Endothelin and Increased Contractility in Adult Rat Ventricular Myocytes

Role of Intracellular Alkalosis Induced by Activation of the Protein Kinase C–Dependent Na^+-H^+ Exchanger

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Endothelin, a 21-amino acid vasoactive peptide, is among the most potent positively inotropic agents yet described in mammalian heart. Having demonstrated that endothelin's inotropic effect is due, in part, to an apparent sensitization of cardiac myofilaments to intracellular calcium, we determined whether this could be due to a rise in intracellular pH (pH_i). In isolated adult rat ventricular cells loaded with the H^+-selective fluorescent probe BCECF, 100 pM endothelin increased contractile amplitude to 190±26% of baseline and pH_i by 0.08±0.02 (n=8), whereas 1 nM endothelin increased pH_i by 0.13±0.03 with little further increase in contractility. Amiloride (10^{-4} M) prevented the increase in pH_i in response to endothelin and reduced the inotropic response by 45%, although the inotropic effect could be readily restored by subsequent NH_4Cl-induced alkalinization. Similarly, inhibitors of protein kinase C (H-7 and sphingosine) diminished or abolished the rise in pH_i after endothelin superfusion while causing a decline in its inotropic effect comparable with that observed with amiloride. Pretreatment with pertussis toxin, which we have demonstrated results in complete ADP-ribosylation of the α-subunits of G_o and G_i GTP-binding proteins and abolition of endothelin's positive inotropic effect, only partially reduced the intracellular alkalinization induced by the peptide, suggesting a complex signal transduction mechanism. Thus, the positive inotropic action of endothelin is due in part to stimulation of the sarcolemmal Na^+-H^+ exchanger by a protein kinase C–mediated pathway, resulting in a rise in pH_i and sensitization of cardiac myofilaments to intracellular Ca^{2+}. (*Circulation Research* 1991;68:269–279)

The regulation of the contractile state of the myocardium under normal physiological conditions has long been assumed to be dependent on the extent of mechanical loading of muscle fibers and the degree of activation of the autonomic nervous system. Endothelin, a potent vasoconstrictor peptide originally derived from media bathing primary cultures of porcine aortic endothelial cells, has recently been reported to act as a positive inotropic agent in mammalian atrial and ventricular muscle. These reports[1–5] suggest that local factors, perhaps released by the microvascular endothelium and/or endocardium, might also directly regulate myocardial contractile function. Indeed, in isolated rat ventricular myocytes, endothelin is the most potent positive inotropic factor we have yet tested, with an EC_{50} in this system of 50 pM,[3] a value that approximates the reported K_d of endothelin for its receptor in either intact heart or in sarcolemmal membranes.[6–8] In addition, the kinetics of endothelin's effect on myocyte contractility are unusual, with a slow onset and sustained duration of action in both single cell and intact tissue preparations, indicating that the peptide may modulate the inotropic responsiveness of the heart over a time frame of minutes to hours, in contrast to the rapid onset and shorter-lived effects of known neurohumoral factors.[3]

The mechanism by which endothelin induces a positive inotropic effect in cardiac myocytes remains controversial. Although an increase in cytosolic calcium has been observed in isolated rabbit myocytes at endothelin concentrations above 10 nM,[9] we have reported that in isolated, freshly dissociated adult rat ventricular myocytes, endothelin increased contractile amplitude with little or no increase in cytosolic calcium, appearing to enhance myofilament respon-

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siveness to calcium via a pertussis toxin–sensitive pathway.[3] At concentrations at or below 1 nM that would elicit a maximal increase in contractile amplitude, there was little or no increase in the peak systolic or diastolic calcium level during intracellular calcium transients in myocytes paced at 1.5 Hz, although some increase in intracellular calcium was variably observed at endothelin concentrations at or above 1 nM.

One potential mechanism by which endothelin could enhance myofilament responsiveness to calcium would be the induction of an intracellular alkalosis, since changes in intracellular pH (pH_i) are known to affect the calcium sensitivity of the contractile apparatus.[10–12] Endothelin has been shown to increase pH_i in glomerular mesangial cells and vascular smooth muscle cells, probably by activation of the Na^+-H^+ antiporter in the plasma membrane.[13,14] The purpose of the present study was to examine the effects of endothelin on pH_i in adult rat cardiac myocytes and to determine whether the increase in contractile amplitude observed with the peptide could be due in part to an increase in sarcoplasmic pH. For the purposes of this report, "endothelin" refers to the originally described porcine/human endothelin, now termed "endothelin-1."[15]

Materials and Methods

Calcium-tolerant isolated rat ventricular cells were prepared using a variation of the methods described by Haworth et al[16] and Cheung et al,[17] as previously described.[18] Briefly, hearts from adult (225–250 g) male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were retrogradely perfused for 5 minutes with Krebs-Henseleit (KH) buffer, followed by a 5-minute perfusion with nominally Ca^{2+}-free KH buffer and, subsequently, Ca^{2+}-free KH buffer containing 0.05% collagenase and 0.03% hyaluronidase for 25 minutes. Ventricles were then minced and incubated in KH buffer containing 1 mM $CaCl_2$, 0.002% trypsin, and 0.002% deoxyribonuclease, and the cells were released by trituration and sedimented in 2% bovine serum albumin. The cells were allowed to attach to 12-mm glass coverslips coated with collagen (Vitrogen, Collagen Corporation, Palo Alto, Calif.) for contractility and fluorescence measurements.

Fluorescence Spectroscopy

Fluorescence measurements were performed in a SPEX CM2 dual excitation spectrofluorometer (SPEX Industries, Edison, N.J.) as previously described.[3,19] Coverslips with attached cells were placed in a water-jacketed chamber (37±0.5°C) on the light-shielded, heated stage of the microscope and superfused with buffer at a rate of 1 ml/min. Coverslips were placed in an aluminum chamber, 25 mm in diameter, with a volume of 300 μl. The contents of the chamber could be efficiently exchanged within 30 seconds, or more quickly if necessary, using transiently higher flow rates. Because of potential adverse effects of constant ultraviolet light exposure, exposures to the excitation light beam (and thus determination of pH_i) were limited to brief "snapshots" of several seconds after each experimental perturbation, with total exposure to the excitation beam limited to several minutes for each experiment.

Freshly isolated adult ventricular myocytes were loaded with the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF, Molecular Probes, Eugene, Ore.) by permitting the cells to attach to coverslips, incubating them in superfusion buffer (see below) with 2 μM BCECF/AM, the acetoxymethyl ester derivative of BCECF, for 20 minutes at 23°C, and then washing them. A 1 mM BCECF/AM stock solution was prepared in dry dimethylsulfoxide and kept frozen in aliquots until use.

After each experiment using a single cell (only one experiment was performed per coverslip), an in situ calibration of the BCECF fluorescent signal was performed within each cell. Myocytes were calibrated by superfusion with two or three calibration buffers as previously described.[19] Calibration buffers differed from the superfusion medium and contained HEPES/KOH (4 mM); EGTA (0.5 mM); pyruvate (5 mM); glucose (5.6 mM); K_2ATP (10 mM); the ionophores nigericin (20 μM), ionomycin (4 μM), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (0.2 μM); and KCl to yield a final K^+ concentration of 150. pH was adjusted at 37°C with KOH or HCl to 6.60 or 7.50, respectively. The pH_i for each cell was then determined from a linear regression of fluorescence ratio versus the pH value of the calibration buffer.

Contractility Measurements

Measurements of contractile amplitude of single isolated rat cardiomyocytes without simultaneous acquisition of pH_i data were made on the stage of an inverted phase-contrast microscope as previously described.[18,19] Myocytes were stimulated at 25% above threshold with a 3-msec square-wave pulse through a platinum wire placed in the superfusion liquid connected to a stimulator (model S88, Grass Instruments, Quincy, Mass.).

After equilibration with the superfusion buffer and stabilization of myocyte contractile amplitude at a stimulation rate of 1.5 Hz, the cardiocytes were superfused with buffers containing 100 pM endothelin for 7 minutes to obtain a maximal increase in contractile amplitude. Consequently, the superfusion time of other drugs was increased proportionately (100 μM amiloride or 10 μM ouabain with or without endothelin), with similar times of exposure to ultraviolet light, as described in the text, table, and figure legends below. Those cells exposed to varying extracellular pH (pH_o), with or without prior exposure to 100 pM endothelin, were exposed to superfusion buffer at each pH_o for 3 minutes. Similarly, cells made alkalotic and subsequently acidotic by initial exposure to, and then washout of, NH_4Cl were first allowed to stabilize for 3 minutes in superfusion buffer at a pH_o of 7.4; stabilization was followed by

exposure to 10 mM NH_4Cl for 3 minutes and then to superfusion buffer alone, pH_o 7.4, for another 6 minutes. A similar protocol was followed in which drugs were added before the NH_4Cl prepulse technique was begun; however, in this case, the cells were allowed to equilibrate in superfusion buffer plus endothelin and/or amiloride or in superfusion buffer plus endothelin and/or ouabain for 12 minutes. Unless otherwise indicated, the superfusion buffer contained 137 mM NaCl, 4.0 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.6 mM glucose, 4 mM HEPES, and 0.05% bovine serum albumin at a pH of 7.40 at 37°C. For cells suspended with a bicarbonate-buffered system, the superfusion buffer was as described above, except that 20 mM HCO_3^- was substituted for HEPES and the NaCl concentration was 120 mM. pH measurements were made using a pH meter (model 140, Corning, Medfield, Mass.) at 37°C, and pH was adjusted with NaOH or HCl.

Materials

Hyaluronidase type II, trypsin, bovine serum albumin (bovine fraction V), amiloride, ouabain, isoproterenol, D-sphingosine, and CCCP were purchased from Sigma, St. Louis; collagenase and deoxyribonuclease I were from Worthington Biochemical, Freehold, N.J.; the protein kinase inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was from Seikagaku Kogyo Co., Tokyo; ionomycin and nigericin were obtained from Calbiochem, La Jolla, Calif.; pertussis toxin was from List Biologicals, Campbell, Calif.; and endothelin (synthetic porcine/human endothelin-1) was from Peninsula Laboratories, Belmont, Calif.

Statistics

Data were analyzed by Student's *t* test for unpaired observations with a modified Bonferroni correction for multiple comparisons, repeated-measures analysis of variance for paired observations, and Student-Newman-Keuls test for multiple comparisons. Data in the text and figures are expressed as mean±SEM. Throughout the article, unless otherwise stated, baseline or "basal" values are expressed as 100% before administration of any drugs or a change in superfusion buffer pH, and all subsequent data are expressed as a percent of baseline or basal values.

Results

Effects of Endothelin on Myocyte pH_i and Contractility

As we have shown previously,[3] endothelin increased contractility in isolated rat ventricular myocytes; typically, no change in contractile amplitude was apparent for approximately 4 minutes, and a maximal response was present at 7 minutes. A response could be observed in all cells at 1 pM, and the EC_{50} was approximately 50 pM; a maximal response was present at 1 nM with an increase of approximately 90–100% over baseline contractility. In the BCECF-loaded cells reported here, the time course

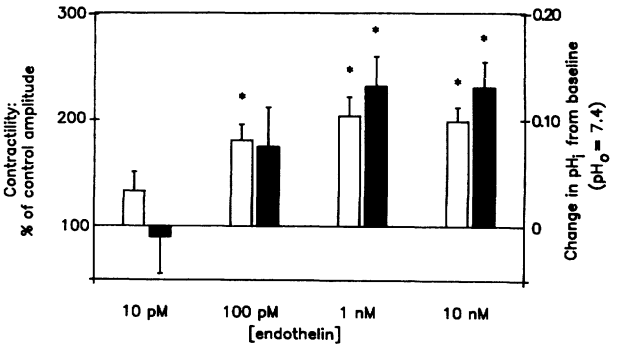

FIGURE 1. *Bar graph showing changes in intracellular pH (pH_i) and contractile amplitude after endothelin exposure. Single cardiac myocytes freshly isolated from adult rat ventricles were loaded with the H^+-specific fluorescent dye BCECF and superfused with physiological media buffered with HEPES at extracellular pH (pH_o) 7.4 over the range of endothelin concentrations indicated. Contractility, expressed as a percentage of the control preendothelin contractile amplitude, at 8 minutes after addition of the peptide is shown in open bars, and the corresponding average change in pH_i at 8 minutes is shown in solid bars. The pH_i in myocytes at pH_o 7.4, stimulated at 1.5 Hz, was 7.09±0.01* (n=*71), and this defines the baseline pH_i value. The BCECF intracellular fluorescence signal was calibrated in situ at the end of each experiment as described in "Materials and Methods." Average data for five to seven cells are given for each point. Changes in pH_i and contractility were maximal at 1 nM.* *p<*0.05 compared with values for contractile amplitude or pH_i before endothelin. The increase in contractility at 10 pM averaged 33% but did not reach statistical significance* (n=*4).*

and magnitude of the contractile response to endothelin were similar to what we have reported in unloaded or fura 2–loaded cells.[3]

In adult rat ventricular myocytes bathed in superfusion buffer at pH 7.4, the baseline pH_i was 7.09±0.01 (n=71 cells) in cells stimulated at 1.5 Hz. The pH_i in resting, nonstimulated cells was approximately 7.14±0.02. As shown in Figure 1, endothelin increased myocyte pH_i. This dose–response relation was shifted somewhat to the right compared with the increase in contractility; there was no significant response in pH_i apparent at 10 pM. The maximal rise in pH corresponded to the maximal increase in contractility at 1 nM. The time course of the change in pH_i also differed marginally from the increase in contractility; some increase in pH_i was always apparent at 2–3 minutes (as shown in Figure 2) although, like the contractility response, the rise in pH_i was not maximal for 6–8 minutes (Figure 2). In contrast, no increase in contractility was apparent for about 4 minutes after beginning the endothelin infusion. When cells were exposed to 1 nM endothelin in a bicarbonate-buffered superfusion medium, the increase in pH_i and contractile amplitude were qualitatively similar to that seen with HEPES-buffered media. pH_i increased 0.072 pH units, and contractile amplitude increased to 207% of control (n=3). Consequently, because of the need for precision

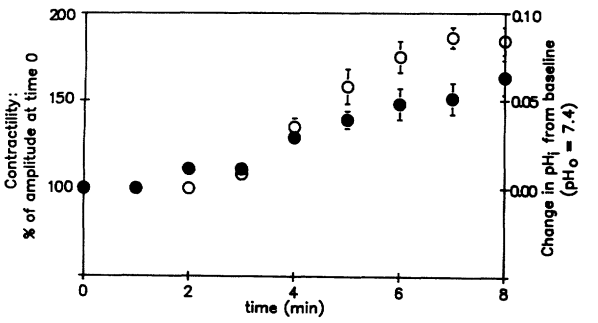

FIGURE 2. *Graph showing time course of changes in intracellular pH (pH$_i$) and contractile amplitude with endothelin. Freshly isolated ventricular myocytes were superfused with physiological buffer at extracellular pH (pH$_o$) 7.4, loaded with the H$^+$-specific fluorescent dye BCECF, stimulated at 1.5 Hz, and exposed to 100 pM endothelin at time 0. Changes in contractile amplitude are represented by open symbols, and changes in pH$_i$ by solid symbols. In neither case was the increase significant until 4 minutes* (n=6).

and ease of reproducibility of superfusion buffer pH, all subsequent experiments were done with HEPES-buffered media. To exclude the possibility that the increase in pH_i with endothelin was secondarily related to an increase in contractility per se in this model, cells were exposed to a concentration of isoproterenol (1.25–5.0 nM) that resulted in an increase in contractile amplitude roughly equivalent to that produced by 100 pM endothelin. Isoproterenol at this concentration had no effect on pH_i (-0.01 ± 0.01 of baseline pH_i, $n=3$). Also, ouabain (10^{-4}–10^{-3}) routinely tended to decrease pH_i in myocytes superfused at pH_o 7.4 despite a comparable increase in contractile amplitude.

Effects of Varying pH$_o$ and NH$_4$Cl on Myocyte Contractility

To examine the relation between pH and changes in myocyte contractile amplitude in isolated rat ventricular myocytes paced at 1.5 Hz, pH_i was manipulated either by varying pH_o or by the NH_4Cl prepulse technique as described in "Materials and Methods." Varying pH_o had a predictable effect on myocyte pH_i; increasing pH_o to 7.9 from 7.4 increased pH_i to 7.26 ± 0.03. The changes in contractile amplitude with varying pH_o are shown in Figure 3A. Extracellular alkalinization resulted in a marked increase in contractile amplitude, with little or no decrease in contractility at a pH_o of 6.9. To determine the effects of prior exposure to endothelin on subsequent intracellular alkalinization or acidification during changes in pH_o, myocytes pretreated with 100 pM endothelin for 7 minutes were subsequently exposed to the same protocol as the control cells described above (Figure 3A). In this figure, the initial contractile amplitude was assigned a value of 100%. The increase in contractile amplitude with endothelin at 7 minutes averaged $190\pm26\%$ of baseline ($n=8$). As illustrated here, endothelin-pretreated cells had only a modest further increase in contractile amplitude after exposure to a pH_o of 7.9 and little or no further decline in contractility when pH_o was reversed to 6.9.

In Figure 3B, the changes in contractile amplitude are plotted as a function of the changes in pH_i induced by exposure to and, subsequently, removal of NH_4Cl. Before exposure to NH_4Cl, cells exposed to 100 pM endothelin increased their pH_i by 0.08 ± 0.02 ($p<0.05$; $n=6$). The subsequent increases in pH_i in both control cells and endothelin-pretreated cells were similar. Interestingly, the negative inotropic effect of a decline in pH_i on NH_4Cl washout was more pronounced in endothelin-pretreated cells, falling to 53% of the contractile amplitude in endothelin-pretreated cells measured just before addition of NH_4Cl (open circle with ##). This was in spite of a lower pH_i in control cells. Subsequently, there was a marked rebound of pH_i in endothelin-pretreated cells after the recovery from NH_4Cl washout–induced intracellular acidosis, resulting in an increase in pH_i to 7.25 ± 0.05 ($n=6$) at 9 minutes after removal of the initial prepulse of NH_4Cl. This rebound intracellular alkalosis was unassociated with any comparable rebound increase in contractility (Figure 3B).

Endothelin and Inhibition of Na$^+$-H$^+$ Exchange

To determine the potential role of the sarcolemmal Na^+-H^+ exchanger in the intracellular alkalinization after endothelin infusion, cells were exposed to 100 pM endothelin with or without pretreatment with 100 μM amiloride. Amiloride alone at this concentration had no effect on the contractility of these cells paced at 1.5 Hz and superfused at pH 7.4 ($103\pm12\%$ of baseline, $n=15$, at 5 minutes). Amiloride also did not significantly affect the pH_i of cells paced at 1.5 Hz at a superfusion buffer pH_o of 7.4 (-0.03 ± 0.03, $n=4$). Unlike the reported effect of amiloride on the fluorescence intensity of 6-carboxyfluorescein, addition of 10^{-4} M amiloride did not affect the fluorescence ratio of BCECF in these cells.[20,21]

The addition of 100 pM endothelin to cells pretreated with 10^{-4} M amiloride resulted in an increase in contractile amplitude that averaged $149\pm17\%$ of baseline ($n=7$), about 45% below that seen with endothelin alone. However, the intracellular alkalinization observed with 100 pM endothelin was completely blocked with amiloride (-0.07 ± 0.03, p=NS, $n=6$). The effects of varying pH_o or of NH_4Cl addition and washout on contractile amplitude of amiloride-pretreated cells, with and without endothelin, are shown in Figures 4A and 4B, respectively. In these figures, baseline contractile amplitude (100%) is the contractile response of isolated rat cardiac myocytes paced at 1.5 Hz after 5 minutes of superfusion with buffer containing 100 μM amiloride. Myocytes pretreated with amiloride alone ($n=7$) showed no enhanced contractile response to superfusion with buffer at pH_o 7.9 in contrast to control cells (Figure 3A). However, in cells pretreated with amiloride and endothelin at pH 7.9, there was a marked increase in contractility at this pH_o that was similar to that seen in nonamiloride-

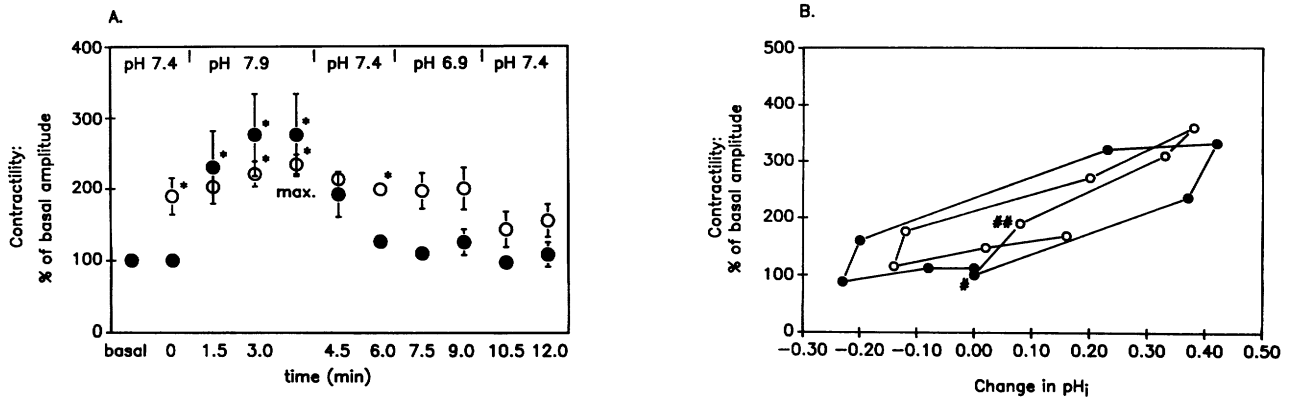

FIGURE 3. *Graphs showing the effect of changing intracellular pH (pH_i) on myocyte contractility. Single isolated ventricular myocytes were loaded with the H^+-specific fluorescent dye BCECF, attached to coverslips for simultaneous acquisition of pH_i and contractility data at 37°C, and stimulated at 1.5 Hz (see "Materials and Methods").* Panel A: *After equilibration with the superfusion buffer, the contractile amplitude was measured and assigned a value of 100% ("basal" contractility). The cardiocytes were then superfused with media alone (●; n=8) or with 100 pM endothelin (○; n=7) at pH 7.4 for 7 minutes (time 0). Endothelin (100 pM) typically increased contractile amplitude to 190–200% of basal levels. Subsequently, the myocytes were exposed to media with or without 100 pM endothelin at the extracellular pH values indicated. *p<0.05 compared with basal contractile amplitude.* Panel B: *After equilibration at extracellular pH 7.4, the myocytes were made alkalotic and then acidotic by initial exposure to 10 mM NH_4Cl for 3 minutes and by its subsequent washout. For both control cells (●; n=8) and cells pretreated with 100 pM endothelin (○; n=8), the relation between pH_i and contractility is shown during the alkalinization and subsequent intracellular acidification. The basal pH_i for all cells (n=16) was 7.09, and this point (#) was assigned a ΔpH_i value of 0.00 and a contractility value of 100%. Eight cells were then exposed to 100 pM endothelin, and pH_i and contractility were allowed to equilibrate for 8 minutes (##) before the addition of NH_4Cl to the superfusion media. The subsequent changes in pH_i and contractile amplitude are illustrated in endothelin-pretreated cells and controls cells during NH_4Cl exposure and washout as described above. pH_i and contractility were recorded at 90-second intervals to minimize exposure to ultraviolet excitation light. Although error bars for each point for both parameters are excluded for clarity of presentation, any change in pH_i greater than 0.05 was significant at the p<0.05 level; the pH_i of a group of cells at a given time point was compared with that group's baseline predrug pH by paired analysis with a standard Newman-Keuls test to determine the level of significance. In contrast, the further increase in contractile amplitude in the endothelin-pretreated cells above that caused by endothelin alone (100 pM; increase in pH_i of 0.08±0.02; before NH_4Cl exposure [##]) was significant only at the point of maximal alkalosis (ΔpH_i of +0.38±0.01; p<0.05) after NH_4Cl exposure. None of the changes in contractile amplitude in either control or endothelin-pretreated cells was significantly different from baseline after NH_4Cl washout.*

pretreated cells (Figure 3A). Subsequent acidification with buffer at pH_o 6.9 resulted in only an insignificant fall in contractile amplitude in both amiloride (alone) and amiloride-plus-endothelin–pretreated cells (Figure 4A).

With intracellular alkalinization induced by superfusion with buffer containing NH_4Cl (Figure 4B), there was an increase in myocyte contractility in amiloride-pretreated cells that was approximately half of that observed in control cells not pretreated with amiloride, as shown in Figure 3B. This is consistent with the fact that amiloride with NH_4Cl blunted the increase in pH_i by about 50% compared with nonamiloride-pretreated cells (note the change in scale on the pH axis in Figure 4B). Intracellular alkalinization with NH_4Cl in endothelin- and amiloride-pretreated cells resulted in an increase in contractile amplitude equal to that seen in endothelin-pretreated cells not exposed to amiloride. This marked increase in contractile amplitude with endothelin occurred despite the fact that amiloride with NH_4Cl blunted the rise in pH_i in endothelin-treated cells, as in control cells, by about half, implying that the effect of intracellular alkalinization on enhancing contractile amplitude was maximal in endothelin-treated cells at a pH_i of approximately 7.30.

Subsequent acidification after NH_4Cl washout resulted in a decline in contractile amplitude in amiloride-treated cells, with or without endothelin, as shown in Figure 4B. This decline in contractile amplitude in amiloride-treated cells was enhanced and prolonged compared with that in control cells (i.e., not amiloride-pretreated cells) and reached a minimum of 33% (amiloride) and 26% (amiloride plus endothelin) of baseline. As expected, amiloride slowed or prevented the recovery from acidosis at 9 minutes after NH_4Cl washout.

It is of interest to note that by use of the experimental protocol described here, in which cells are pretreated with amiloride, the amplitude of the rise in pH_i during superfusion with NH_4Cl was blunted by the inhibitor of Na^+-H^+ exchange; the rate of decline back to baseline pH_i was likewise blunted by this inhibitor. As described by Boron and DeWeer[22] in their study of intracellular pH transients caused by NH_3, the degree of initial intracellular alkalinization

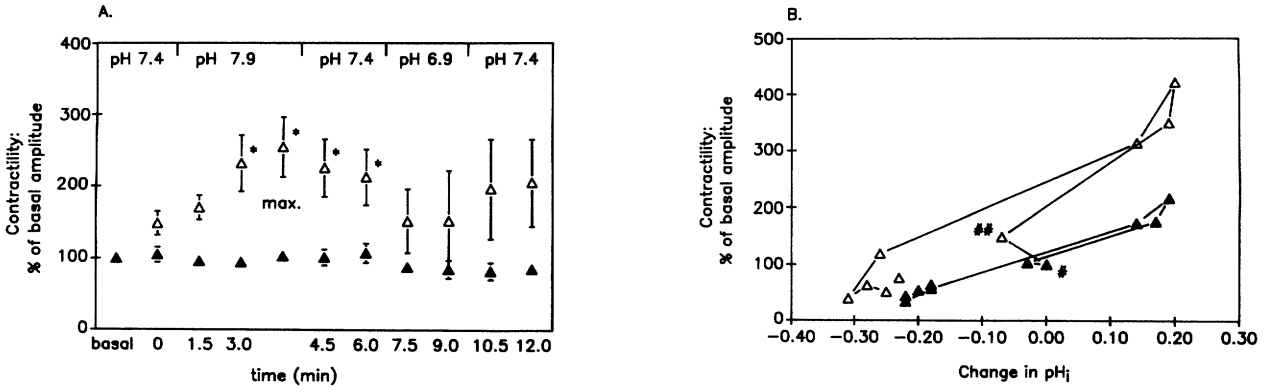

FIGURE 4. *Graphs showing endothelin and inhibition of Na^+-H^+ exchange.* Panel A: *Single isolated ventricular myocytes were monitored for changes in contractile amplitude while the extracellular pH of the superfusion buffer was varied as shown. All cells were exposed to 100 μM amiloride for 12 minutes before beginning the protocol at time 0; seven of these cells were also exposed to 100 pM endothelin for the final 7 minutes (△), and eight of these cells were exposed to amiloride alone (▲). Amiloride pretreatment alone had no significant effect on either pH_i or baseline contractile amplitude of myocytes paced at 1.5 Hz at extracellular pH 7.4 (−0.03±0.03; p=NS). Amiloride pretreatment reduced the positive inotropic response to endothelin by about 45% (to 149±17% of the contractile amplitude in amiloride-alone cells). The mean contractile amplitude of both groups of cells after 12 minutes of preincubation with either amiloride alone or amiloride plus endothelin, pH 7.4, was assigned a value of 100% at time 0. *p<0.05 in the amiloride- and endothelin-pretreated cells compared with baseline contractility.* Panel B: *As described in the legend to Figure 3B, the relation between changes in intracellular pH (pH_i) and contractile amplitude is shown for myocytes pretreated with amiloride alone (▲) or amiloride and endothelin (△) during exposure to 10 mM NH_4Cl for 3 minutes and after its abrupt withdrawal (see "Materials and Methods"). For 10 amiloride-pretreated cells, the initial value for ΔpH_i was 0.00 and the value for contractility was 100% (this point is indicated by #). Six of these cells were then exposed to 100 pM endothelin for 8 minutes (##) before NH_4Cl was added to the superfusion media. At this point, despite the initial statistically insignificant decline in pH_i with endothelin in amiloride-pretreated cells (−0.07±0.03), contractility increased to 144±10% of control (p<0.05). Both groups of cells (▲ and △) were then exposed to 10 mM NH_4Cl and subsequent washout, and recordings of pH_i and contractility were made at 90-second intervals. As in Figure 3B, error bars at each point for both parameters have been excluded for clarity. Any change in ΔpH greater than 0.08 was significant (p<0.05). Importantly, the increases in contractile amplitude with endothelin after exposure to NH_4Cl were highly significant and equaled the absolute increases in contractile amplitude seen in cells exposed to 100 pM endothelin without amiloride (open circles in Figure 3B). The subsequent decline in pH_i and fall in contractile amplitude with NH_4Cl washout was significant and did not recover in the continuing presence of 10^{-4} M amiloride in both groups of cells.*

with NH_4Cl is dependent on the capacity of the cell to buffer subsequent changes in cellular H^+ content, because some intracellular accumulation of H^+ would have occurred during amiloride pretreatment in the absence of another H^+ extrusion mechanism, as in the nominal absence of bicarbonate under these experimental conditions. The slower decline to baseline pH_i in amiloride-pretreated cells continuously superfused with NH_4Cl is also explained in part by H^+ accumulation, since this decline is dependent on the electrochemical gradient for NH_4^+ as well as its permeability, which, although less than NH_3, is still substantial in these cells.[23,24]

Ouabain, Endothelin, and Myocyte pH_i

To compare the response of endothelin-treated isolated rat ventricular myocytes to changes in pH_i with their response after pretreatment with another positive inotropic agent, myocytes were superfused with either 10^{-5} M ouabain for 12 minutes or 10^{-5} M ouabain for 5 minutes; superfusion was followed by the addition of 100 pM endothelin for an additional 7 minutes (data not shown). Higher concentrations of ouabain alone (10^{-4}–10^{-3} M) resulted in initial increases in myocyte contractile amplitude to 160–200% of baseline; these increases were not associated with any significant fall in pH_i but often were followed by the development of contracture either before completion of the protocols or during calibration of the BCECF fluorescence signal. After a stable increase in myocyte contractile amplitude had been achieved, myocytes were exposed to superfusion buffer containing either 10^{-5} M ouabain alone or ouabain plus endothelin; the sequential changes in buffer pH_o were as described in Figure 3A, and NH_4Cl prepulse and washout were as described in Figure 3B. Ouabain alone at 10^{-5} M increased contractile amplitude to 112±7% of baseline (n=11), but there was no significant change in pH_i (−0.02±0.02, n=4). Addition of 100 pM endothelin resulted in an additive increase in contractile amplitude to 191±22% of that seen with ouabain (10^{-5} M) alone.

The increase in myocyte contractility on alkalinizing the cell interior by either raising pH_o or adding NH_4Cl in cells pretreated with ouabain alone was reduced compared with the increase in control cells, even when the slightly higher baseline contractility with ouabain is considered. This may have been due to a tendency toward lower cytoplasmic pH during

alkalinization in ouabain-pretreated cells compared with control cells. For example, the maximum increase in pH_i that was achieved after exposure to NH_4Cl was +0.42±0.05 in control cells, compared with +0.35±0.02 in ouabain-treated cells.

Of interest was a sustained increase in contractile amplitude in ouabain- and endothelin-pretreated cells that subsequently underwent intracellular acidification by either acidification of the external medium or NH_4 washout (data not shown). This is in contrast to the response of control cells or cells treated with endothelin alone, which demonstrated a fall in contractile amplitude toward or below baseline during intracellular acidification. This suggests that the combination of ouabain and endothelin results in a gradual increase in contractile amplitude with time that is relatively unresponsive to further swings in pH_i.

Endothelin, pH_i, and Protein Kinase C

Endothelin increases cytoplasmic pH in isolated adult rat cardiac myocytes via an amiloride-sensitive mechanism, presumably the Na^+-H^+ exchanger at the sarcolemmal membrane, as documented above. Since activation of the Na^+-H^+ antiporter in a variety of cell types is known to be mediated by protein kinase C, two inhibitors of this enzyme, H-7 and the structurally unrelated compound sphingosine, were used to determine the involvement of protein kinase C in the sequence of events leading to intracellular alkalinization with endothelin. The increase in pH_i after exposure to 1 nM endothelin was completely inhibited by a 45-minute preincubation of myocytes in superfusion buffer with 50 μM H-7 (n=4), as shown in Figure 5. Pretreatment of myocytes with 200 μM sphingosine for 10 minutes followed by 1 nM endothelin reduced the subsequent alkalinization of the cell interior to 0.053±0.006 pH units (40.2% of the response of cells treated with endothelin alone, Figure 5). Incubation with this concentration of sphingosine was restricted to 10 minutes; this time limit was set because our own experience has shown that toxic effects of this drug occur during incubations lasting longer than 20 minutes. The increase in contractile amplitude in H-7–pretreated and sphingosine-pretreated cells subsequently exposed to endothelin was 156±21% and 136±7% of control, respectively, approximately half of that seen with endothelin alone, as shown in Figure 5.

We[3] have previously demonstrated that pertussis toxin completely inhibits the increase in contractility observed with endothelin. To determine its effect on the intracellular alkalinization induced by endothelin, quiescent cells were pretreated with 100 ng/ml pertussis toxin for 3 hours. This protocol had no effect on the contractile amplitude of cells paced subsequently at 1.5 Hz and resulted in an augmentation of the contractile response to β-agonists, as expected. Pertussis toxin pretreatment also has been shown to reduce subsequent [^{32}P]ADP-ribosylation of an endogenous G protein α-subunit by 98% in these cells.[3] Pertussis toxin incubation blunted the rise in pH_i in response to 1 nM endothelin to 0.070±0.032 pH units (n=5), although this was not significantly different from the increase noted without pertussis toxin. However, as documented previously,[3] the amplitude of contraction in response to 1 nM endothelin after pertussis toxin pretreatment did not increase significantly over baseline at 7 minutes (119±20%, p=NS).

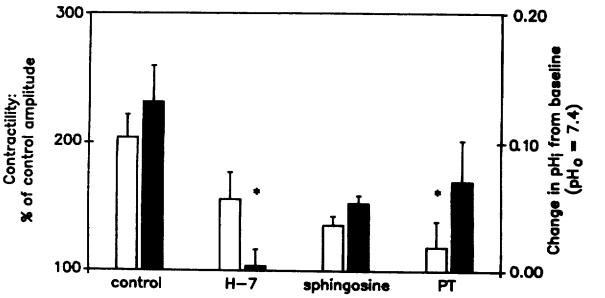

FIGURE 5. *Bar graph showing role of protein kinase C and resistance to pertussis toxin (PT) in the intracellular pH (pH_i) response to endothelin. pH_o, extracellular pH. Freshly isolated adult rat ventricular myocytes were exposed to 1 nM endothelin, and contractility (open bars) and pH_i (filled bars) were recorded at 8 minutes. Eight cells were preincubated with either 50 μM H-7 or 200 μM sphingosine to determine whether these inhibitors of protein kinase C would affect the alkalinization response to endothelin. H-7 completely blocked the rise in pH_i with 1 nM endothelin (*p<0.05), although neither the decline in contractility with H-7 nor the changes in pH_i and contractility with sphingosine reached statistical significance after corrections for multiple comparisons. Pertussis toxin (100 ng/ml for 3 hours) had a nonsignificant effect on pH_i but reproducibly inhibited the contractile response to endothelin (p<0.01).*

Discussion

Measurement of pH_i in Cardiomyocytes

Since the introduction of second-generation fluorescent dyes such as fura 2 and BCECF for the measurement of intracellular ion activities, the accurate calibration of their fluorescence signal has been hampered by several methodological problems. With the in situ calibration technique described above and in "Materials and Methods," the intracellular BCECF signal can be confidently calibrated over the range of pH_i values achieved in this report, as documented previously.[19] The value obtained for baseline pH_i in this study for adult rat ventricular cells superfused at pH_o 7.4 (37°C) and stimulated at 1.5 Hz was 7.09±0.01, which is in accord with values recently reported by Wallert and Fröhlich[23] and by Eisner et al[25] in these cells and in other cardiac tissue preparations. The slightly lower baseline pH_i reported here compared with values obtained by others in resting (quiescent) cells is likely due to the decline in pH_i that accompanies pacing, as described by Bountra et al.[26,27]

The observations reported here were made on unloaded cells; that is, the cells underwent shortening against an internal load that is presumed to be constant (isotonic). However, recent observations by Lee and Allen[28] and Kent et al[29] support the utility of the isolated myocyte model as relevant in the study of cardiac contractility. Finally, our laboratory has demonstrated that in isolated rat myocytes subjected to a staircase pacing protocol from 0.5–3.0 Hz the amplitude of contraction correlated closely with the increase in the velocity of contraction (S. Borzak, J.D. Marsh, personal communication, September 1990).

Myocyte Contractility and pH_i

Reports from several laboratories,[30–33] including our own,[3] have now confirmed the original observations of Ishikawa et al,[2] who determined that endothelin is a potent inotropic agent in cardiac muscle that appears to sensitize cardiac myofilaments to intracellular Ca^{2+}. One potential mechanism for this effect is an increase in intracellular pH. At a given level of intracellular Ca^{2+}, in muscle not maximally contracted and in the presence of metabolic substrates, contractile amplitude and developed tension increased upon alkalinization of the milieu surrounding the myofilaments and fell in response to acidosis.[10] With the advent in the last decade of ion-selective microelectrodes and fluorescent dyes, these observations have been confirmed and expanded.[11,24–26,34–37]

In the isolated rat ventricular myocyte model reported here, control cells underwent a significant increase in contractile amplitude with induction of an intracellular alkalosis, although the subsequent intracellular acidosis did not result in a fall below baseline in contractile amplitude in the absence of amiloride pretreatment (see Figures 2 and 3). However, when the intracellular acidosis was not preceded by a significant alkalosis, as in the data shown in Figure 3B for amiloride-pretreated cells, a large and sustained fall in contractile amplitude occurred. Thus, prior alkalinization of the cell blunts the negative inotropic effect of a subsequent intracellular acidosis, resulting in a hysteresis phenomenon in the relation of contractile amplitude to pH_i, as has been noted previously in Purkinje fibers[34] and single isolated rat ventricular myocytes.[25] Interestingly, the hysteresis in contractile function was also apparent in myocytes pretreated with a concentration of ouabain (10^{-5} M) sufficient to cause only a 10–15% rise in baseline contractile amplitude (data not shown). Presumably, this can be explained by a rise in intracellular Na^+ activity that decreased the sarcolemmal Na^+ gradient and resulted in both a slowed recovery from the intracellular acid load after NH_4Cl washout and a reduced Ca^{2+} efflux (or increased Ca^{2+} influx) via Na^+-Ca^{2+} exchange, thereby maintaining contractile amplitude despite the intracellular acidosis. Indeed, several reports have noted that the negative inotropic effect of a low pH_i can be modified considerably or reversed by a rise in cell Na^+ and, consequently, Ca^{2+}.[24,26,35–39]

Endothelin, Myocyte Contractility, and Na^+-H^+ Exchange

Endothelin has been shown to increase pH_i in several tissues including mesangial cells[13] and vascular smooth muscle[14] by activating the plasmalemmal Na^+-H^+ exchanger. We did not observe the transient early acidification reported in mesangial cells by Simonson et al,[13] which was only observed at concentrations of endothelin above 10 nM. Endothelin has also been shown to activate phospholipase C and phosphoinositide hydrolysis in a number of tissues, including cardiac cells, with subsequent activation of protein kinase C[30,40–42]; this activation pattern presumably leads to phosphorylation of the Na^+-H^+ antiporter and results in the observed intracellular alkalinization.

As shown in Figure 1, endothelin does increase pH_i in cardiac myocytes, with a half-maximal response of 0.08 pH units at approximately 100 pM in this model. Endothelin increased the rate of recovery of pH_i from NH_4Cl washout–induced acidosis and also caused a significant overshoot of pH_i after NH_4Cl washout, beyond the baseline increase in pH_i induced by the peptide alone. The time course of the increase in pH_i after addition of endothelin to the superfusion media overlaps the time course of the contractile response to the peptide; although a small increase in pH could be detected within 1–2 minutes, no increase in contractility was apparent for about 4 minutes. Amiloride pretreatment with subsequent exposure to endothelin prevented the rise in pH_i and produced a concomitant 45% decrease in the maximal contractile response compared with endothelin alone.

The concentration of amiloride used in these experiments (100 μM) was chosen because it was approximately 10-fold greater than the reported IC_{50} of the Na^+-H^+ exchanger in the sarcolemmal membrane of these cells at 140 mM Na^+[23] and because it was sufficient to largely prevent the recovery of pH_i upon washout of NH_4Cl in control cells. Although the concentration of amiloride used here is somewhat lower than that reported to affect other sarcolemmal Na^+ carriers, including Na^+-Ca^{2+} exchange, we cannot exclude other nonspecific effects of amiloride in these cells that might also have affected myocyte contractile amplitude. Nevertheless, it is likely that both the decline in pH_i and the reduced inotropic response to endothelin in amiloride-pretreated cells were related, since two inhibitors of protein kinase C, H-7 and sphingosine, that produced complete or partial inhibition of the endothelin-induced rise in pH_i also caused a proportionate reduction in the inotropic effect of the peptide.

Despite the evidence that a rise in pH_i contributed to the inotropic action of endothelin, there is a clear dissociation between changes in pH_i and the peptide's inotropic effect. This dissociation is indicated by the 40–50% increase in contractile amplitude and the absence of an increase in pH_i in amiloride-

pretreated cells after exposure to 100 pM endothelin. In addition, no change in pH_i could be detected at concentrations of endothelin below 10 pM, a concentration that reproducibly causes a 10–25% increase in contractile amplitude in these cells at 8 minutes. Also, a large increase in contractile amplitude in endothelin-treated cells was achieved with NH_4Cl superfusion in the presence of amiloride (Figure 4B); this increase in contractility was comparable with that seen in the absence of amiloride (Figure 3B) despite the lower maximal pH_i with NH_4Cl in amiloride-pretreated cells. This implies that although an intracellular alkalinization contributes to the inotropic effect of endothelin, this effect is maximal after an increase in pH of approximately 0.20. Finally, alkalinization can occur in the absence of an increase in contractility, as occurred in pertussis toxin–treated cells (Figure 5). Thus, although it is neither the essential nor the sole modulator of the increase in contractile state, the rise in pH_i with endothelin contributes to the maximal increase in contractile amplitude with the peptide and appears to be initiated by a pertussis toxin–resistant second-messenger pathway, while the increase in contractile amplitude is mediated via a G_o or G_i GTP-binding protein.

Increased activity of the Na^+-H^+ exchanger could lead to conditions that would favor reduced Ca^{2+} efflux or increased influx by Na^+-Ca^{2+} exchange, although again we were unable to detect an increase in either systolic or diastolic Ca^{2+} concentration in myocytes exposed to less than 1 nM endothelin.[3] Interestingly, a fall in intracellular calcium transients has been described during NH_4Cl-induced intracellular alkalosis, perhaps due to increased Ca^{2+} uptake by the sarcoplasmic reticulum and/or reduced Na^+-H^+ exchange after introduction of the weak base.[43] Thus, the failure of our previous work[3] to document a change in calcium transients accompanying the inotropic effect of endothelin could have been due to the offsetting effects of a small rise in intracellular calcium with endothelin and a fall in Ca^{2+} due to the intracellular alkalinization induced by the peptide. However, this explanation is unlikely; unlike the introduction of a weak base, the mechanism by which endothelin increases pH_i involves stimulation of Na^+-H^+ exchange, which, as noted above, should lead to a modest increase in cytosolic Ca^{2+}.

Endothelin's Role in Cardiac Myocyte Cell Biology and Function

Intracellular alkalinization could also have other roles in normal myocyte physiology aside from the effects on contractility described here. A rise in pH_i is clearly associated with a mitogenic response in many cells and with hypertrophy in vascular smooth muscle.[42,44,45] In addition, endothelin has been shown to have mitogenic effects in vascular smooth muscle cells[46] and other cell types.[47–50] Importantly, the mitogenic effects of endothelin and, where they have been measured, the effects of endothelin on pH and on the expression of certain proto-oncogenes, such as c-*fos* and c-*jun* associated with some forms of hypertrophic growth in cardiac myocytes,[51] occur at relatively low concentrations (i.e., <1 nM), comparable with the concentrations used here that induce maximal increases in contractile amplitude and pH_i. Activation of phospholipase C by endothelin has been well documented in both established cell lines and primary cultures of vascular smooth muscle cells,[52] endothelial cells,[49] and glial cells,[53] although only at higher concentrations, typically with an EC_{50} of 10 nM or higher.

The response of a variety of cell lines and primary cultures pretreated with pertussis toxin and then exposed to endothelin also implicates a complex and perhaps tissue-specific signal transduction mechanism for this peptide, comprising several independently regulated pathways. In freshly isolated rat ventricular cells, as documented here, pertussis toxin completely inhibited the inotropic effect after exposure to endothelin, while variably reducing the concomitant intracellular alkalinization by no more than 50% (Figure 5). In contrast, pertussis toxin had no effect on the endothelin-induced rise in inositol trisphosphate (EC_{50}, 10 nM) in freshly dissociated rat atrial cells.[54] A dissociation between phospholipase C and phospholipase A_2 activation in vascular smooth muscle cells has been noted based on their response to pertussis toxin,[41] and a similar large increase in phospholipase A_2 activiation with endothelin was seen in mesangial cells, but only at concentrations near 0.1 μM.[50] This is well above the dose range necessary for inositol trisphosphate generation (1–10 nM) or for the induction of mitogenesis, intracellular alkalinization, and the stimulation of c-*fos* (<1 nM).

Thus, it is possible that other actions of endothelin on ventricular myocytes might be demonstrable at concentrations greater than 1 nM. It is unknown what local tissue concentrations of endothelin are relevant. Although the reported K_d for receptor binding of endothelin-1 to cardiac tissue is in the subnanomolar range,[6–8] much higher concentrations could occur under pathological conditions. Nevertheless, the data reported here indicate that endothelin, in amounts well below 1 nM, will result in increased contractile function and an intracellular alkalinization that could facilitate the development of myocyte hypertrophy.

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