05 pH units/min; $n = 3$ experiments), indicating that the VSM cells actively recovered from the acid load. The recovery of pH_i after acid loading was blocked by addition of 3 μM EIPA 10 min before and during the ammonium chloride pulse (rate < 0.01 pH units/min; $n = 3$); similar inhibition was produced by 10, 35 and 100 μM EIPA (results not shown). Upon removal of NH_4Cl , cells exposed to 3 μM EIPA exhibited greater

Figure 2 VSM cell pH_i in response to increasing concentrations of ET-1 (0.1–50 nM)

Changes in pH_i ($\pm$ S.D.) were determined as the difference between baseline, steady-state pH_i values (determined after 5–10 min perfusion with Hepes-PSS) and pH_i values obtained 5 min after perfusion with ET-1 in Hepes-PSS. Coverslips (3–5) from at least two different primary cell cultures were exposed to a single dose of ET-1. Number of coverslips: 3 (0.1 nM ET-1); 3 (1.0 nM ET-1); 5 (10 nM ET-1); 4 (50 nM ET-1). * $P < 0.05$; ** $P < 0.01$ versus baseline. The ability of cells to maintain pH_i at steady-state values during at least 20 min of perfusion with Hepes-PSS was confirmed for each primary culture (results not shown).

acidification than control cells, with the minimum pH_i approximating 6.4 (results not shown). These data indicate the presence of an EIPA-inhibitable membrane system for active extrusion of acid and pH_i regulation in the VSM cells.

Changes in pH_i were also examined in cells perfused with Na^+ -free Hepes-PSS, as an alternate manipulation to inhibit Na^+ - H^+ exchange [13]. Replacement of Na^+ with *N*-methyl-D-glucamine or with choline chloride abolished the alkalization mediated by 10 nM ET-1: $\Delta pH_i = 0.01 \pm 0.01$ in *N*-methyl-D-glucamine media versus 0.11 ± 0.01 in Na^+ -replete media ($n = 3$, $P < 0.01$); $\Delta pH_i = 0.0 \pm 0.0$ in choline chloride media versus 0.11 ± 0.02 in Na^+ -replete media ($n = 3$, $P < 0.01$). In a fashion similar to the effect of EIPA, perfusion with Na^+ -free Hepes-PSS solutions produced significant cytosolic acidification in otherwise unstimulated cells (results not shown). These results further confirm that primary cultures of coronary VSM cells perfused with Hepes-PSS respond to ET-1 with a dose-dependent, sustained cytosolic alkalization, resulting from activation of Na^+ - H^+ exchange.

Role of Na^+ - H^+ exchange in ET-1-mediated $[Ca^{2+}]_i$ response

A typical $[Ca^{2+}]_i$ response of coronary VSM cells to 10 nM ET-1 is illustrated in Figure 3(a). Coronary VSM cells in the presence of Hepes-PSS ($1.25 \text{ mM } [Ca^{2+}]_o$) responded to ET-1 with a transient increase in $[Ca^{2+}]_i$, followed by a lower but sustained plateau phase of increased $[Ca^{2+}]_i$. In the presence of $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ typically peaked within 30 s and then declined to a steady-state plateau level (sustained component) within 5 min. A variable percentage (0–75 %) of cells exhibited spontaneous oscillatory $[Ca^{2+}]_i$ spiking activity during the plateau phase of the response to ET-1, resulting in fluctuations of the average signal. The plateau phase persisted after washout of ET-1 (results not shown). Peak transient $[Ca^{2+}]_i$ responses increased with increasing concentrations of ET-1 (0.1–50 nM; $P < 0.05$; Table 1). Although a clear dose-dependence of the mean plateau $[Ca^{2+}]_i$ response was not demonstrated (Table 1), plateau $[Ca^{2+}]_i$ responses were significantly elevated above baseline $[Ca^{2+}]_i$ levels at all ET-1 concentrations ($P < 0.05$). The mean transient increase in $[Ca^{2+}]_i$

Figure 3 Effect of ET-1 on $[Ca^{2+}]_i$ in VSM cells perfused with Hepes-PSS

$[Ca^{2+}]_i$ was quantified as described in the Materials and methods section. Calibration, *ex vivo*, indicated a ratio of 1.0 to approximate 600 nM $[Ca^{2+}]_i$. (a) Control $[Ca^{2+}]_i$ response to 10 nM ET-1 ($n = 22$ cells). (b) Effect of pretreatment with $3 \mu\text{M}$ EIPA on $[Ca^{2+}]_i$ response to 10 nM ET-1. EIPA was added to the perfusate before and during the addition of ET-1 ($n = 20$ cells).

in response to 10 nM ET-1 persisted in Ca^{2+} -free Hepes-PSS $[\Delta[Ca^{2+}]_i(\text{peak response} - \text{baseline}) \text{ in } Ca^{2+}\text{-free media } ([Ca^{2+}]_o < 125 \text{ nM}) = 575 \pm 50 \text{ nM}$ versus $470 \pm 48 \text{ nM}$ in Ca^{2+} -replete media ($1.25 \text{ mM } [Ca^{2+}]_o$); $n = 3$ experiments; $P = 0.18$). The sustained phase of the $[Ca^{2+}]_i$ response to ET-1 was absent in nominally Ca^{2+} -free medium and thus was $[Ca^{2+}]_o$ -dependent, whereas the transient primarily reflects mobilization of Ca^{2+} from intracellular stores.

Direct comparison of pH_i and $[Ca^{2+}]_i$ changes in a given cell was not possible because simultaneous measurements of pH_i and $[Ca^{2+}]_i$ were not obtained. It appears, however, that the initial rise in $[Ca^{2+}]_i$ precedes the initial rise in pH_i and reaches a maximum more rapidly. In a variety of cells, it has been shown that the initial agonist-mediated increase in $[Ca^{2+}]_i$ distinctly precedes the increase in pH_i , indicating that cytosolic alkalization is not involved in the transient (inositol 1,4,5-triphosphate-mediated) component of Ca^{2+} release from internal stores [18,31].

Initiation of $[Ca^{2+}]_i$ responses before alkalization would not preclude the possibility that activation of the Na^+ - H^+ antiport modulates a component of the $[Ca^{2+}]_i$ response. However, the biphasic $[Ca^{2+}]_i$ response to 10 nM ET-1 was not significantly altered in cells exposed to $3 \mu\text{M}$ EIPA (Figure 3b; Table 1; $P =$

Table 1 VSM [Ca²⁺]_i as a function of increasing concentrations of ET-1

Cells were loaded with fura 2 AM and [Ca²⁺]_i was measured as described in the Materials and methods section. Increases in [Ca²⁺]_i (Δ [Ca²⁺]_i; nmol/l) were calculated by subtracting the baseline from the peak response to ET-1 (transient), and by subtracting the baseline from the mean response during a 5-min interval beginning 10 min post-transient (plateau). Values are means $\pm$ S.E.M. Coverslips (3–6) were exposed to a single dose of ET-1 as indicated by the numbers in parentheses. 3 μ M EIPA + 10 nM ET-1: pretreatment of VSM cells with 5-(*N*-ethyl-*N*-isopropyl)amiloride did not significantly affect the [Ca²⁺]_i response to ET-1 relative to controls [EIPA vehicle (DMSO) + 10 nM ET-1]. Na⁺-free media [choline Cl⁻ or *N*-methyl-D-glucamine chloride (NmDg) Cl⁻] + 10 nM ET-1: isosmotic replacement of NaCl by choline chloride or *N*-methyl-D-glucamine chloride in Hepes-PSS for 10 min before, and during, exposure of cells to ET-1 did not significantly affect the [Ca²⁺]_i response to ET-1 relative to controls (Hepes-PSS).

Treatment	Δ [Ca ²⁺] _i $\pm$ S.E.M. (transient) (nM)	Δ [Ca ²⁺] _i $\pm$ S.E.M. (plateau) (nM)
0.1 nM ET-1	306 $\pm$ 174 (3)	104 $\pm$ 5 (3)
10 μ M ET-1	520 $\pm$ 46 (6)	99 $\pm$ 7 (6)
50 nM ET-1	718 $\pm$ 70 (3)	184 $\pm$ 19 (3)
3 mM EIPA + 10 nM ET-1	445 $\pm$ 51 (4)	77 $\pm$ 11 (4)
EIPA vehicle + 10 nM ET-1	472 $\pm$ 65 (4)	89 $\pm$ 21 (4)
Na ⁺ -free (choline Cl ⁻) + 10 nM ET-1	515 $\pm$ 109 (4)	100 $\pm$ 27 (4)
Hepes-PSS + 10 nM ET-1	484 $\pm$ 40 (4)	96 $\pm$ 18 (4)
Na ⁺ -free (NmDg Cl ⁻) + 10 nM ET-1	440 $\pm$ 108 (3)	76 $\pm$ 13 (3)
Hepes-PSS + 10 nM ET-1	460 $\pm$ 110 (3)	70 $\pm$ 26 (3)

0.74). EIPA at a concentration of 3 μ M was sufficient to block the alkalization response to ET-1 (Figure 1) and the pH_i recovery from ammonia prepulse acidification. This concentration of EIPA is higher than the reported IC₅₀ range for the Na⁺-H⁺ antiport in VSM (0.06–1.0 μ M) [32,33], yet is substantially less than the reported IC₅₀ for the Na⁺-Ca²⁺-exchanger or inward Ca²⁺ currents (approx. 130 μ M) [33]. These data provide evidence contrary to the hypothesis that activation of Na⁺-H⁺ exchange modulates increases in [Ca²⁺]_i mediated by ET-1 in VSM. Blockade of histamine-induced alkalization by 3 μ M EIPA was also without effect on the histamine-induced [Ca²⁺]_i response, indicating that Na⁺-H⁺ exchange does not significantly modulate [Ca²⁺]_i responses to a different agonist (results not shown).

Although 3 μ M EIPA was sufficient to block Na⁺-H⁺ exchange without effect on ET-1-mediated [Ca²⁺]_i responses, higher levels of EIPA (10, 35 and 100 μ M) produced a progressive inhibition of the transient and sustained components of the [Ca²⁺]_i response to 10 nM ET-1; 100 μ M EIPA was > 70% inhibitory (results not shown). Cytosolic acidification by EIPA is not the cause of the inhibition of ET-1-mediated [Ca²⁺]_i responses because 3 μ M EIPA produced a maximal EIPA-inducible acidification (pH_i = 6.6) without affecting [Ca²⁺]_i responses. Additionally, we did not observe any significant effect of any of the EIPA concentrations used on basal fura 2 fluorescence. It has also been shown in VSM that the dissociation constant (*K*_d) for Ca²⁺ and fura 2 does not increase markedly until pH_i is reduced below 6.5 [4]. Thus, higher doses of EIPA exert a [Ca²⁺]_i-inhibitory effect that is not related to its ability to inhibit Na⁺-H⁺ exchange.

Additional experiments examined [Ca²⁺]_i responses evoked by ET-1 in cells perfused with Na⁺-free Hepes-PSS (Na⁺ isosmotically replaced with *N*-methyl-D-glucamine or with choline chloride), a manipulation used to inhibit Na⁺-H⁺ exchange [11]. As summarized in Table 1, [Ca²⁺]_i responses evoked by 10 nM ET-1 were not inhibited by Na⁺-free media relative to Na⁺-replete media. This further indicates that Na⁺-H⁺ exchange

activation does not modulate ET-1-mediated Ca²⁺ responses in coronary VSM.

Replacement of NaCl with choline chloride (or *N*-methyl-D-glucamine) in Hepes-PSS caused a small but significant rise in [Ca²⁺]_i before treatment with ET-1 (choline chloride: Δ [Ca²⁺]_i = 35 $\pm$ 11 nM; *n* = 4; *P* < 0.01). This [Ca²⁺]_i rise was not detectable in Na⁺-free, Ca²⁺-free Hepes-PSS (results not shown). Furthermore, it was not inhibited by pretreatment of cells with 200 μ M ouabain but was reversed by washing with Na⁺-containing Hepes-PSS (results not shown). The elevation of [Ca²⁺]_i under these conditions may be mediated by the Na⁺-Ca²⁺ exchanger, as previously demonstrated [8].

Further experiments were performed in Ca²⁺-free Hepes-PSS in the presence and absence of extracellular Na⁺. By minimizing the influence of [Ca²⁺]_o, by its removal, these experiments examined whether or not activation of Na⁺-H⁺ exchange by ET-1 affected intracellular release of Ca²⁺ (transient component of the [Ca²⁺]_i response to ET-1). The ET-1-mediated Ca²⁺ transient was not diminished by the absence of extracellular Na⁺ in Ca²⁺-free Hepes-PSS [Δ [Ca²⁺]_i (peak response – baseline) in Ca²⁺-free, Na⁺-replete media ([Ca²⁺]_o < 125 nM) = 540 $\pm$ 7 nM versus 492 $\pm$ 73 Ca²⁺-free, Na⁺-free (choline chloride) media; *n* = 4; *P* = 0.58]. These data suggest that, in the presence of [Ca²⁺]_o, the initial elevation of [Ca²⁺]_i resulting from [Na⁺]_o removal was not masking a possible decrease in ET-1-mediated [Ca²⁺]_i transient responses mediated by Na⁺-H⁺ exchange inhibition.

ET-1 and other agonists have been shown to increase [Na⁺]_i [34,35]. Na⁺ influx accompanying ET-1 stimulation, even in the absence of a significant pH_i change, could initiate changes in [Ca²⁺]_i by membrane depolarization and activation of voltage-gated Ca²⁺ channels or by activation of the Na⁺/Ca²⁺ exchanger. It has been suggested that the sustained component of the ET-1-induced [Ca²⁺]_i response is generated by an increase in intracellular Na⁺, arising from stimulation of the Na⁺-H⁺ antiport, which subsequently inhibits Na⁺-Ca²⁺ exchange-mediated extrusion of [Ca²⁺]_i [9]. However, our results, under Na⁺-free conditions, suggest that extracellular Na⁺ is not a prerequisite for ET-1-mediated [Ca²⁺]_i responses and that activation of Na⁺-H⁺ exchange is not involved in the cascade of ET-1-mediated events leading to increased [Ca²⁺]_i in coronary VSM. The possibility remains that VSM [Ca²⁺]_i responses are dependent upon Na⁺-H⁺ exchange at lower concentrations of ET-1.

In platelets, inhibition of the Na⁺-H⁺ exchanger markedly attenuated the rise in [Ca²⁺]_i induced by thrombin [17,20]. The levels of EIPA used in these studies, however, were higher than that necessary to block the Na⁺-H⁺ exchanger. Similar to our results, lower concentrations of EIPA blocked the exchanger without having a significant effect on [Ca²⁺]_i [21,36]. Replacement of Na⁺ by choline was shown to have no significant effect on the platelet rise in [Ca²⁺]_i evoked by thrombin or platelet-activating factor in the presence or absence of [Ca²⁺]_o [37]. Furthermore, the Na⁺-H⁺ antiport does not play a detectable role in platelet [Ca²⁺]_i responses to ADP or arginine vasopressin [38].

[Ca²⁺]_i-dependence of the ET-1-mediated pH_i response

A third series of experiments examined whether an elevation of [Ca²⁺]_i is a prerequisite for activation of the Na⁺-H⁺ exchanger by ET-1. The alkalization response of VSM cells to 10 nM ET-1 was not significantly altered by removal of extracellular Ca²⁺ (Δ pH_i = 0.090 $\pm$ 0.015 in Ca²⁺-free media ([Ca²⁺]_o < 125 nM) versus 0.093 $\pm$ 0.012 in Ca²⁺-replete media (1.25 mM [Ca²⁺]_o; *n* = 3, *P* = 0.87). These data indicate that the [Ca²⁺]_o-dependent, sustained component of the ET-1-mediated [Ca²⁺]_i response is not strictly required for activation of the Na⁺-H⁺ exchanger.

Figure 4 Effect of the intracellular calcium chelator MAPTAM on $[Ca^{2+}]_i$ and pH_i responses to ET-1

For $[Ca^{2+}]_i$ measurements, MAPTAM (100 μ M) was added to the fura 2 AM loading medium for the final 30 min of the loading procedure. Cells were then washed and treated as described in the Materials and methods section. For pH_i measurements, MAPTAM (100 μ M) was added 5 min after the addition of BCECF AM and was maintained in the loading medium for 30 min before washing. Values represent mean responses $\pm$ S.E.M. (a) Control $[Ca^{2+}]_i$ -time-course response to 10 nM ET-1 in cells perfused with HEPES-PSS (1.25 mM Ca^{2+}) ($n = 15$ cells). (b) Control pH_i -time-course response to 10 nM ET-1 in cells perfused with HEPES-PSS (1.25 mM Ca^{2+}) ($n = 29$ cells). (c) $[Ca^{2+}]_i$ -time-course response to 10 nM ET-1 in cells preloaded with MAPTAM and perfused with HEPES-PSS (1.25 mM Ca^{2+}) ($n = 29$ cells). (d) pH_i -time-course response to 10 nM ET-1 in cells preloaded with MAPTAM and perfused with HEPES-PSS (1.25 mM Ca^{2+}) ($n = 24$ cells). (e) $[Ca^{2+}]_i$ -time-course response to 10 nM ET-1 in cells preloaded with MAPTAM and perfused with Ca^{2+} -free HEPES-PSS (nominally Ca^{2+} -free plus 0.5 mM EGTA) ($n = 34$ cells). (f) pH_i -time-course response to 10 nM ET-1 in cells preloaded with MAPTAM and perfused with Ca^{2+} -free HEPES-PSS (nominally Ca^{2+} -free plus 0.5 mM EGTA) ($n = 24$ cells).

The cell-permeable Ca^{2+} chelator, MAPTAM, was used to investigate the influence of $[Ca^{2+}]_i$ mobilization on Na^+H^+ exchange activity induced by ET-1. The results are illustrated in Figures 4(a)–4(f). Figures 4(a) and 4(b) show typical $[Ca^{2+}]_i$ and pH_i responses to ET-1 respectively for cells perfused with Ca^{2+} -containing HEPES-PSS. Cells preloaded with 100 μ M MAPTAM in the presence of 1.25 mM $[Ca^{2+}]_o$, and then exposed to 10 nM ET-1, exhibited marked inhibition of the transient increase in $[Ca^{2+}]_i$ plus partial inhibition of the sustained $[Ca^{2+}]_i$ response (Figure 4c) and partial inhibition of pH_i responses (Figure 4d). When MAPTAM was preloaded and the cells perfused with

Ca^{2+} -free HEPES-PSS, all components of the ET-1-induced $[Ca^{2+}]_i$ response were blocked (Figure 4e). Under these conditions, no increment in pH_i was observed following addition of ET-1 (Figure 4f). To preclude the possibility that MAPTAM may have directly affected the Na^+H^+ exchanger, rates of recovery from intracellular acidification (induced after a 20 mM NH_4Cl prepulse) were assessed in Ca^{2+} -free HEPES-PSS in the presence and absence of MAPTAM. No significant difference in recovery rates was observed [recovery rate (pH units/min) = 0.19 ± 0.02 control versus 0.17 ± 0.003 with 100 μ M MAPTAM; $n = 3$; $P = 0.45$]. This indicates that the ability of the Na^+H^+ exchanger to

respond to an acid load was preserved in the presence of MAPTAM.

Our data demonstrate that an increment in $[\text{Ca}^{2+}]_i$ is a prerequisite for activation of the $\text{Na}^+\text{-H}^+$ exchanger by ET-1 in VSM. In primary cultures of rat VSM cells, preventing the thrombin-induced $[\text{Ca}^{2+}]_i$ transient by $[\text{Ca}^{2+}]_i$ chelation with quin-2 inhibited activation of $\text{Na}^+\text{-H}^+$ exchange [14]. Likewise, Poch et al. [31] have reported that $[\text{Ca}^{2+}]_i$ chelation with MAPTAM blocked the platelet $[\text{Ca}^{2+}]_i$ and pH_i increases induced by thrombin. Hendley et al. [39] observed that thrombin-induced alkalinization in fibroblasts is dependent upon increased $[\text{Ca}^{2+}]_i$ derived from internal stores. In contrast, stimulation of the $\text{Na}^+\text{-H}^+$ exchanger can occur in the absence of measureable $[\text{Ca}^{2+}]_i$ changes in fibroblasts [28]. Although our data show that a $[\text{Ca}^{2+}]_i$ increase is necessary for ET-1-induced stimulation of the $\text{Na}^+\text{-H}^+$ exchanger in coronary VSM, our study did not examine whether increases in $[\text{Ca}^{2+}]_i$ in the absence of agonists are sufficient to prompt activation of the exchanger. Whether increases in $[\text{Ca}^{2+}]_i$ alone are sufficient to stimulate the exchanger may vary with cell type [14,31,39].

VSM cellular response to ET-1 in a $\text{CO}_2/\text{HCO}_3^-$ buffer system

The previous experiments used a nominally HCO_3^- -free buffer system in order to isolate $\text{Na}^+\text{-H}^+$ exchange activity from HCO_3^- -dependent pH_i regulatory systems. Because controversy exists concerning the ability of Ca^{2+} -mobilizing agonists to induce alkalinization in a $\text{CO}_2/\text{HCO}_3^-$ buffer system, we also assessed the pH_i response to ET-1 in the presence of HCO_3^- -PSS. Figure 5(a) shows a typical ET-1-mediated pH_i response for cells perfused with nominally HCO_3^- -free Hepes-PSS. Figure 5(b) reveals that cells perfused with HCO_3^- -PSS did not display an equivalent rise in pH_i in response to 10 nM ET-1. pH_i values did not differ from time-controlled pH_i values obtained in the absence of ET-1 (results not shown). Comparison of Figures 5(a) and 5(b) also reveals that the baseline pH_i before addition of ET-1 was significantly higher in the presence of HCO_3^- -PSS than in the presence of Hepes-PSS [$\text{pH}_i(\text{HCO}_3^-\text{-PSS}) = 7.00 \pm 0.02$ versus $\text{pH}_i(\text{Hepes-PSS}) = 6.84 \pm 0.02$; $P < 0.05$]. The higher basal pH_i in HCO_3^- -PSS was partially reduced by addition of 0.5 mM 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS) to the media (results not shown), implying that basal activity of a HCO_3^- -dependent acid extrusion mechanism is contributing to pH_i regulation [10,23].

Finally, we observed that cells perfused with HCO_3^- -PSS elicited biphasic $[\text{Ca}^{2+}]_i$ responses upon exposure to 10 nM ET-1, similar to those observed in Hepes-PSS [$[\text{Ca}^{2+}]_i$ (nM): baseline = 229 ± 20 ; $\Delta[\text{Ca}^{2+}]_i$ (peak transient - baseline) = 420 ± 63 ; $\Delta[\text{Ca}^{2+}]_i$ (plateau - baseline) = 131 ± 35].

Our findings that ET-1-mediated alkalinization in coronary VSM was not observed in the presence of $\text{CO}_2/\text{HCO}_3^-$ whereas $[\text{Ca}^{2+}]_i$ responses occurred in the presence or absence of $\text{CO}_2/\text{HCO}_3^-$, suggest the lack of involvement of cytosolic alkalinization in the mechanism by which ET-1 increases $[\text{Ca}^{2+}]_i$ in coronary VSM. The present study did not examine whether or not the $\text{Na}^+\text{-H}^+$ exchanger is stimulated by ET-1 in the presence of $\text{CO}_2/\text{HCO}_3^-$. However, activation of this antiport has been shown to occur during agonist stimulation in VSM in the presence of $\text{CO}_2/\text{HCO}_3^-$ [15,23].

In summary, we conclude that in VSM: (1) ET-1 mediates a biphasic increase in $[\text{Ca}^{2+}]_i$ and a sustained activation of $\text{Na}^+\text{-H}^+$ exchange; (2) a concomitant alkalinization occurs only in the absence of HCO_3^- ; (3) ET-1-mediated $[\text{Ca}^{2+}]_i$ responses are not dependent upon or influenced by activation of the $\text{Na}^+\text{-H}^+$ exchanger; and (4) conversely, activation of the $\text{Na}^+\text{-H}^+$ ex-

Figure 5 Effect of bicarbonate on pH_i responses to 10 nM ET-1 as a function of time

Values represent means $\pm$ S.E.M. (a) Control pH_i response to ET-1 in cells perfused with Hepes-PSS ($n = 33$ cells). (b) pH_i response to ET-1 in cells perfused with HCO_3^- -PSS (without Hepes) and gassed with 5% CO_2 /room air to maintain $\text{pH}_o = 7.4$ as described in the Materials and methods section ($n = 29$ cells).

changer by ET-1 requires an increment in $[\text{Ca}^{2+}]_i$. These data suggest that $\text{Na}^+\text{-H}^+$ exchange does not play a primary role in $[\text{Ca}^{2+}]_i$ -dependent transduction of signals elicited by ET-1 in coronary VSM.

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