# Endothelin-1 and PKC Induce Positive Inotropy Without Affecting $pH_i$ in Ventricular Myocytes

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**It has been proposed that intracellular alkalinization underlies the enhanced contractility of ventricular myocytes exposed to endothelin (ET)-1. The effects of ET-1 on the contractility and intracellular pH ($pH_i$) were examined here in cultured adult rat ventricular myocytes by employing the pH-sensitive fluorescent dye SNARF-1. Variable $pH_i$ changes were observed on ET-1 stimulation. Most myocytes ($n = 20$ of 32) did not alkalinize, but showed an approximate 60% increase in twitch amplitude in response to ET-1. In the remaining myocytes (12 of 32), ET-1 induced an increase in $pH_i$ by 0.05 ± 0.02 pH units with a similar approximate 60% increase in twitch amplitude. Therefore, there was no strong correlation between ET-1–mediated positive inotropy (enhanced contractility) and intracellular alkalinization. To determine whether ET-1 contractile and $pH_i$ responses were mediated by protein kinase C (PKC), yellow fluorescent protein (YFP)–fused dominant negative (dn) PKC constructs were used as isoform specific inhibitors. In dn-PKC-ε-YFP–expressing myocytes, the ET-1–mediated positive inotropic response was greatly diminished to 13 ± 15%, but alkalinization was still observed. Expression of dn-PKC-δ-YFP also did not block alkalinization, but in this case the positive inotropic response was still observed. In a previous study, we showed that expression of PKC-δ and PKC-ε caused a strong positive inotropy on stimulation with phorbol 12,13-dibutyrate (PDBu). Using this system, PDBu failed to affect $pH_i$ in the majority of PKC expressing myocytes despite increases in twitch amplitudes of >60%. Overall, the poor correlation of positive inotropic responses and alkalinization was observed for ET-1 with and without dn-PKC constructs and for PDBu with and without wild-type PKC constructs. These results suggest that ET-1 produces positive inotropy via PKC-ε by mechanisms other than intracellular alkalinization.** Exp Biol Med 231:865–870, 2006

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## Introduction

Endothelin (ET)-1, a potent vasoconstrictor peptide, has acute positive inotropic effects in mammalian heart. However, the mechanism by which ET-1 induces positive inotropy in cardiac myocytes remains unclear. It has been postulated that the rise in intracellular pH ($pH_i$) plays a role in the establishment of the positive inotropic response by increasing myofilament sensitivity to $Ca^{2+}$. In the present study, we have investigated the relationship between ET-1's inotropic response and $pH_i$ in adult rat ventricular myocytes and further examined whether protein kinase C (PKC) is involved in these processes. PKC-dependent $Na^+/H^+$ exchanger stimulation led to intracellular alkalosis and modulated contractile function in various cell systems (1, 2). However, direct PKC activation using caged diacylglycerol in ventricular myocyte was not accompanied by a pH change (3), and vasopressin V1-receptor activation produced a positive inotropic response without intracellular alkalinization in papillary muscles (4).

Recently, we found that phorbol 12,13-dibutyrate (PDBu)-induced negative inotropism was converted to strong positive inotropism when either PKC-δ or PKC-ε was expressed in ventricular myocytes. Moreover, PKC-δ and PKC-ε each promoted a robust positive inotropic response in conjunction with translocation to intracellular sites such as the Golgi apparatus or transverse tubules (5). Identifying whether intracellular alkalinization is involved in this strong positive inotropic response would further expand our understanding of the mechanisms of ET-1, PKC, and positive inotropism in heart.

## Materials and Methods

**Materials.** All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless noted otherwise.

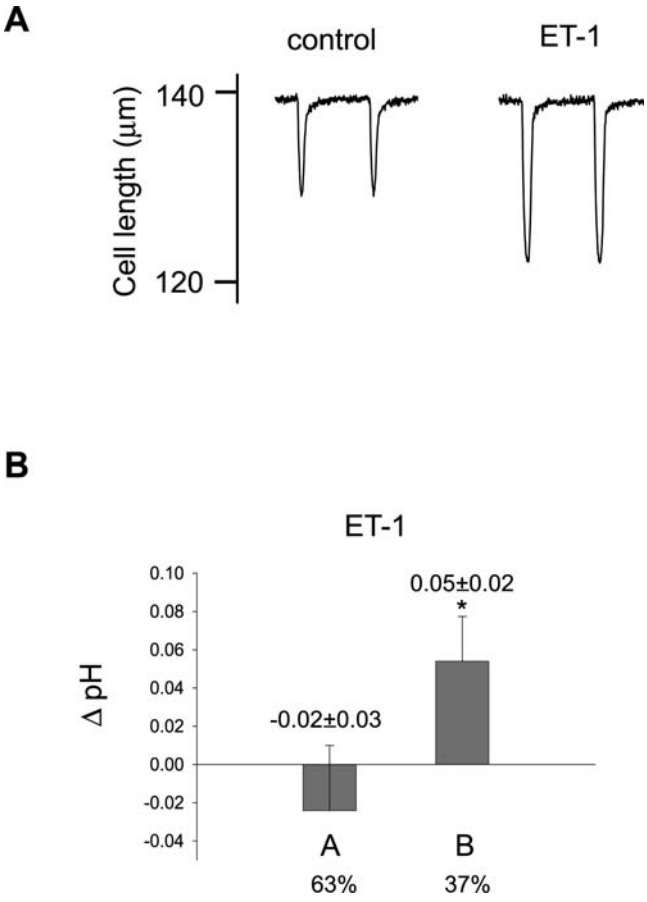


**Figure 1.** Effects of ET-1 on twitch amplitudes and $pH_i$. (A) Representative twitch traces from a single myocyte before (left) and 15 minutes after treatment with 10 n*M* ET-1 (right). (B) Steady-state $pH_i$ change caused by 10 n*M* ET-1. Myocytes were loaded with SNARF-1 and fluorescence ratio (640 nm/580 nm) was obtained by analyzing fluorescence intensity using Lasersharp software. Two different responses were obtained: group A ($n = 14$) had no effect on pH and group B ($n = 10$) showed an alkalinization. * $P < 0.05$ vs. group A.

Carboxy SNARF-1 was from Molecular Probes (Eugene, OR).

**Fluorescent PKC Constructs.** Full-length rabbit PKC-ε and mouse PKC-δ were fused to the C-terminus of green fluorescent protein (GFP) in a pShuttle vector driven by cytomegalovirus promoters (Stratagene). To alter PKC isoform activity in cardiac myocytes, dominant negative (dn) mutant PKC cDNA were constructed through site-directed mutagenesis of wild-type (wt) PKC cDNAs. Dn-PKC constructs were generated through a double mutation by converting K to R at the ATP binding site (amino acid 376 for PKC-δ and amino acid 436 for PKC-ε) and A to E at the pseudosubstrate site (amino acid 147 for PKC-δ and amino acid 159 for PKC-ε) (6). This double mutation permanently impairs the ATP-binding site of the enzyme, but still allows the enzyme to compete for anchoring sites, thereby effectively attenuating the activity of each PKC isoform. All PKC clones were fully sequenced and tested for protein expression by Western blotting and then generated as adenoviruses. Generation of recombinant adenoviruses was accomplished using AdEasy adenoviral vector system (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

**Cardiac Myocyte Adenoviral Infection.** Animal handling practices used in this study have been reviewed by and received approval from the Animal Care Committee of the University of Wisconsin. Ventricular myocytes were isolated from 3-month-old male Sprague-Dawley rats with enzymatic digestion, then plated onto laminin-coated coverslips and infected with adenoviruses as described previously (1).

**Twitch Measurements.** Cell twitches were initiated by electric field stimulation with a SD9 stimulator (Grass Instrument, Quincy, MA) in a modified PH1 chamber (Warner Instrument, Hamden, CT) mounted on a Zeiss inverted microscope. The stimulation protocol was 0.4 Hz, 10-ms duration, and 60 V at 22°C. Individual myocytes were monitored with a model VED 104 video edge detector (Crescent Electronics, Sandy, UT) and cell shortening was recorded using Felix software (Photon Technology International, West Sussex, UK).

**Intracellular pH Measurement Using SNARF-1.** Myocytes expressing fluorescent PKCs were cultured for 30–40 hours before observation. The $pH_i$ of single myocytes was measured using the pH-sensitive fluorophore carboxy-SNARF-1. Myocytes were loaded with the membrane-permeable acetoxy-methylester form of carboxy-SNARF-1 (1 μ*M*) for 10–20 minutes. Confocal images were acquired with a Bio-Rad Radiance 2100 laser scanning confocal microscope to monitor pH changes before and after agonist stimulation. The fluorophore was excited by light at 514 nm, and the emitted fluorescence signals were measured simultaneously at 580 nm and 640 nm. The 640 nm/580 nm emission ratio was converted to a pH value using the nigericin calibration technique (7). All experiments were performed at room temperature (21°C–23°C).

## Results

In the present study, we tested the effect of ET-1 on contractile function in cultured ventricular myocytes. ET-1 stimulation induced about a 60% increase in twitch amplitude (Fig. 1A, Table 1). To investigate whether the effect of ET-1 on twitch amplitude is associated with an intracellular alkalinization, we measured $pH_i$ after ET-1 stimulation using pH indicator dye SNARF-1. The simultaneous measurements of $pH_i$ and contraction were performed during electrical field stimulation under basal conditions and after stimulation with 10 n*M* ET-1. Basal $pH_i$ values for cultured ventricular myocytes were $6.99 \pm 0.06$ when perfused at room temperature (21°C–23°C) in Ringer's solution buffered with HEPES to pH 7.4 ($n = 16$). More than 90% of myocytes responded to ET-1 with positive inotropism. Different from ET-1's consistent inotropic effect, addition of ET-1 caused variable $pH_i$ changes

**Table 1.** Summary of ET-1 and PDBu Effects on Twitch Amplitude[a]

| Activator[b] | Cell type[c] | Twitch amplitude (% change)[d] | *n* |
|---|---|---|---|
| ET-1 | Control | 59 ± 21 | 32 |
| | dn-PKC-δ-YFP | 72 ± 53 | 10 |
| | dn-PKC-ε-YFP | 13 ± 15[e] | 10 |
| PDBu | Control | −46 ± 19 | 5 |
| | wt-PKC-δ-GFP | 93 ± 38 | 12 |
| | wt-PKC-ε-GFP | 65 ± 31 | 6 |

[a] Data presented as mean ± SD ($n$ = number of myocytes), with negative responses indicated by a minus sign.
[b] ET-1 at 10 n*M* and PDBu at 100 n*M*.
[c] Control myocytes (no fluorescent protein expressed); wt-PKC-δ-GFP myocytes at 3- to 10-fold overexpression; wt-PKC-ε-GFP myocytes at 6- to 10-fold overexpression.
[d] Data presented as percent change compared with untreated control in the parameter of interest.
[e] $P < 0.05$ vs. dn-PKC-δ-YFP.

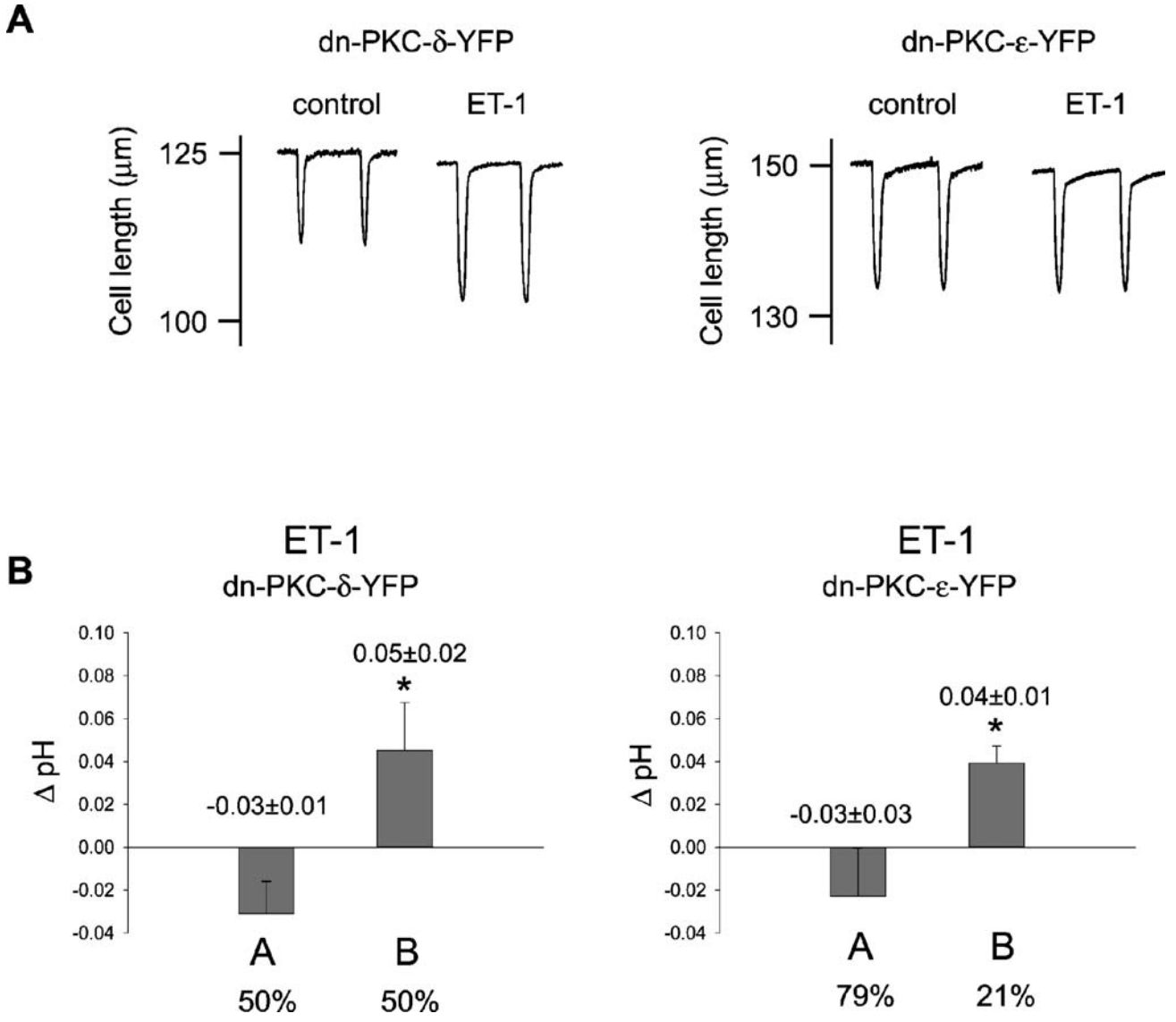


**Figure 2.** Effects of ET-1 on twitch amplitude and $pH_i$ in dn-PKC-YFP–expressing myocytes. (A) Representative twitch traces in dn-PKC-δ-YFP and dn-PKC-ε-YFP expressed myocytes before and after treatment with 10 n*M* ET-1. ET-1–mediated positive inotropic response was blunted by dn-PKC-ε-YFP expression. (B) Steady-state $pH_i$ changes caused by 10 n*M* ET-1 were measured using SNARF-1. In both dn-PKC-δ-YFP and dn-PKC-ε-YFP expressed myocytes, there were two different groups showing either no pH change or alkalinization after 10 n*M* ET-1 stimulation. ($n = 14$ for each construct). *$P < 0.05$ vs. group A.

showing either alkalinization or no pH change. Among myocytes showing positive inotropic responses to ET-1, 63% of the population ($n = 20$) did not alkalinize (Fig. 1B, Group A). Only 37% of cells showing positive inotropy were associated with an intracellular alkalinization with a mean of 0.05 ± 0.02 pH units ($n = 12$) (Fig. 1B, Group B). These data show a poor correlation of alkalinization and positive inotropic responses in ET-1 stimulated cells.

We further examined whether inhibition of PKC function changed the relationship between contractility and $pH_i$ in response to ET-1. Three major PKC isozymes are expressed in a rat adult heart: PKC-α, -δ, and -ε. Because organic inhibitors of PKC-α have no effect on ET-1's inotropic responses, we examined the role of PKC-ε and PKC-δ isoforms using dn-PKC constructs. Yellow fluorescent protein (YFP) fusion to dn-PKC allows us to indicate the expression level and the enzyme's localization. Within 40 hours of adenovirus infection, fluorescent PKC constructs were well expressed and visualized under the confocal microscope. There was an isoform-dependent preference in anchoring sites in constitutively translocating dn-PKCs (M. Kang, J. W. Walker, unpublished data). Differentially localizing dn-PKC–expressing myocytes were stimulated with ET-1, and changes in contractility and $pH_i$ were monitored. In dn-PKC-ε-YFP–expressing myocytes, the positive inotropic response to ET-1 was minimal (13 ± 15% increase in twitch amplitude). In contrast, myocytes expressing dn-PKC-δ-YFP behaved like controls cells expressing GFP alone or no fluorescent protein showing about a 70% increase in twitch amplitude (Fig. 2A, Table 1). Thus inhibition of ET-1–mediated positive inotropy was PKC-ε isoform specific. Again, a poor correlation of alkalinization and inotropic responses was observed in the presence of dn-PKC isoforms. Dn-PKC-δ-YFP–expressing cells induced positive inotropic responses to ET-1, but only half showed alkalinization (mean 0.05 ± 0.02 pH units, Fig. 2B, Group A and B). Also, although there was no positive inotropy in dn-PKC-ε-YFP–expressing cells with ET-1 stimulation, intracellular alkalinization was detected in 21% of tested cells (Fig. 2B). This reinforces the idea that ET-1–induced positive inotropy is not mediated by intracellular alkalinization.

Next, we tested how negative inotropic responses might affect $pH_i$, and PDBu was chosen as a negative inotropic agonist. Myocytes stimulated with 100 n*M* PDBu presented about a 46% decrease in twitch amplitude (Fig. 3A, Table 1). Interestingly, PDBu-stimulated cells showing negative inotropy also had a mixed response on $pH_i$. Sixty percent of the population showed no significant change in $pH_i$, but 40% of tested cells showed intracellular alkalinization with a mean of 0.04 ± 0.02 pH units after PDBu stimulation (Fig. 3B, Group A and B). These data indicate intracellular alkalinization occurs even when the overall twitch amplitude is inhibited. When PKC-δ or PKC-ε was introduced into myocytes, PDBu no longer generated a negative inotropy, but instead gave a robust positive inotropic

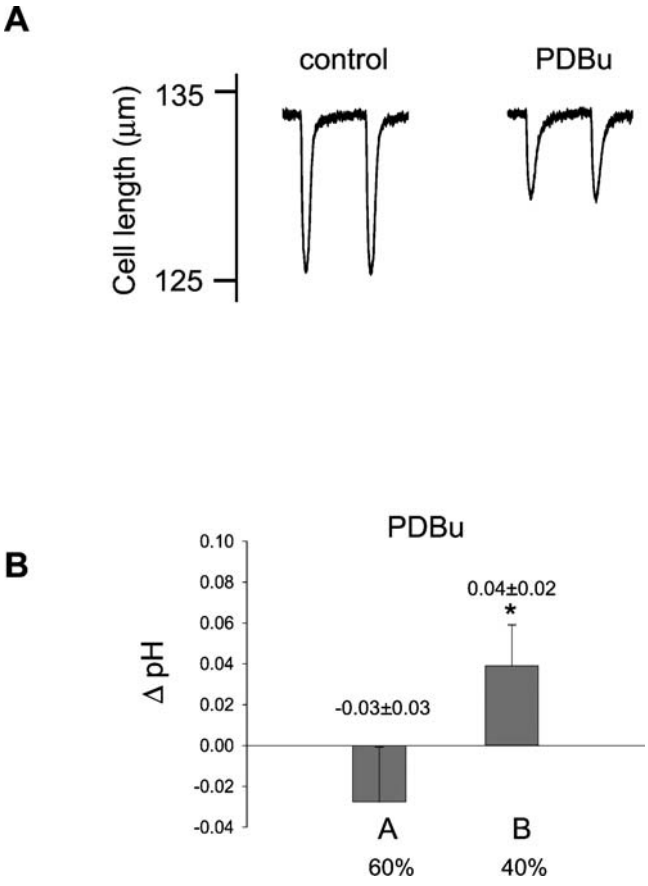


**Figure 3.** Effects of PDBu on twitch amplitudes and $pH_i$. (A) Representative twitch traces from a single myocyte before (left) and 10 minutes after treatment with 100 n*M* PDBu (right). (B) Steady-state $pH_i$ changes caused by 100 n*M* PDBu were measured using SNARF-1. PDBu stimulation also triggered two different responses: group A ($n = 6$) showed no significant change in $pH_i$ and group B ($n = 4$) showed an alkalinization by 0.05 pH units. $^*P < 0.05$ vs. group A.

response (5). To determine whether intracellular alkalinization is more consistent in PDBu-induced positive inotropic responses, we measured $pH_i$ after PDBu activation in PKC-expressing cells. Addition of PDBu showed positive inotropic responses from both PKC-δ– and PKC-ε–expressed cells (93% and 65% increase in twitch amplitude, respectively) (Fig. 4A, Table 1). However, fewer cells showed alkalinization on PDBu stimulation compared with ET-1 stimulation, although bigger positive inotropic responses were observed. Most cells showing positive inotropy presented no significant pH change with PDBu. The mean pH change values are summarized in Fig. 4B, including control cells without PKC expression. These data further support the idea that positive inotropic responses mediated by ET receptor activation or direct PKC activation were not caused by intracellular alkalinization.

## Discussion

Reports from several laboratories (8–10), including our own (11), have shown that ET-1 is a potent inotropic agent in cardiac muscle. However, the detailed mechanism of ET-1's

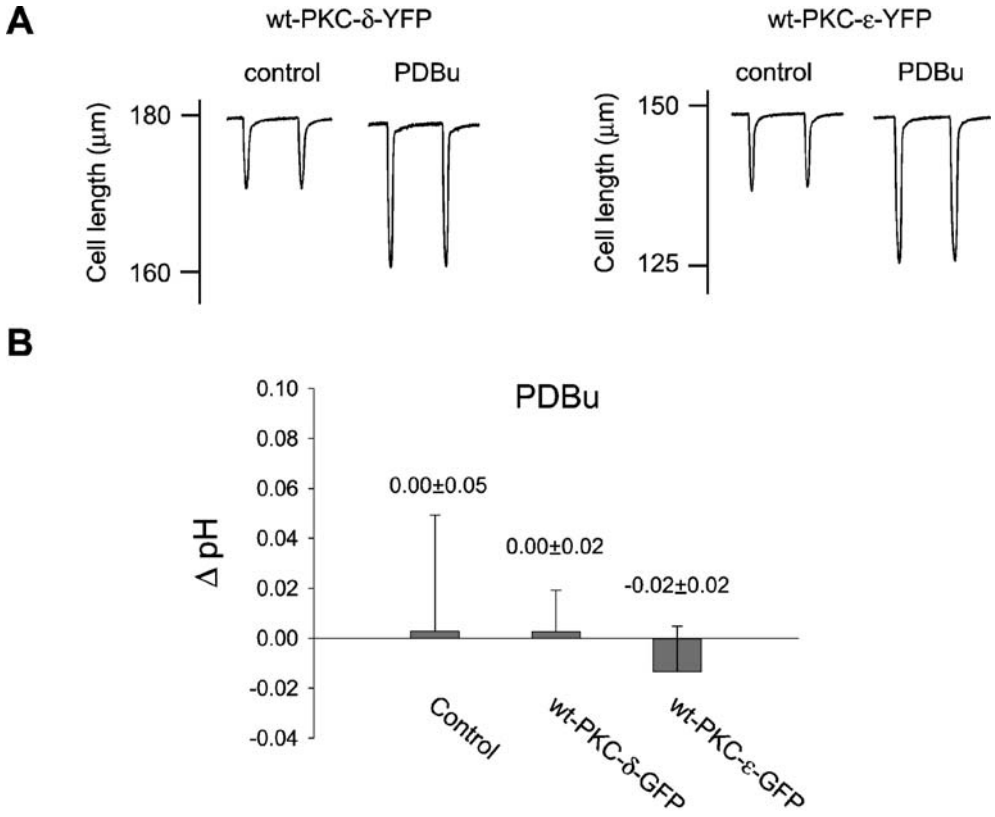


**Figure 4.** Effects of PDBu on twitch amplitudes and $pH_i$ in wt-PKC-GFP expressing myocytes. (A) Positive inotropic responses in wt-PKC-δ-GFP and wt-PKC-ε-GFP–expressing myocytes stimulated with 100 n*M* PDBu. (B) Summary of mean $pH_i$ changes caused by 100 n*M* PDBu in controls (no fluorescent proteins expressed), wt-PKC-δ-GFP, and wt-PKC-ε-GFP–expressing myocytes.

inotropic effect remains unclear. Induction of an intracellular alkalosis is one potential mechanism for this effect. The importance of pH change in muscle function has been demonstrated by Fabiato and colleagues (12), who showed that acidosis produced a decrease in myofilament $Ca^{2+}$ sensitivity and depressed the maximum tension developed in skinned cardiac muscles. Therefore, we tested the hypothesis that ET-1 would have a positive inotropic effect associated with alkalinization. In cultured adult rat myocytes, more than 90% of cells responded to ET-1 with positive inotropism, but changes in $pH_i$ were quite variable. Most myocytes (63%) showing positive inotropy were not associated with intracellular alkalinization. We found a 0.05 pH unit increase in pH after ET-1 stimulation in only 37% of tested cells revealing considerable heterogeneity within the population. Using pharmacologic approaches, Kramer and colleagues (2) showed that the positive inotropic action of ET-1 is due in part to stimulation of the $Na^+/H^+$ exchanger by activation of the PKC-dependent pathway, resulting in alkalinization and sensitization of cardiac myofilaments to intracellular $Ca^{2+}$. However, despite the evidence that a rise in $pH_i$ contributed to the inotropic action of ET-1, there was a clear dissociation between changes in $pH_i$ and the inotropic effect. The dissociation was that cells pretreated with amiloride, a $Na^+/H^+$ exchanger inhibitor, still showed ET-mediated positive inotropic response in the absence of alkalosis. Also, alkalinization can occur in the absence of an increase in contractility, which was found in the present study (Fig. 2B; Group B in dn-PKC-ε-YFP–expressing cells).

Because ET-1 induces positive inotropy through G protein–coupled receptor activation, we also compared pH changes in positive inotropic responses mediated by more direct PKC activation. The role of PKC in the regulation of $pH_i$ has been proposed to occur via ERK activation (13). Phosphorylation-mediated stimulation of $Na^+/H^+$ exchanger activity is caused by a conformational change altering interactions of the regulatory domain with the transport domain resulting in increased $H^+$ affinity of the proton sensor in $Na^+/H^+$ exchanger (14). Here we tested how PKC activation affects contractility and $pH_i$. Overexpression of either PKC-δ or PKC-ε produced a robust positive inotropy by PDBu stimulation (Fig. 4A). Still, we did not find a consistent alkalinization with positive inotropic responses. The mean changes in $pH_i$ was less than 0.02 pH units, indicating direct PKC activation was not associated with alkalosis. It has been well documented that addition of PDBu produces a negative inotropy in fresh and cultured myocytes (Fig. 3A) (15, 16). In this study, we found that 40% of PDBu-stimulated myocytes also showed an alkalinization with negative inotropic response, adding more evidence to the poor correlation of inotropy and pH

changes (Fig. 3B). Accordingly, factors other than alkalinization may be involved in ET-1–mediated positive inotropy. In this context, it is noteworthy that phosphorylation of myofilament proteins by ET-1 actively regulates twitch dynamics and myofilament $Ca^{2+}$ sensitivity in mouse ventricular myocytes (17). Pi and colleagues (18) provided evidence that phosphorylation of PKC sites on cardiac troponin I plays a central role in depressing myofilament ATPase activity and in enhancing myofilament $Ca^{2+}$ sensitivity. In addition, increases in cytosolic $Ca^{2+}$ and prolonged action potential duration might be other key factors affecting positive inotropy mediated by ET-1. These possibilities are now under investigation.

Numerous studies indicate that ET-1 signals through ET receptor/$G\alpha_q$/phospholipase C/PKC signaling cascade. Among PKC isozymes, PKC-ε has received attention as a major PKC isoform mediating neurohumoral responses to angiotensin, phenylephrine, epinephrine, and ET in heart (19–21). An important finding in this study is that selective inhibition of the PKC-ε isoform blocked ET-1–mediated inotropic responses. This is the first report directly showing PKC-ε is the isoform that induces positive inotropy on ET-1 stimulation. Inhibition of PKC-δ isoform in the same novel PKC family was not effective to block the ET-1 response. Taken together, the data indicate that ET-1–mediated positive inotropic responses are PKC-ε dependent, but that intracellular pH is not consistently changed on ET-1 or PKC stimulation of ventricular myocytes.


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