Endothelin and Angiotensin II Stimulation of Na^+-H^+ Exchange Is Impaired in Cardiac Hypertrophy

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Abstract

We compared the effects of endothelin-1 (ET-1) on intracellular pH, intracellular $[Ca^{2+}]_i$, and cell contraction in hypertrophied adult ventricular myocytes from ascending aortic banded rats and age-matched controls. Intracellular pH (pH_i) was measured in individual myocytes with SNARF-1, and $[Ca^{2+}]_i$ was measured with indo-1, simultaneous with cell motion. Experiments were performed at 36°C in myocytes paced at 0.5 Hz in Hepes-buffered solution (pH_o 7.40) containing 1.2 mM $CaCl_2$. At baseline, calibrated pH_i, diastolic and systolic $[Ca^{2+}]_i$ values, and the amplitude of cell contraction were similar in hypertrophied and control myocytes. Exposure of the control myocytes to 10 nM ET-1 caused an increase in the amplitude of cell contraction to 163±22% of baseline ($P < 0.05$), associated with intracellular alkalinization (pH_i + 0.08±0.02 U, $P < 0.05$) and a slight increase in peak systolic $[Ca^{2+}]_i$ (104±1% of baseline, $P < 0.05$). In contrast, in the hypertrophied myocytes, exposure to ET-1 did not increase the amplitude of cell contraction or cause intracellular alkalinization (−0.01±0.02 U, NS). Similar effects were observed in the hypertrophied and control myocytes in response to exposure to 10 nM angiotensin II. ET-1 also increased the rate of recovery from intracellular acidosis induced by the washout of NH_4Cl in the control cells, but did not do so in the hypertrophied cells. In the presence of 10 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride, which inhibits Na^+-H^+ exchange, ET-1 did not cause a positive inotropic effect or intracellular alkalinization in control cells. The activation of protein kinase C by exposure to phorbol ester caused intracellular alkalinization and it increased the rate of recovery from intracellular acidification induced by an NH_4Cl pulse in control cells but not in hypertrophied cells. ET-1, as well as angiotensin II, and phorbol ester, fail to stimulate forward Na^+-H^+ exchange in adult hypertrophied myocytes. These data suggest a defect in the coupling of protein kinase C signaling with Na^+-H^+ exchange in adult hypertrophied myocytes. (*J. Clin. Invest.* 1997. 99: 125–135.) Key words: hypertrophy • myocytes • endothelin-1 • angiotensin II

Introduction

Cardiac pressure overload hypertrophy is associated with changes in gene expression and function that recapitulate a fetal pattern of cellular biology (1, 2). For example, we have shown that hypertrophied myocytes from aortic banded rats show a fetal pattern of gene programming and metabolism (3, 4). Recent studies suggest that the vasoactive peptides endothelin-1 (ET-1)[1] and angiotensin II may play an important role as trophic growth factors during the hypertrophic growth response in immature myocytes (5, 6). We have shown that the effects of these vasoactive peptides on immediate early cardiac gene induction and protein synthesis differ in normal and hypertrophied adult hearts (7–9), suggesting the potential for differing intracellular signal transduction pathways in adult hypertrophied myocytes.

Previous studies have demonstrated that ET-1, as well as angiotensin II, cause a positive inotropic effect in isolated adult ventricular myocytes from rabbits, which appears to be mediated by intracellular alkalinization which sensitizes the myofilaments to Ca^{2+} rather than by changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) (10, 11). In adult rabbit and rat myocytes, this intracellular alkalosis appears to be due in part to the stimulation of the Na^+-H^+ exchanger (12–14). In contrast, both ET-1 and angiotensin II fail to increase contractility and induce a slight intracellular acidosis in immature cultured ventricular myocytes from both neonatal rat and embryonic chick hearts (10).

The objective of this study was to test the hypothesis that the effects of ET-1 on cell contraction and intracellular pH in adult hypertrophied myocytes differ from those in adult normal myocytes. Therefore, we compared the effects of ET-1, as well as angiotensin II, on cell contraction, intracellular pH, and $[Ca^{2+}]_i$ in isolated normothermic adult ventricular myocytes from aortic-banded rats and normal age-matched controls. We also examined the effects of ET-1 and angiotensin II on the recovery from intracellular acidification and the potential mechanistic role of the stimulation of forward Na^+-H^+ exchange. Our findings indicate that there is a defect in the coupling of protein kinase C activation with enhanced Na^+-H^+ exchange in hypertrophied myocytes.

Methods

Preparation of aortic-banded rats. Weanling male Wistar rats (Charles River Breeding Laboratories, Wilmington, DE) were banded at the age of 3–4 wk (body weight, 75–100 grams) by placing a stainless steel

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1. *Abbreviations used in this paper:* 2DG, 2-deoxyglucose; $[Ca^{2+}]_i$, intracellular calcium; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; ET-1, endothelin-1; TMA-Cl, trimethylamine hydrochloride; TPA, 12-0-tetradecanoylphorbol-13-acetate.

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clip of 0.6 mm internal diameter on the ascending aorta via a thoracic incision. Age-matched control rats underwent a sham-operation. The rats were fed normal rat chow and water ad libitum, and were used 12 wk after banding. We have shown at this stage after aortic banding this model of pressure-overload hypertrophy is characterized by an increase in left ventricular weight relative to sham-operated controls, concentric hypertrophy, and the absence of chamber dilatation (4, 15–17).

Simultaneous measurement of $[Ca^{2+}]_i$ and cell contraction. Left ventricular myocyte dissociation was performed by a modification of methods of Capogrossi et al. (18) and Haddad et al. (19) and has been described in detail elsewhere (3). $[Ca^{2+}]_i$ was measured with Ca^{2+}-sensitive fluorescence indicator indo-1 AM (20) (Molecular Probes, Inc., Eugene, OR) as described previously (3, 11) using a modification of the method of duBell et al. (21) and Ikenouchi et al. (22). Myocytes were attached on coverslips with cell adhesive (Cell-Tak; Collaborative Research, Inc., Waltham, MA) and loaded with 5 μM indo-1 AM in Hepes-buffered solution at room temperature for 30 min. The coverslip was rinsed with indo-1 AM free buffer solution and placed in a flow-through heated (36°C) cell superfusion chamber on the stage of an inverted microscope (Nikon, Tokyo, Japan). The instrumentation for fluorescence measurement has been described in detail elsewhere (3, 11, 22–24). The excitation source was a high-pressure Hg-arc lamp which provides an intense emission peak at 360 nm. Further selection of this excitation was made with narrow band width interference filters. The excitation beam was chopped at 360 Hz to reduce bleaching and the myocyte was illuminated via epifluorescent optics using Fluor ×40 objective lens (Nikon). The fluorescence light was collected by the objective lens and transmitted to a custom-modified spectrofluorometer (FM-1000; Rincon Scientific Instruments, Santa Barbara, CA) for simultaneous measurement of both 400 and 500 nm wavelengths using two separate photomultiplier tubes. The spectrofluorometer provided analogue signals representing the fluorescence intensity at both wavelengths and the ratio of emitted fluorescence (400/500 nm). The subtraction of background autofluorescence was done by offsetting the photomultiplier tube outputs during the measurement of fluorescence from an unloaded myocyte at the beginning of each experiment. An adjustable iris was used to restrict the optical image to only one myocyte of interest in each experiment to minimize background fluorescence from other myocytes. The image of the beating myocyte was obtained by illumination via the 50-W standard microscope light source passed through a 645-nm band-pass filter. This wavelength was long enough not to interfere with the fluorescence detection at 400 and 500 nm. The motion of the myocyte was monitored using a solid state camera (GP-CD60; Panasonic, Secaucus, NJ) and a custom-modified video detector system (24) (Crescent Electronics, Sandy, UT). The analogue output of the cell motion signal was monitored and recorded continuously with the analogue signal of the $[Ca^{2+}]_i$-sensitive fluorescence ratio (F400/500 nm). Two platinum electrodes placed in the bathing fluid were connected to a stimulator (SD9G; Grass Instruments, Quincy, MA) and used to stimulate the myocyte at 0.5 Hz with 3-ms pulses.

In these experiments, myocytes from 51 hearts from sham-operated rats and 40 hearts from aortic-banded rats were studied. Two to four experiments were performed in sequence from separate coverslips of myocytes isolated from one heart. We have documented recently the stability of both cell motion and $[Ca^{2+}]_i$ for the 15-min duration of the protocols used in this study and demonstrated minimal compartmentation of indo-1 in intracellular organelles in chemically skinned myocytes (3). To determine the absolute values of peak-systolic and end-systolic $[Ca^{2+}]_i$ under baseline conditions in hypertrophied and control myocytes, we performed calibration studies described (3) by a modification of the methods of Cheung et al. (25) and Borzak et al. (26). The calibration methods have been described in detail elsewhere (3).

Measurement of intracellular pH. Intracellular pH (pH_i) was measured with the pH-sensitive indicator SNARF-1 AM (Molecular probes, Inc.) as described recently (10–12, 22). First, 50 μg of SNARF-1 AM was added to 50 μl of dimethyl sulfoxide, which was mixed with 450 μl of fetal bovine serum. Loading of SNARF-1 AM was done by exposing the myocytes on coverslips of a final concentration of 8 μM SNARF-1 AM for 15 min at 36°C. The coverslip was rinsed with SNARF-1 AM free solution and placed on a flow-through cell chamber as described above. Excitation was performed at 540 nm, and fluorescence emission was collected simultaneously at 580 and 640 nm using the same optics system as described above except for the substitution of different dichroic mirror and interference filters. To improve the signal-to-noise ratio, the 640/580 fluorescence ratio signal was filtered by a high cutoff filter at 1.0 Hz. At the end of the experiment, the emission ratio from each cell was calibrated in situ by exposing cells to solutions of varying pH. Each solution contained (mM): K^+ 140 (adjusted to keep $[K^+]$ constant), $MgCl_2$ 1.0, Hepes 4.0, EGTA 2.0, 2,3-butanedione monoxime 30, BAPTA-AM 50 μM (Molecular Probes Inc.) and nigericin 14 μM (Sigma Chemical Co., St. Louis, MO), and was titrated to various pH values (6.7, 6.85, 7.0, 7.2, 7.4) using 1.0 N KOH. Since changes in pH are not linear to the ratio of fluorescence emission (23), pH_i was calculated by the equation, $pH_i = (ax + c)/(1 + bx)$; where x is the measured emission ratio, and a, b, and c are constant parameters (12, 23).

Experimental protocol: effects of ET-1 on cell contraction and $[Ca^{2+}]_i$. Hypertrophied ($n = 21$) and control ($n = 17$) myocytes were superfused with oxygenated Hepes-buffered normal Tyrode solution of the following composition (mM): NaCl 137, KCl 3.7, $MgCl_2$ 0.5, Hepes (free acid) 4.0, $CaCl_2$ 1.2, glucose 11, and probenecid 0.5 with a final pH of 7.40. Probenecid, a blocker of organic anion transport, was added as it has been shown to inhibit secretion of both indo-1 and fura-2 free acids from loaded cells (27, 28). The temperature of the myocytes was maintained at 36–37°C. The myocytes were placed at 0.5 Hz. After recording baseline data, the cells were superfused with oxygenated Hepes-buffered solution containing ET-1 (Sigma) over a concentration range of 10^{-10} to 10^{-6} M. Each concentration was studied in a separate group of myocytes (5–7 cells per group) for 10 min. The analogue cell motion signals and the F400:500 nm analogue signals were recorded simultaneously. The F400/500 values were converted to $[Ca^{2+}]_i$ using the procedure described in the previous section.

In separate experiments, we also studied the effects of angiotensin II (Sigma) on cell motion and intracellular calcium handling in additional control ($n = 23$) and hypertrophied myocytes ($n = 17$). First, myocytes were superfused with the same Hepes-buffered solution used for baseline superfusion in the ET-1 protocol, then with the same solution containing angiotensin II over a concentration range of 10^{-10} to 10^{-6} M for 10 min. Each concentration was studied in separate groups of myocytes (4–7 cells per group). The temperature, pH, and the pacing rate were the same as those in the ET-1 protocol.

Effects of ET-1 on intracellular pH. To investigate whether the increased amplitude of cell contraction in myocytes exposed to 10 nM ET-1 and the lack of an inotropic effect in hypertrophied control myocytes is due to changes in the pH_i, separate experiments were performed in hypertrophied ($n = 12$) myocytes and control myocytes ($n = 11$) loaded with SNARF-1. Myocytes were first superfused with the same Hepes-buffered solution used for baseline superfusion to eliminate effects of Cl^-/HCO_3^- exchange and Na^+/HCO_3^- exchange on pH_i. The superfusate was then switched to solution with 10 nM ET-1 for 10 min. In additional experiments in hypertrophied ($n = 8$) and control ($n = 8$) myocytes, the effects of an NH_4Cl pulse in the presence and absence of 10 nM ET-1 was examined by abrupt exposure and washout of Hepes-buffered solution containing 10.0 mM NH_4Cl prepared by equimolar replacement of NaCl with NH_4Cl. As previously described (12, 23), the abrupt exposure to NH_4Cl initially increases pH_i as basic NH_3 rapidly enters the myocyte. The pH_i then falls as charged NH_4^+ enters the cell via K^+ channels and dissociates. Upon washout of extracellular NH_4Cl, intracellular acidosis is created as internal NH_3 leaves the cell, causing the intracellular retention of H^+. Previous studies have clarified that the initial rate of recovery from this intracellular acidosis depends predominantly on forward

Na^+-H^+ exchange (12, 29). In additional experiments, the same protocol was done in SNARF-1 loaded hypertrophied ($n = 4$) and control ($n = 8$) myocytes in the presence and absence of 10 nM angiotensin II.

Contribution of Na^+-H^+ exchange. To further assess the contribution of Na^+-H^+ exchange to ET-1–induced alkalosis, additional SNARF-1–loaded control ($n = 9$) and hypertrophied myocytes ($n = 6$) were perfused with 10 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) (Sigma), which inhibits Na^+-H^+ exchange. As described above, all experiments were performed using Hepes-buffered solutions with pH_o of 7.40. Intracellular pH was measured at baseline and after 6 min of exposure to 10 μM EIPA, followed by 6 min of exposure to 10 nM ET-1 in the presence of EIPA. The identical protocol was performed in additional control ($n = 4$) and hypertrophied ($n = 4$) myocytes to examine effects of exposure to 10 nM angiotensin II in the presence of EIPA. The same protocols were performed in an additional series of myocytes to measure cell contractions, since optical considerations do not permit simultaneous measurement of SNARF-1 fluorescence and detection of cell motion. Because ET-1 causes pH_i to fall in both control and hypertrophied myocytes during inhibition of Na^+-H^+ exchange, the possibility of metabolic acid production was evaluated by preincubating cells in glucose-free solution containing 15.0 mM 2-deoxyglucose (2DG) to inhibit glycolysis with 5 mM acetate as substrate for mitochondrial oxidative phosphorylation (3, 12) and 10 μM EIPA. The effects of exposure to 10 nM ET-1 in the presence of 2DG and 10 μM EIPA were examined in SNARF-1–loaded control ($n = 4$) and hypertrophied ($n = 4$) myocytes.

Calculation of intrinsic intracellular buffering power (B_i). B_i was experimentally estimated using step reductions in trimethylamine hydrochloride (TMA-Cl; Sigma) to cause stepwise changes in pH_i as previously described in isolated myocytes (12, 30, 31). To eliminate transsarcolemmal flux of acid equivalents via Cl^-/HCO_3^- exchange, Na^+-HCO_3^- transport, and Na^+/H^+ exchange, all cell bathing solutions used for B_i measurements contained no added HCO_3^-, CO_2, or sodium, and were buffered with Hepes. Sodium-free solutions were prepared by equal replacement of NaCl with *N*-methyl-D-glucamine (Sigma), using 1.0 N HCl to adjust pH to 7.40. To minimize $[Ca^{2+}]_i$ accumulation via Na^+/Ca^{2+} exchange, the solutions also contained no added calcium. The final composition of the sodium-free solutions (mM) was: *N*-methyl-D-glucamine 137, KCl 3.7, glucose 11, Hepes, 4.0, MgCl 0.5. These solutions were gassed with 100% O_2 through a column of $Ba(OH)_2$ crystals to remove any residual bicarbonate. The solutions containing TMA-Cl of 15, 10, 5, 1, and 0 mM were prepared by equimolar replacement of *N*-methyl-D-glucamine with TMA-Cl, respectively. pH_i, was allowed to stabilize in sodium-free solution before applying TMA-Cl. The relationship between B_i and pH_i, where pH_i was taken at the midpoint of each pH_i step, was plotted as line fit by least squares, linear regression for the control myocytes (27 measurement points from 7 myocytes), and hypertrophied myocytes (31 measurement points from 8 myocytes). B_i (mM/pH) was calculated as described previously (12, 30, 31):

$$B_i = \Delta [TMAH^+]_i / \Delta pH_i ;$$

where ΔpH_i is the measured change in pH_i, and $\Delta[TMAH^+]_i$ is the change in intracellular concentration of trimethylamine ions, calculated according to:

$$[TMAH^+]_i = \frac{[TMA]_0 10^{pH_0 - pH_i}}{1 + 10^{pH_0 - pK}} ;$$

where $[TMA]_o$ is the total extracellular concentration of trimethylamine, pH_o is extracellular pH, with the dissociation constant (pK) taken as 9.8 (30). Since all experiments were conducted in the nominal absence of CO_2 and HCO_3^-, intracellular HCO_3^- buffering was negligible and total intracellular buffering (B_T) was equal to B_i.

Effects of protein kinase C on Na^+-K^+ exchange. To examine the potential role of the activation of protein kinase C in control and hypertrophied myocytes, the effects of 80 nM 12-0-tetradecanoylphorbol-13-acetate (TPA; Sigma) on pH_i were compared in control ($n = 7$) and hypertrophied myocytes ($n = 5$) loaded with SNARF-1. In addition, the effect of preincubation with 80 nM TPA on the rate of recovery from intracellular acidification induced by an NH_4Cl pulse was studied in control ($n = 5$) and hypertrophied ($n = 5$) cells. Following the observation that TPA caused intracellular alkalinization in control myocytes, the effect of the inactive phorbol ester 4-α phorbol 12,13-didecanoate (100 nM, Research Biochemicals Inc., Natick, MA) on pH_i was also studied ($n = 4$ cells). In addition, we studied the effects of 10 nM ET-1 ($n = 6$) or 10 nM angiotensin II ($n = 5$) on pH_i in the presence of 30 nM GF-109203X, a selective protein kinase C inhibitor (Research Biochemicals Inc.), in control cells. Intracellular pH was measured at baseline and after 6 min of exposure to 30 nM GF-109203X, followed by 6 min of exposure to 10 nM ET-1 or 10 nM angiotensin II in the presence of GF-109203X.

To determine if protein kinase C also mediates the intracellular acidification that is seen when Na^+-H^+ exchange is blocked, the effect of TPA on pH_i after inhibition of Na^+-H^+ exchange with 10 μm EIPA was studied in control ($n = 5$) and hypertrophied ($n = 6$) myocytes.

Statistical analysis. Two-way ANOVA with repeated measures was used for the comparisons between the control versus hypertrophied myocytes, or control versus hypertrophied perfused hearts, of values measured over time in response to exposure to ET-1, or to angiotensin II. Unpaired Student's *t* test was used for comparisons of the values between the groups at baseline. $P < 0.05$ was considered significant. Results are expressed as mean±SEM.

Results

Characteristics of isolated hypertrophied and normal myocytes. Both left ventricular weight and left ventricular weight-to-body weight ratio were significantly increased in left ventricles from aortic-banded rats compared with those from sham-operated rats (Table I). Table II shows baseline function of left ventricular control myocytes from sham-operated rats and hypertrophied myocytes from the aortic-banded rats used in the experimental protocols. Myocytes were superfused with oxygenated Hepes-buffered solution (pH 7.40) containing 1.2 mM $CaCl_2$ and were paced at 0.5 Hz at 36–37°C. There was a significant increase in myocyte cell length in hypertrophied myocytes compared with controls. The amplitude of cell contraction and percent fractional shortening were not statistically different between the two groups. Both time to peak shortening and the time to 50% relengthening were slightly prolonged in the hypertrophied myocytes relative to the control myocytes. Table II shows the results of the intracellular Ca^{2+} calibration. Under the baseline experimental conditions of this study, systolic and diastolic $[Ca^{2+}]_i$ levels were similar between control and hypertrophied myocytes. The calibrated values of

Table I. Body Weight and Left Ventricular Weight of Control and Hypertrophied Rats

	BW	LVW	LVW/BW
	grams	*mg*	
Controls ($n = 51$)	535±12	1895±45	3.44±0.06
LVH ($n = 40$)	520±13	2410±50*	4.71±0.10*

Mean±SEM. *$P < 0.05$ versus control rats. *BW*, body weight; *LVW*, left ventricular wet weight; *LVW/BW*, the ratio of left ventricular wet weight to body weight; *LVH*, hypertrophied aortic-banded rats.

Table II. Baseline Characteristics of Myocyte Function and Intracellular Ca^{2+}

	Controls	Hypertrophy
	(n = 51)	*(n = 43)*
Myocyte length (μm)	136±2	149±2*
Fractional cell shortening (%)	4.46±0.30	4.52±0.31
Amplitude cell shortening (μm)	6.2±0.3	6.8±0.3
Peak positive first derivative of cell motion (μm/s)	169±10	158±15
Peak negative first derivative of cell motion (μm/s)	−141±11	−136±13
Time to peak shortening (ms)	54±2	75±2*
Time to 50% relengthening (ms)	33±1	43±2*
Peak-systolic $[Ca^{2+}]_i$ (nm)	659±71	738±40
End-diastolic $[Ca^{2+}]_i$ (nm)	96±14	99±12
Time to peak $[Ca^{2+}]_i$ (ms)	33±1	37±1*
Time to 50% decline in $[Ca^{2+}]_i$ from peak (ms)	95±3	111±3*
Intracellular pH (pH_i)	(n = 40)	(n = 25)
	7.07±0.02	7.03±0.02

Mean±SEM. *$P < 0.05$ versus control myocytes. All data were obtained under baseline perfusion conditions of 36°C, perfusate $[Ca^{2+}]$ 1.2 mM, and paced rate of 0.5 Hz. $[Ca^{2+}]_i$ was measured using cells loaded with the Ca^{2+}-sensitive indicator indo-1. Intracellular pH was measured using the pH-sensitive indicator SNARF-1.

diastolic and systolic $[Ca^{2+}]_i$ in the normothermic paced rat myocytes are similar to those previously reported by us and others (3, 26, 32). The time to peak $[Ca^{2+}]_i$ and time to 50% decline in $[Ca^{2+}]_i$ from peak were slightly prolonged in the hypertrophied myocytes (12 and 17% above control myocyte values, respectively). As shown in Table II, the calibrated values of pH_i using the indicator SNARF-1 were similar in control ($n = 20$) and hypertrophied myocytes ($n = 19$), and these values are comparable with measurements reported by others in isolated ventricular myocytes (22, 23).

Effects of ET-1 in isolated hypertrophied and normal myocytes. ET-1 caused a maximal positive inotropic effect at a concentration of 10 nM in the control myocytes, manifest as an increase in the amplitude of contraction to 163±22% of baseline ($P < 0.05$). However, ET-1 did not increase the amplitude of cell contraction at any concentration in the hypertrophied myocytes (102±7% of baseline, NS, in response to 10 nM ET-1). The effects of 10 nM ET-1 on fractional cell shortening and peak systolic $[Ca^{2+}]_i$, relative to baseline are illustrated in Fig. 1. Perfusion with 10 nM ET-1 caused a slight but consistent increase in peak systolic $[Ca^{2+}]_i$ in the control and hypertrophied myocytes (104±1%, $P < 0.05$; and 103±1% of baseline, $P < 0.05$, respectively). There was no change in diastolic $[Ca^{2+}]_i$ or diastolic cell length in either group in response to 10 nM ET-1.

The effects of angiotensin II were studied in additional control and hypertrophied myocytes. Angiotensin II also caused a maximal positive inotropic effect at a concentration of 10 nM in the control myocytes, manifest as an increase in the amplitude of cell contraction to 119±4% of baseline ($P < 0.05$). However, angiotensin II did not increase the amplitude of cell contraction at any concentration in the hypertrophied myocytes (105±4% of baseline, NS, in response to 10 nM angiotensin II). Similar to the effects of ET-1, angiotensin II caused only a slight increase in peak systolic $[Ca^{2+}]_i$ in the control and hypertrophied myocytes (105±0.6% of baseline, $P < 0.05$; and 108±3.0% of baseline, $P < 0.05$, respectively) without any change in diastolic $[Ca^{2+}]_i$ levels. In addition, exposure to 10^{-6} M BMS 186-295, a highly specific angiotensin AT_1 receptor subtype blocker (Bristol-Myers Squibb), completely inhibited the positive inotropic effect of 10 nM angiotensin II in control cells ($n = 8$, 108±9% of baseline, NS) and had no effect in the hypertrophied cells ($n = 5$, 94±4% of baseline, NS).

Effects of ET-1 and angiotensin II on intracellular pH. As illustrated in Fig. 2, 10 nM ET-1 caused a significant increase in pH_i in the control myocytes (+0.08±0.02 U, $P < 0.05$). How-

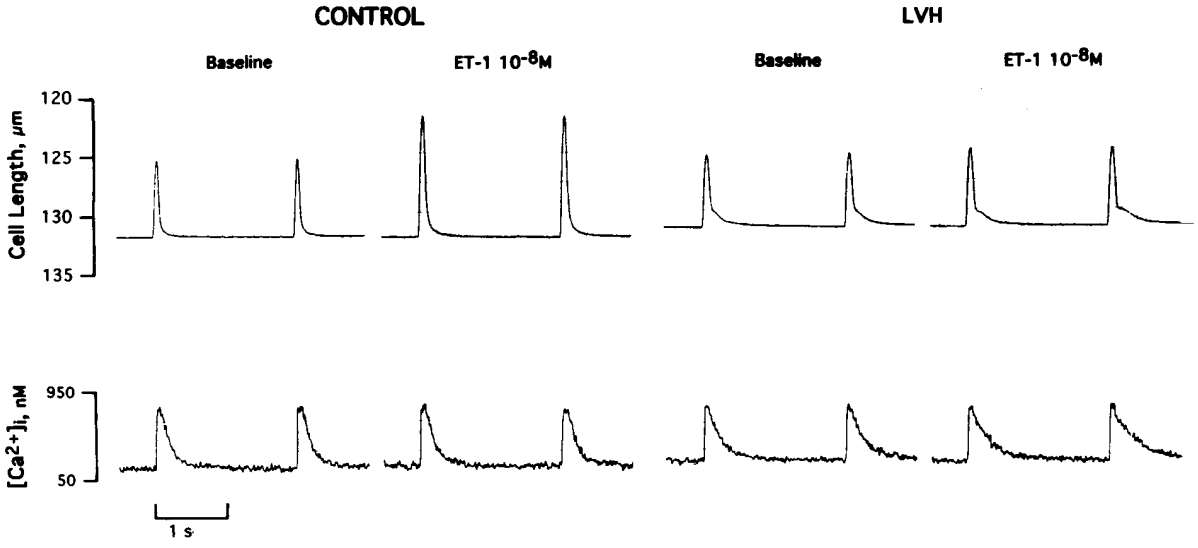

Figure 1. Representative traces of cell motion and $[Ca^{2+}]_i$-sensitive ratio of fluorescence intensities of 400 to 500 nM (F400/500) during baseline condition and in response to 10 nM ET-1 in a control myocyte (*left*) and in a hypertrophied myocyte (*right*). These representative recordings were selected from the control ($n = 7$) and hypertrophied ($n = 7$) myocyte studies. Tracings are displayed with the convention that systolic myocyte shortening is displayed as an upward deflection of the cell motion trace. Note that ET-1 increased the amplitude of cell shortening in the control myocyte, with minimal change in $[Ca^{2+}]_i$, but did not cause a positive inotropic effect in the hypertrophied myocyte.

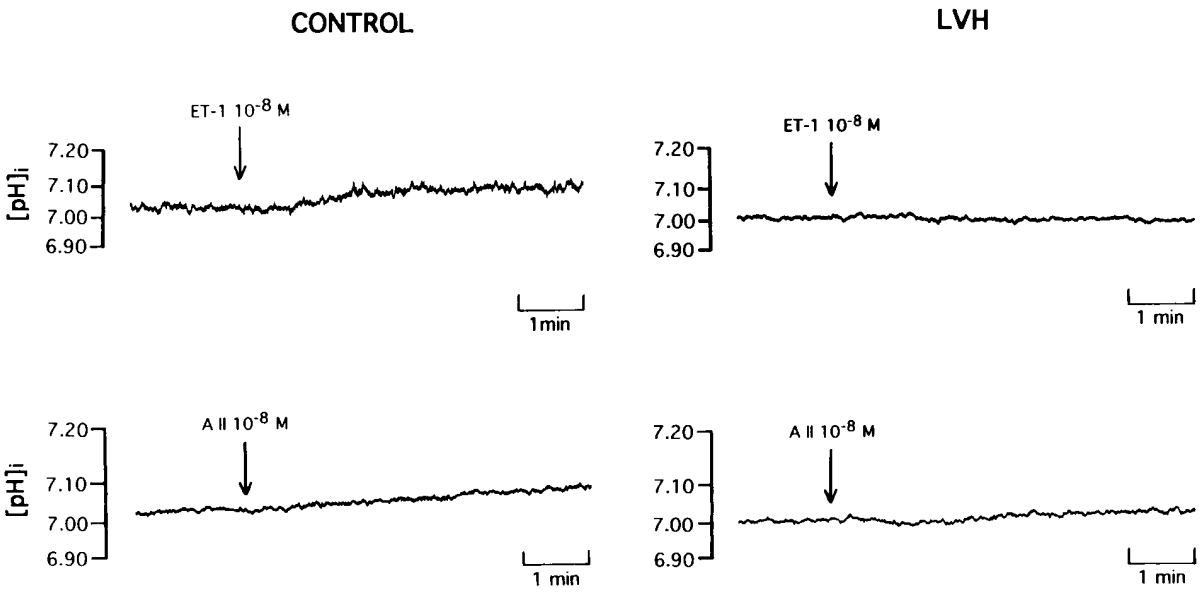

Figure 2. Representative traces of the effects of 10 nM ET-1 and 10 nM angiotensin II on pH_i in a control myocyte (*left*) and on hypertrophied myocyte (*right*) loaded with the pH-sensitive fluorescence indicator, SNARF-1. ET-1 and angiotensin II caused intracellular alkalinization in the control myocyte. In contrast, there was no change in pH_i in the hypertrophied myocyte in response to ET-1 and angiotensin II.

ever, the hypertrophied myocytes showed no increase or a slight fall in pH_i in response to 10 nM ET-1 (-0.01 ± 0.0 U, NS). The average effects of ET-1 on pH_i are shown in Fig. 3 *A*. The effect of 10 nM angiotensin II on pH_i was studied in additional control and hypertrophied cells (Fig. 3 *B*). Although the effect was not as marked as that observed with ET-1, angiotensin II also caused an intracellular alkalinization in pH_i in control cells ($+0.02\pm0.01$ U, $P < 0.05$) and no change in hypertrophied myocytes ($+0.001\pm0.01$ U, NS).

To assess the potential contribution of ET-1 to the recovery from acidosis, additional control and hypertrophied myocytes were exposed to an abrupt pulse and washout of NH_4Cl in the absence and in the presence of 10 nM ET-1. As illustrated in Fig. 4, *aA* and *bA*, the NH_4Cl pulse caused an initial intracellular alkalosis and secondary intracellular acidosis. The magnitude and time course of recovery from intracellular acidification was similar in the control and hypertrophied myocytes. In the presence of 10 nM ET-1, the recovery from intracellular acidification and from the associated negative inotropic effect was enhanced in the control myocytes but not in the hypertrophied myocytes (Fig. 4). The average effects of ET-1 on the increase in pH_i during the initial 3 min of recovery from maximal intracellular acidification are shown in Fig. 5 and Table III. The effect of 10 nM angiotensin II on the initial rate of recovery from intracellular acidification was also studied in additional control and hypertrophied myocytes (Fig. 5 and Table

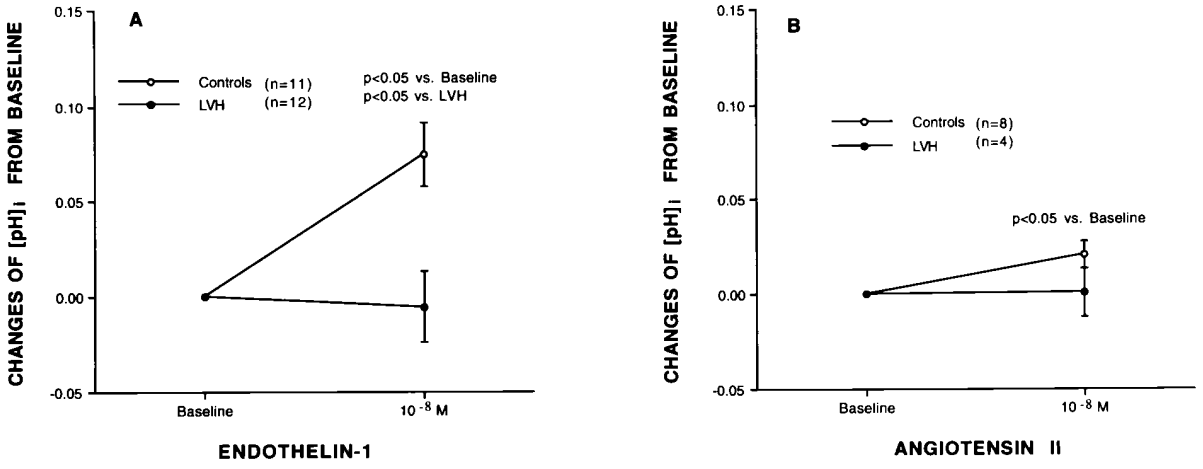

Figure 3. Average effects of 10 nM ET-1 (*A*) and 10 nM angiotensin II (*B*) on intracellular pH in hypertrophied and control myocytes. Values are normalized relative to baseline for each myocyte. At baseline, pH_i was similar in both groups. Both ET-1 and angiotensin II caused intracellular alkalinization in the control cells, whereas no change or a slight fall in pH_i was observed in the hypertrophied myocytes. Values are reported under steady state conditions 6 min after exposure to drug.

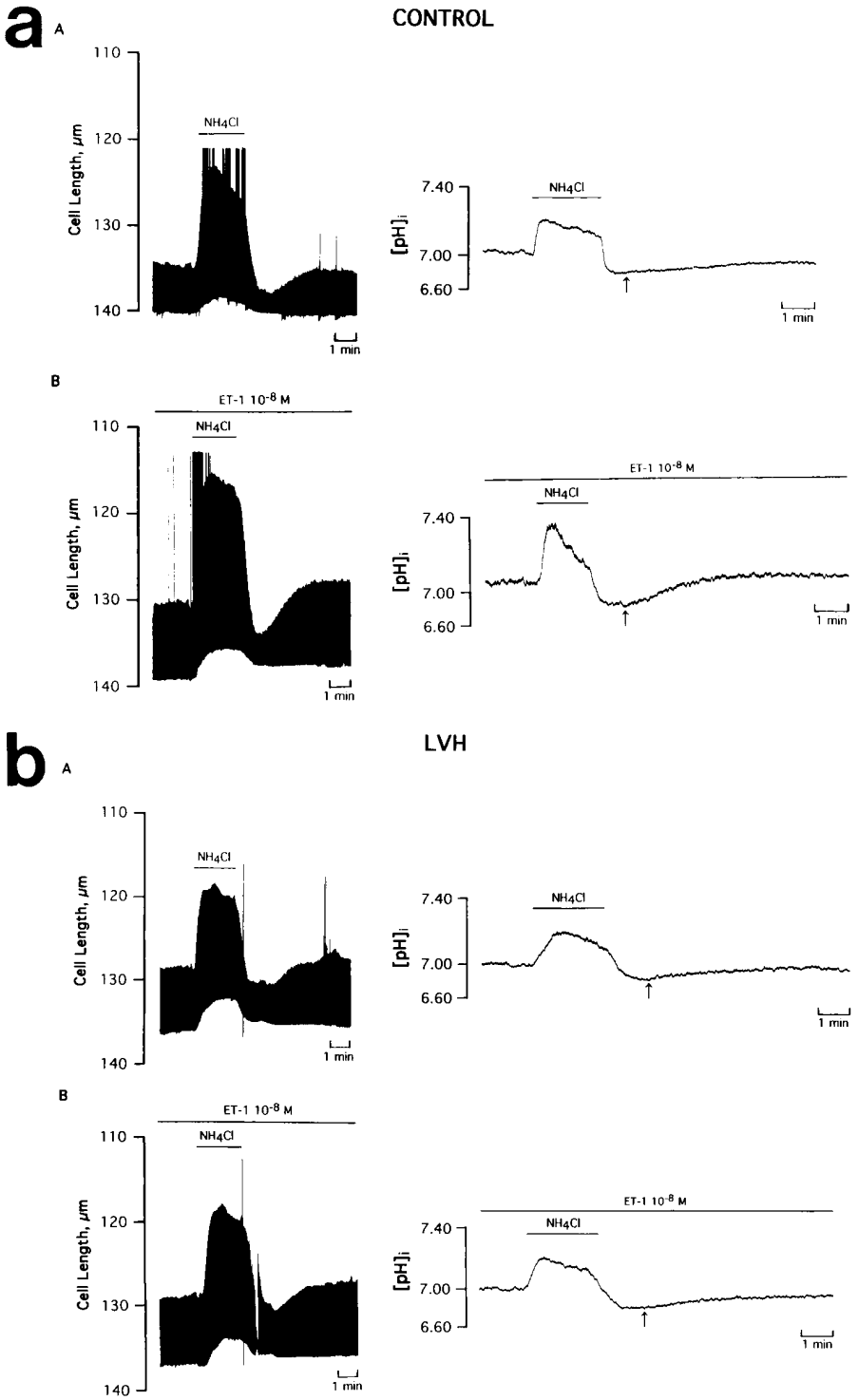

Figure 4. Representative effects of exposure and washout of NH_4Cl in the absence (*aA* and *bA*) and presence (*aA* and *bB*) of 10 nM ET-1 in control (*a*) and hypertrophied (*b*) myocytes on myocyte shortening (cell length) and pH_i. In the absence of ET-1 (*aA* and *bA*), the NH_4Cl pulse caused an initial intracellular alkalinization and secondary acidification which was of similar time course and magnitude in the control and hypertrophied myocytes. Arrows denote the onset of recovery from acidification. Note that alkalinization was accompanied by an increase in the amplitude of cell contraction and incomplete relaxation, whereas acidification caused a negative inotropic effect. Panel *aB* illustrates that in the control myocytes, the presence of ET-1 during the NH_4Cl pulse was associated with an exaggerated initial alkalinization, and acceleration of the recovery of pH_i from the secondary phase of acidification, implicating the stimulation of Na^+-H^+ exchange. In contrast, in the hypertrophied myocytes (*bB*), ET-1 did not modify the changes in pH_i induced by the NH_4Cl pulse.

III). Exposure to angiotensin II enhanced the rate of recovery from intracellular acidification induced by an NH_4Cl pulse in control myocytes but not in the hypertrophied myocytes.

In these experiments using the NH_4Cl pulse, differences in the effects of ET-1 and angiotensin II on recovery from intracellular acidosis in Hepes-buffered control and hypertrophied cells could be related to differences in the stimulation of H^+ flux via Na^+-H^+ exchange or to differences in intrinsic buffering power (B_i). As shown in Fig. 6, the intrinsic buffering power of hypertrophied and control myocytes was similar under the experimental conditions of this study.

Na^+-H^+ exchange in hypertrophied myocytes. To assess the contribution of Na^+-H^+ exchange to the effects of ET-1, control and hypertrophied myocytes were exposed with 10 μM EIPA for 6 min to inhibit Na^+-H^+ exchange and then exposed to the 10 nM ET-1 for 6 min during continuous exposure to EIPA. EIPA alone did not modify pH_i in control or hypertrophied cells (Table IV). In the presence of EIPA, ET-1 did not

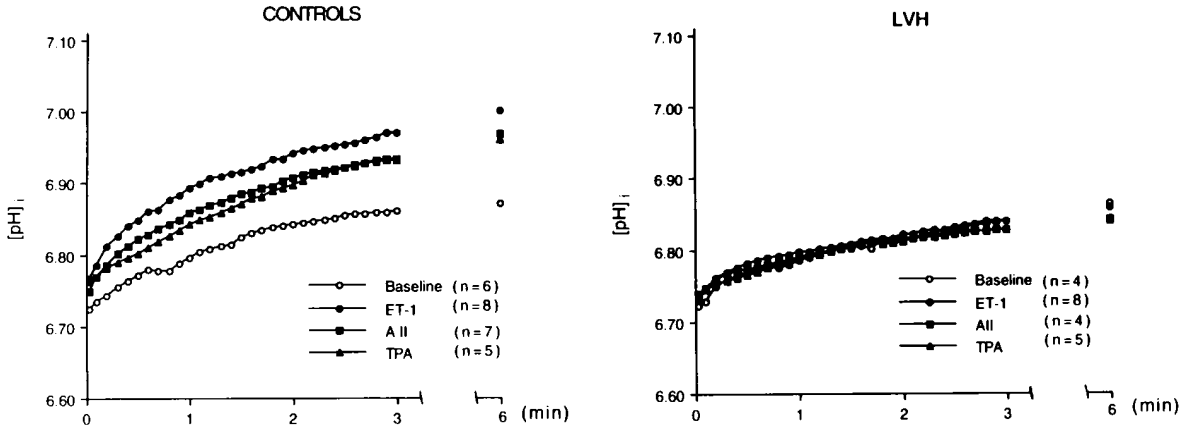

Figure 5. The time course of recovery from maximal intracellular acidosis elicited by washout of a 10.0 mM NH_4Cl pulse in control and hypertrophied cells. The final recovery value 6 min after washout of NH_4Cl is shown. The left panel shows data for control myocytes at baseline in the absence of drug and in the presence of 10 nM ET-1, 10 nM angiotensin II, and 80 nM TPA. The right panel shows data for hypertrophied myocytes. Five to eight cells were studied per group. For clarity, the standard error bars are not shown. In comparison with no drug, ET-1, angiotensin II, and TPA each increased the rate of recovery from intracellular acidification in the control cells ($P < 0.05$). In contrast, these interventions had no effect on the rate of recovery from intracellular acidification in the hypertrophied cells (P = NS), suggesting a failure of the interventions to stimulate Na^+-H^+ exchange.

cause intracellular alkalinization in the control myocytes; instead, ET-1 caused intracellular acidification in both control and hypertrophied myocytes (Table IV). The effects of 10 nM angiotensin II on pH_i during inhibition of Na^+-H^+ exchange with 10 μM EIPA were also studied in additional control and hypertrophied myocytes. Angiotensin II in the presence of EIPA caused intracellular acidification in both control and hypertrophied myocytes (Table IV). To examine the possibility that the intracellular acidification induced by ET-1 in the presence of the inhibition of Na^+-H^+ exchange is in part related to metabolic acid production (12), the effects of 10 nM ET-1 on pH_i were studied in control and hypertrophied cells in the presence of solution containing 10 μM EIPA and 15 mM 2DG to inhibit glycolysis with 5.0 mM acetate as substrate for oxidative phosphorylation. The presence of 2DG completely prevented the ET-1–induced intracellular acidification during exposure to EIPA in both control (−0.03±0.02 U, NS) and hypertrophied (−0.02±0.02 U, NS) cells.

Activation of protein kinase C. Krämer et al. (13) and Kohmoto et al. (10) have suggested previously that the effects of ET-1 on intracellular alkalinization may be mediated in part by activation of protein kinase C. Therefore, we compared the effects of the phorbol ester TPA on pH_i in additional control and hypertrophied cells. Exposure to 80 nM TPA increased pH_i in control cells (+0.06+0.01 U, $P < 0.05$) but not in hypertrophied cells (+0.01±0.01 U, NS). Exposure to the inactive phorbol ester 100 nM 4-α phorbol 12,13-didecanoate had no effect on pH_i in the control cells (+0.01±0.01 U, NS). In addition, we studied the effects of 10 nM ET-1 and 10 nM angiotensin II on pH_i in the presence of specific inhibition of protein kinase C with 30 nM GF-109203X in control cells. In the presence of GF-109203X, ET-1 (−0.01±0.01 U, NS) and angiotensin II (−0.03±0.02 U, NS) had no effect on pH_i. To examine the effects of TPA on Na^+-H^+ exchange, the rate of recovery from intracellular acidification induced by an NH_4Cl pulse in the presence of 10 nM TPA was compared in additional control and hypertrophied cells. The results are shown in Fig. 5 and Table III. In the control cells, 80 nM TPA enhanced the initial rate of recovery from intracellular acidification; in contrast, TPA had no effect on the initial rate of recovery from intracellular acidification in the hypertrophied cells.

Table III. Effects of Interventions on the Initial Recovery of Intracellular pH from NH_4Cl-induced Acidification

	No drug	ET-1	A II	TPA
Controls	Δ0.12±0.01	Δ0.20±0.02*‡	Δ0.19±0.02*‡	Δ0.18±0.02*‡
LVH	Δ0.12±0.01	Δ0.11±0.02	Δ0.11±0.02	Δ0.11±0.01

Mean±SEM. *$P < 0.05$ versus no drug, ‡$P < 0.05$ versus LVH. The ΔpH_i (pH units) was measured during the first 3 min of recovery from maximal acidification induced by washout of NH_4Cl pulse in presence and absence of drug. Each intervention was performed in separate groups of cells (4–8 cells/group). ET-1, exposure to 10 nM ET-1; *A II*, exposure to 10 nM angiotensin II; *TPA*, exposure to 80 nM TPA.

The effect of TPA on pH_i was also examined after inhibition of Na^+-H^+ exchange with EIPA to determine if protein kinase C also mediates the intracellular acidification that is seen when Na^+-H^+ exchange is blocked (Table IV). Following inhibition of forward Na^+-H^+ exchange, TPA caused intracellular acidification rather than alkalinization in both control cells and hypertrophied cells. This suggests that the defect in protein kinase C signaling in hypertrophied cells is not generalized beyond the defect in coupling between protein kinase C and Na^+-H^+ exchange. Thus, it is apparent that the effects of ET-1, angiotensin II, and TPA on the stimulation of Na^+-H^+ exchange are similar in control cells, but differ in adult hypertrophied cells.

Discussion

In this study, exposure of normal adult rat ventricular myocytes to ET-1 caused an increase in the amplitude of contraction which was mediated by intracellular alkalinization rather than by a predominant increase in $[Ca^{2+}]_i$. The responses of adult hypertrophied myocytes were strikingly different since

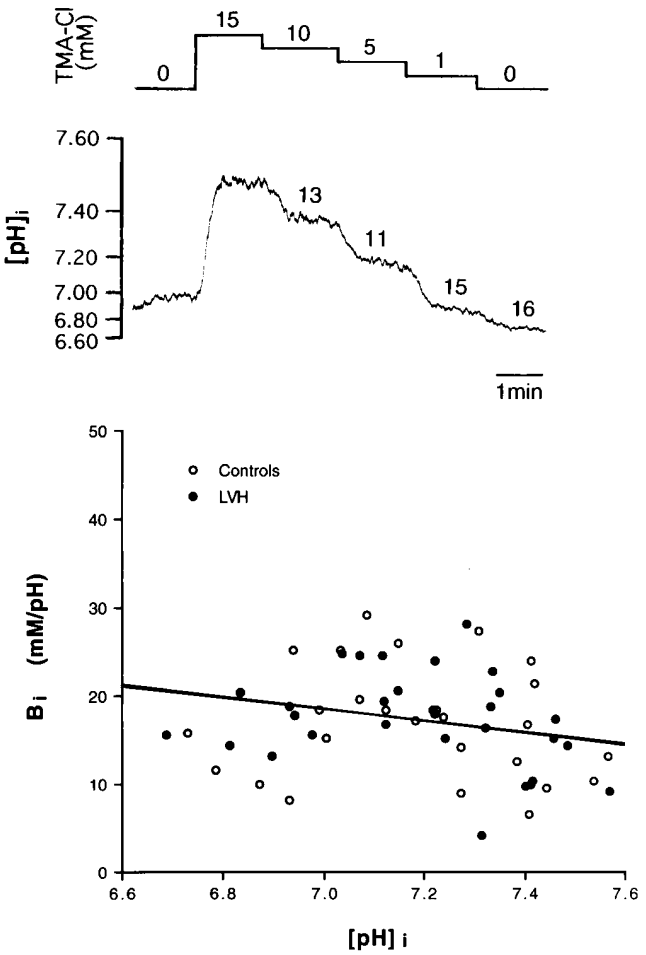

Figure 6. The intrinsic buffering power (B_i) relative to pH_i measured in control and hypertrophied ventricular myocytes (pH_o = 7.40). All cell bathing solutions contained *N*-methyl-D-glucamine as replacement for sodium, zero sodium, and were buffered with Hepes. As illustrated in the top panel, B_i was calculated during stepwise changes in pH_i that were elicited by application and removal of TMA-Cl. The line fits for data obtained from the control and hypertrophied cells are displayed and are virtually superimposed, showing that B_i is similar in control and hypertrophied cells over the range of pH_i shown (*bottom*). Line fits by least squares, linear regression: B_i (mM/pH) = 63.14–6.39 pH_i (n = 27 measurements from seven control myocytes); B_i = 65.55–6.72 pH_i (n = 31 measurements from eight LVH myocytes).

ET-1 did not increase contractility or cause intracellular alkalinization. Similar effects on contractility, intracellular pH, and $[Ca^{2+}]_i$ were observed in response to angiotensin II as in response to ET-1. It is well established that the effects of the vasoactive peptides ET-1 and angiotensin II may vary with the preparation which is studied and with the stage of development. In intact isolated heart preparations, both positive and negative inotropic effects of ET-1 and angiotensin II have been reported in adult rabbits and rat hearts (10, 11, 16, 29, 33). These conflicting responses may be related to the effects of these vasoactive peptides on coronary vasomotor tone, thus confounding the assessment of direct myocardial actions. Recent studies on isolated normothermic paced adult rabbit myocytes have demonstrated that ET-1 and angiotensin II cause a

Table IV. Effects of EIPA on Intracellular pH

	Controls	LVH
Baseline	(n = 18) 7.07±0.02	(n = 17) 7.05±0.02
EIPA alone	(n = 18) 7.05±0.02	(n = 17) 7.02±0.03
EIPA with ET-1	(n = 9) 6.96±0.03*	(n = 8) 6.89±0.02*
EIPA with AT II	(n = 4) 6.92±0.04*	(n = 4) 6.89±0.03*
EIPA with TPA	(n = 5) 6.97±0.03*	(n = 5) 6.94±0.02*

Mean±SEM. *$P < 0.05$ versus baseline. *EIPA*, exposure to 10 mM EIPA, an inhibitor of Na^+-H^+ exchange for 6 min; *ET-1*, exposure to 10 nM ET-1 for 6 min during continued perfusion with EIPA; *A II*, exposure to 10 nM angiotensin II for 6 min during continued perfusion with EIPA. *TPA*, exposure to 80 nM TPA for 6 min during continued perfusion with EIPA. Intracellular pH was measured at baseline, 6 min after exposure to EIPA, and 6 min after the addition of ET-1, AT II, or TPA. In the presence of EIPA, ET-1, AT II, and TPA each caused intracellular acidification in both the control and LVH cells.

minimal change in intracellular Ca^{2+} transients and promote an increase in the amplitude of cell contraction which is mediated predominantly by intracellular alkalinization (10, 11). In contrast, the exposure of immature embryonic or neonatal chick and rat ventricular myocytes to ET-1, as well as angiotensin II, has been reported to cause an absent or negative inotropic effect and a slight intracellular acidosis (10, 34). Thus, it appears that the intracellular signal transduction pathways of these vasoactive peptides are strikingly heterogeneous in myocytes of the same species at different stages of development. This has implications for understanding the cellular mechanisms of these peptides as trophic growth factors in immature versus adult myocardium.

In this study, we used adult dissociated myocytes from ascending aortic-banded rats killed 12 wk after banding. At this stage of chronic pressure-overload, the animals are characterized by concentric left ventricular hypertrophy with preserved systolic shortening and indices of contractile function, the absence of chamber dilation, a predominant increase in myocyte width, and slight increase in myocyte length (4, 15, 17). At this stage of pressure overload, the animals are characterized by a fetal pattern of gene reprogramming including the enhanced expression of multiple genes characteristic of the immature program including atrial natriuretic factor, α-skeletal actin, β myosin heavy chain, as well as cardiac ACE and angiotensinogen (4, 16, 35), with preservation of normal message levels of SR Ca^{2+} ATPase (4). Our interest in examining the signaling of ET-1 and angiotensin II was stimulated by our recent observations that the acute induction of protooncogenes and new protein synthesis in response to these vasoactive peptides are impaired in adult hypertrophied hearts in comparison with normal hearts (7–9).

Under the basal normothermic perfusion conditions of this study, both intracellular pH and diastolic and systolic $[Ca^{2+}]_i$ levels were similar in normal and hypertrophied myocytes. In these experiments, we observed a slight increase in peak systolic $[Ca^{2+}]$, induced by both ET-1 and angiotensin II in control and hypertrophied myocytes. A small and somewhat inconsistent increase in adult myocyte peak systolic $[Ca^{2+}]_i$ has also been found after exposure to angiotensin II or ET-1 in previous studies (13, 36, 37) and may be due to a stimulatory effect of intracellular alkalosis on the L-channel Ca^{2+} current

(38). It is not clear if this mechanism could account for the effect of these peptides on systolic Ca^{2+} in hypertrophied myocytes, in which an effect of intracellular pH was not observed. In immature cultured neonatal rat ventricular myocytes, angiotensin II induces an immediate but transient increase in diastolic $[Ca^{2+}]$ (6, 39) which appears due to an inositol triphosphate–mediated release of Ca^{2+} from sarcoplasmic reticulum stores (39). This is followed by a reduction in the amplitude of the Ca^{2+} transient, an effect ascribed to diacylglycerol-induced activation of protein kinase C (39). A similar effect of angiotensin II on release of Ca^{2+} from the sarcoplasmic reticulum was not found in embryonic chick myocytes, where only a steady state reduction in the amplitude of the Ca^{2+} transient was observed after exposure to ET-1, angiotensin II, or phorbol ester (10). In the present experiments, the effects of these peptides on the steady state $[Ca^{2+}]$ transient (slight increase or no change) in hypertrophied myocytes resembled those seen in normal adult myocytes rather than immature cultured myocytes.

Our experiments in normal adult rat ventricular myocytes confirmed prior observations that ET-1 and angiotensin II induce sensitization of the myofilaments to calcium by intracellular alkalinization which is mediated in part by activation of Na^+-H^+ exchange (12–14) and provided the novel observation that these effects do not occur in adult hypertrophied myocytes. Exposure of the normal myocytes to ET-1, as well as to angiotensin II, in the presence of EIPA to inhibit Na^+-H^+ exchange prevented the expected intracellular alkalinization and increase in contraction amplitude. In the presence of EIPA, ET-1 and angiotensin II caused an intracellular acidosis and depression of contractility which could be blocked by 2DG, which inhibits glycolysis (3, 22, 40). This finding is congruent with recent observations in adult rabbit myocytes by Matsui et al. (12) which suggest that angiotensin II may stimulate metabolic acid production, an effect that is unmasked when Na^+-H^+ exchange is blocked.

To gain further understanding of the regulation of intracellular pH in normal and hypertrophied adult myocytes and its modification by ET-1 and angiotensin II, we measured changes in pH_i and cell contraction in response to a sudden intracellular alkalosis and subsequent acidosis induced by abrupt exposure to and subsequent removal of NH_4Cl (12, 30, 31). These experiments yielded two insights. First, in the absence of ET-1 or angiotensin II, exposure to NH_4Cl caused a similar magnitude and time course of initial alkalinization and subsequent acidosis in control and hypertrophied myocytes. Second, in the presence of ET-1, as well as angiotensin II, control myocytes exhibited an exaggerated initial alkalosis and faster rate of recovery from the secondary acid load at the same level of pH_i. In contrast, in the hypertrophied myocytes, we observed no effect of either ET-1 or angiotensin II on the speed of recovery from intracellular acidification induced by NH_4Cl challenge.

In these experiments using washout of the NH_4Cl pulse in Hepes-buffered cells to induce an intracellular acid load, transsarcolemmal flux of acid equivalents via Cl^-/HCO_3^- transport was eliminated because the cell bathing solutions contained no added HCO_3^- and were buffered with Hepes. The rate of switching of solutions was a constant in all experiments. Thus, the rate of recovery from an intracellular acidosis depends on both the net H^+ efflux via Na^+-H^+ exchange as well as the intracellular buffering capacity. Therefore, we measured intrinsic buffering power (B_i) in control and hypertrophied cells. Cell bathing solutions contained no added sodium and were buffered with Hepes to eliminate transsarcolemmal flux of acid equivalents via Cl^-/HCO_3^- exchange, Na^+/HCO_3^- or Na^+-H^+ exchange. B_i tended to increase slightly as pH_i declined, and values of B_i were similar to determinations by others in ventricular myocytes (12, 30). We observed that intrinsic buffering power was similar in the control and hypertrophied myocytes. Thus, it is reasonable to conclude that the faster rate of recovery from intracellular acidification which was observed in control cells but not in hypertrophied cells in response to Et-1 and angiotensin II was related to the stimulation of H^+ efflux via Na^+-H^+ exchange.

Differences in the stimulation of Na^+-H^+ exchange in response to ET-1 and angiotensin II in hypertrophied and normal myocytes could be related to differences at the level of the receptor or downstream signaling. We and others have observed a reduction in angiotensin II receptor density in hypertrophied rat myocardium (33, 36) and no change in ET-1 receptor density (36). Prior studies in isolated normal adult rat ventricular myocytes have supported the notion that protein kinase C activation mediates the stimulation of Na^+-H^+ exchange by ET-1 and angiotensin II (12–14). In the present experiments, ET-1 and angiotensin II failed to cause intracellular alkalinization in the presence of a selective protein kinase C inhibitor in normal cells. MacLeod and Harding (29) have reported previously that the recovery from an acute acid load in isolated rat myocytes is partially inhibited by amiloride or by removing extracellular Na^+. They observed that exposure to phorbol ester 12-myristate 13-acetate (which activates protein kinase C) exaggerated the changes in intracellular pH and associated changes in cell shortening in normal rat and guinea pig myocytes in response to NH_4Cl challenge, which suggests the stimulation of Na^+-H^+ exchange. We observed that rapid application of the phorbol ester TPA in normal myocytes increased the rate of recovery from intracellular acidification induced by an NH_4Cl pulse, consistent with the stimulation of Na^+-H^+ exchange by the activation of protein kinase C. Exposure to an inactive phorbol ester did not cause intracellular alkalinization in normal myocytes. The failure of active phorbol ester, as well as ET-1 and angiotensin II, to cause intracellular alkalinization in hypertrophied myocytes suggests that downregulation of ET-1 and angiotensin II receptors does not account for the results; instead, the defect lies in the coupling between activation of protein kinase C and stimulation of Na^+-H^+ exchange.

The physiologic significance of the abnormal signaling of protein kinase C and its coupling to Na^+-H^+ exchange in hypertrophied hearts is not yet known. Angiotensin II is locally released during ischemia, and we have shown that low-flow ischemia causes greater contractile dysfunction in hypertrophied hearts than in normal hearts (41). This may be related in part to differences in the stimulation of Na^+-H^+ exchange by angiotensin II during ischemia. In addition, we have shown that the early growth responses (protooncogene induction and protein synthesis) are blunted in adult hypertrophied hearts in response to stimulation with angiotensin II, α-adrenergic agonists, as well as mechanical load (7, 8). The present investigation raises the possibility that defective coupling of protein kinase C and Na^+-H^+ exchange may contribute to the alteration of growth responses in the hypertrophied heart.

Summary. We have confirmed that ET-1, as well as angiotensin II, promotes an increase in contractility in normal adult

rat myocytes via intracellular alkalinization which is partly mediated by activation of Na^+-H^+ exchange. This study shows that the effects of ET-1, as well as angiotensin II, differ in adult hypertrophied myocytes and simulate the fetal response of the absence of a positive inotropic effect and the failure to develop intracellular alkalinization. In contrast with normal adult myocytes, exposure to ET-1, angiotensin II, and the phorbol ester TPA fails to stimulate Na^+-H^+ exchange in hypertrophied myocytes. These studies suggest that intracellular signal transduction pathways of ET-1 and angiotensin II which stimulate Na^+-H^+ exchange via activation of protein kinase C differ in normal and hypertrophied adult myocytes.

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