Phenylephrine acts via IP_3-dependent intracellular NO release to stimulate L-type Ca^{2+} current in cat atrial myocytes

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This study determined the effects of α_1-adrenergic receptor (α_1-AR) stimulation by phenylephrine (PE) on L-type Ca^{2+} current ($I_{Ca,L}$) in cat atrial myocytes. PE (10 μM) reversibly increased $I_{Ca,L}$ (51.3%; $n = 40$) and shifted peak $I_{Ca,L}$ activation voltage by −10 mV. PE-induced stimulation of $I_{Ca,L}$ was blocked by each of 1 μM prazocin, 10 μM L-NIO, 10 μM W-7, 10 μM ODQ, 2 μM H-89 or 10 μM LY294002, and was unaffected by 10 μM chelerythrine or incubating cells in pertussis toxin (PTX). PE-induced stimulation of $I_{Ca,L}$ also was inhibited by each of 10 μM ryanodine or 5 μM thapsigargin, by blocking IP_3 receptors with 2 μM 2-APB or 10 μM xestospongin C or by intracellular dialysis of heparin. In field-stimulated cells, PE increased intracellular NO (NO_i) production. PE-induced NO_i release was inhibited by each of 1 μM prazocin, 10 μM L-NIO, 10 μM W-7, 10 μM LY294002, 2 μM H-89, 10 μM ryanodine, 5 μM thapsigargin, 2 μM 2-APB or 10 μM xestospongin C, and unchanged by PTX. PE (10 μM) increased phosphorylation of Akt, which was inhibited by LY294002. Confocal microscopy showed that PE stimulated NO_i release from subsarcolemmal sites and this was prevented by 2 mM methyl-β-cyclodextrin, an agent that disrupts caveolae formation. PE also increased local, subsarcolemmal SR Ca^{2+} release via IP_3-dependent signalling. Electron micrographs of atrial myocytes show peripheral SR cisternae in close proximity to clusters of caveolae. We conclude that in cat atrial myocytes PE acts via α_1-ARs coupled to PTX-insensitive G-protein to release NO_i, which in turn stimulates $I_{Ca,L}$. PE-induced NO_i release requires stimulation of both PI-3K/Akt and IP_3-dependent Ca^{2+} signalling. NO stimulates $I_{Ca,L}$ via cGMP-mediated cAMP-dependent PKA signalling. IP_3-dependent Ca^{2+} signalling may enhance local SR Ca^{2+} release required to activate Ca^{2+}-dependent eNOS/NO_i production from subsarcolemmal caveolae sites.

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α_1-Adrenergic receptor (α_1-AR) stimulation plays important roles in the regulation of cardiac contraction, cell growth, hypertrophy and cardioprotection (Li *et al.* 1997). Classically, α_1-ARs are coupled via pertussis toxin (PTX)-insensitive G-proteins (G_q), although coupling to G_i-protein has been reported (Steinberg *et al.* 1985; Han *et al.* 1989; Keung & Karliner, 1990; Perez *et al.* 1993). In general, α_1-AR stimulation activates phospholipase C (PLC) to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) and in cardiac muscle IP_3 enhances intracellular Ca^{2+} release (Nosek *et al.* 1986; Fabiato, 1986; Zima & Blatter, 2004). Atrial myocytes express functional IP_3 receptors (IP_3R type-2) at 6–10 times higher levels than in ventricular myocytes and IP_3Rs colocalize with ryanodine receptors in the subsarcolemmal space (Lipp *et al.* 2000; Mackenzie *et al.* 2002). In permeabilized cat atrial myocytes, exposure to IP_3 stimulates local Ca^{2+} release, i.e. Ca^{2+} sparks, from the sarcoplasmic reticulum (SR) (Zima & Blatter, 2004). Although IP_3 signalling has been implicated in atrial excitation–contraction coupling and/or atrial arrhythmias (Lipp *et al.* 2000; Mackenzie *et al.* 2002; Zima & Blatter, 2004), the functional role of IP_3 signalling in atrial muscle is still not clear.

In general, phenylephrine (PE), an α_1-AR agonist, exerts positive inotropic effects in adult ventricular muscle (Hartmann *et al.* 1988; Hescheler *et al.* 1988; Ertl *et al.* 1991; Terzic *et al.* 1992) as well as in rat (Ertl *et al.* 1991; Jahnel *et al.* 1994) and human (Schumann *et al.* 1978; Skomedal *et al.* 1985; Jahnel *et al.* 1992*a*) atrial muscle. The majority of studies indicate that the positive inotropic effect of

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α_1-AR stimulation is not primarily mediated via increases in $I_{Ca,L}$ (Ertl *et al.* 1991). However, in guinea pig ventricular myocytes PE acts via PKC signalling to stimulate $I_{Ca,L}$ and contractile amplitude (Woo & Lee, 1999). Also, in rat ventricular myocytes PE can increase $I_{Ca,L}$, possibly via PKC activation when the intracellular environment is not disturbed by intracellular dialysis (Zhang *et al.* 1998). In neonatal rat ventricular myocytes (Liu *et al.* 1994), PE increases $I_{Ca,L}$ (Liu *et al.* 1994) and chronic exposure to PE in culture induces hypertrophy and increases $I_{Ca,L}$ (Gaughan *et al.* 1998). PE may increase myofilament Ca^{2+} sensitivity (Terzic *et al.* 1992) possibly via PKC activation. In rat atria, PE increases intracellular Ca^{2+} uptake via cAMP-dependent stimulation of voltage-dependent Ca^{2+} channels (Jahnel *et al.* 1994), and possibly in part via secondary changes in Na^+/Ca^{2+} exchange (Jahnel *et al.* 1991, 1992*b*, 1994). In ventricular muscle, the positive inotropic effect of α_1-AR stimulation does not correlate with increases in cellular cAMP levels (Schumann *et al.* 1975; Brodde *et al.* 1978; Bogoyevitch *et al.* 1993). Clearly, the cellular mechanisms underlying α_1-AR stimulation in heart are diverse and not entirely understood.

In cat atrial myocytes, stimulation of β_2-ARs (Dedkova *et al.* 2002) or muscarinic receptors (Wang *et al.* 1998; Dedkova *et al.* 2003) stimulates G_i-mediated release of intracellular NO (NO_i). In human (Kirstein *et al.* 1995) and cat (Wang *et al.* 2002) atrial myocytes, NO stimulates $I_{Ca,L}$ via cGMP-induced inhibition of phosphodiesterase (PDE) type III activity to enhance endogenous cAMP-dependent PKA activity. The purpose of the present study was to determine whether α_1-AR stimulation regulates $I_{Ca,L}$ in cat atrial myocytes and if so, whether NO_i signalling plays a role. The results indicate that α_1-ARs act via PTX-insensitive G-proteins to increase $I_{Ca,L}$ via NO_i signalling. Moreover, α_1-AR stimulation activates NO_i release via PI-3K/Akt and IP_3-dependent signalling mechanisms. IP_3-dependent Ca^{2+} signalling may enhance local subsarcolemmal SR Ca^{2+} release required to activate Ca^{2+}/calmodulin (CaM)-dependent eNOS. A portion of this work has been presented in abstract form (Lipsius *et al.* 2003).

Methods

Adult cats of either sex were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, I.P.). Once fully anaesthetized, a bilateral thoracotomy was performed, and the heart was rapidly excised and mounted on a Langendorff perfusion apparatus. After the heart was enzymatically (collagenase; type II, Worthington Biochemical Corp., Lakewood, NJ, USA) digested, atrial myocytes were isolated as previously reported (Wu *et al.* 1991). Thirty-three hearts were used to isolate atrial myocytes. The animal protocols used in this study were approved by and in accordance with the Institutional Animal Care and Use Committee of Loyola University of Chicago, Stritch School of Medicine. The number of animals used in this study was limited to a minimum.

Atrial myocytes used for electrophysiological studies were transferred to a small tissue bath (0.3 ml) on the stage of an inverted microscope (Nikon Diaphot) and superfused with a Hepes-buffered modified Tyrode solution containing (mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, Hepes 5, glucose 11 and titrated with NaOH to a pH of 7.4. Solutions were perfused by gravity and electrophysiology experiments were performed at $35 \pm 1^{\circ}$C. In general, voltage and ionic currents were recorded using a nystatin (150 μg ml^{-1})-perforated patch whole-cell recording method. The internal pipette solution contained (mM): caesium glutamate 100, KCl 40, $MgCl_2$ 1.0, Na_2-ATP 4, EGTA 0.5, Hepes 5 and titrated with KOH to pH 7.2. CsCl (5 mM) also was added to all external solutions to block K^+ conductances. In one series of experiments, a ruptured patch recording method was used to dialyse heparin intracellularly. The internal pipette solution contained (mM): caesium glutamate 100, CsCl 40, $MgCl_2$ 1, Na_2-ATP 4, EGTA 0.5, Hepes 10, and titrated with CsOH to pH 7.2. A single suction pipette recorded either voltage (bridge mode) or ionic currents (discontinuous voltage clamp mode) using an Axoclamp 2A amplifier (Axon Instruments, Union City, CA, USA). Computer software (pCLAMP; Axon Instruments) was used to deliver voltage protocols, acquire and analyse data. L-type Ca^{2+} current ($I_{Ca,L}$) was activated by depolarizing pulses from a holding potential of -40 mV to 0 mV for 200 ms every 5 s. Peak $I_{Ca,L}$ amplitude was measured in relation to steady-state current.

Immunoblots were used to analyse PE-induced phosphorylation of Akt (protein kinase B) using phospho-Akt antibody (Ser^{473}). Isolated atrial cells were treated with either control medium (M199), 10 μM PE, or PE plus 10 μM LY294002 before harvesting. Cells were incubated with LY294002 for 15 min followed by a 2 min exposure to PE.

Measurements of intracellular NO (NO_i) production were obtained by incubating cells with the fluorescent NO-sensitive dye 4,5-diaminofluorescein (DAF-2) (Kojima *et al.* 1998; Nakatsubo *et al.* 1998), as previously described (Wang *et al.* 2002; Dedkova *et al.* 2003). Cells were exposed to the membrane-permeant DAF-2 diacetate ([DAF-2 DA] = 5 μM; Calbiochem, San Diego, CA, USA) for 10 min in 1 ml standard Tyrode solution. Cells were subsequently washed for 10 min in Tyrode solution containing 100 μM L-arginine. Solutions were perfused by gravity and NO_i measurements were performed at room temperature. DAF-2 fluorescence was excited at 480 nm (F_{480}) and emitted cellular fluorescence was recorded at 540 nm. Single cell fluorescence signals were recorded with a photomultiplier tube (model R2693,

Hamamatsu Corp.) by masking individual cells with an iris positioned in the emission path. Changes in cellular DAF-2 fluorescence intensities (F) in each experiment were normalized to the level of fluorescence recorded prior to stimulation (F_o), and changes in $[NO]_i$ are expressed as F/F_o. DAF-2 is not sensitive to changes in physiological $[Ca^{2+}]$ (Suzuki *et al.* 2002). Activation of DAF-2 by NO is irreversible and therefore fluorescence intensity remains constant even if NO_i levels decrease. In the experiments designed to measure NO_i, solutions contained 100 μM L-arginine. L-Arginine was omitted when L-NIO was used to block endothelial NO synthase (eNOS). Changes in NO_i induced by PE were measured at 5 min of exposure, unless stated otherwise. Cells were field-stimulated at 1 Hz by 3 ms duration suprathreshold rectangular voltage pulses delivered through a pair of extracellular platinum electrodes.

Fast 1-dimensional (1-D) linescan imaging of local intracellular Ca^{2+} release, i.e. Ca^{2+} sparks, was performed at room temperature using a confocal laser scanning unit (Bio-Rad Radiance 2100) attached to an inverted microscopy (Nikon TE2000-u) with a × 40 oil-immersion objective lens (Plan fluor, n.a. = 1.3, Nikon). Fluo-4 (fluo-4/AM; 20 μM, incubation time 20 min) was excited with the 488 nm line of an argon ion laser and emitted fluorescence was collected at > 515 nm. The scan line was positioned parallel with the longitudinal axis of the cell within the subsarcolemmal space and was scanned repetitively at 1.4 ms intervals. Linescan profiles are presented as background-subtracted F/F_o. Ca^{2+} spark

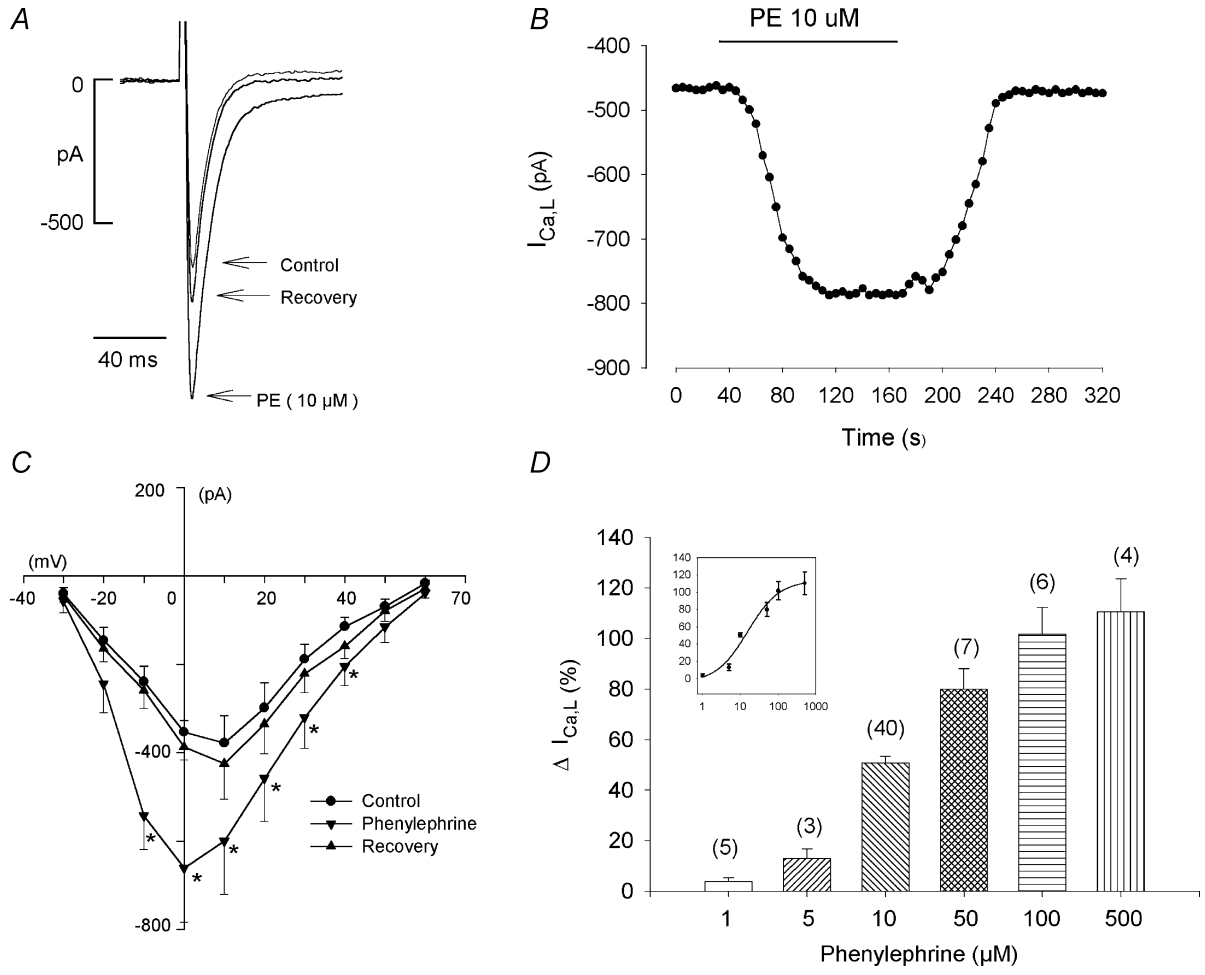

Figure 1. Effects of phenylephrine (PE; 10 μM) on $I_{Ca,L}$
A, PE reversibly increased peak $I_{Ca,L}$. *B*, consecutive measurements of peak $I_{Ca,L}$ amplitude before, during and after exposure to PE shows the time course of PE-induced stimulation of $I_{Ca,L}$. *C*, the current–voltage relationship shows that PE reversibly increased $I_{Ca,L}$ from −30 to +40 mV and shifted the voltage of maximum $I_{Ca,L}$ activation by −10 mV without affecting the reversible potential. *D*, dose–response relationship of PE (1–500 μM) to stimulate $I_{Ca,L}$. The inset shows a sigmoidal dose–response relationship fitted with a Boltzmann equation. The numbers in parentheses indicate the number of cells tested in each experiment. $^*P < 0.05$.

frequency was measured with custom-made software (Spark Laboratory; generously provided by Dr J. Puglisi) and is expressed as the number of observed Ca^{2+} sparks s^{-1} $(100\ \mu m)^{-1}$ of scanned distance in the linescan mode.

Two-dimensional (2-D) imaging was performed at room temperature using a confocal scanning unit (LSM 410, Carl Zeiss, Germany) attached to an inverted microscope (Axiovert 100, Zeiss) fitted with a $\times 40$ oil-immersion objective lens (Plan-Neofluar, n.a. = 1.3, Zeiss). Atrial myocytes were loaded with the NO-sensitive indicator DAF-2 as described above. DAF-2 fluorescence was excited with a 488 nm line of an argon laser and the emitted fluorescence was collected at wavelengths > 515 nm.

Drugs and chemicals in this study include: phenylephrine, prazocin, LY294002, pertussis toxin (PTX), L-N^5-1-iminoethylornithine (L-NIO), N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W-7), 1H-[1,2,4] oxadiazolo [4,3-α] quinoxaline-1-one (ODQ), H-89, ryanodine, 2-aminoethoxydiphenyl borate (2-APB), xestospongin C, heparin, thapsigargin, chelerythrine, methyl-β-cyclodextrin, spermine/NO (all from Sigma Chemical Co., St Louis, MO, USA), and 4,5-diaminofluorescein diacetate (DAF-2 DA) (Calbiochem). Inhibition of G_i-protein by PTX (3.5 $\mu g\,ml^{-1}$ at 36°C for at least 3 h) was confirmed by the ability of PTX to block ACh-induced increases in K^+ conductance.

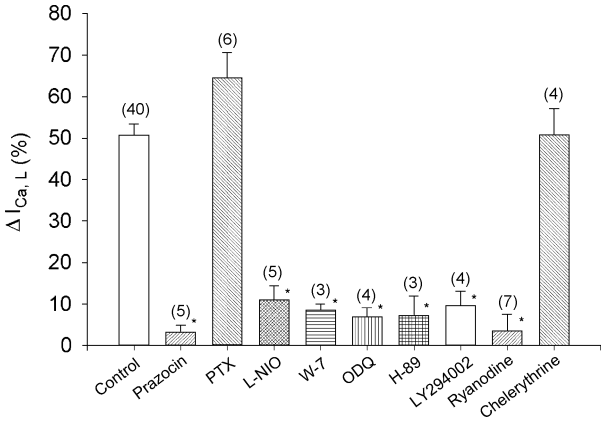

Figure 2. Pharmacological analysis of the signalling mechanisms responsible for PE-induced stimulation of $I_{Ca,L}$
Each experiment was performed by testing PE in the absence (control) and presence (test) of each drug in myocytes isolated from the same hearts. The control values from each experiment ($n = 40$) are grouped together for clarity. Compared with control (open bar), prazocin (1 μM), L-NIO (10 μM), W-7 (10 μM), ODQ (10 μM), H-89 (2 μM), LY294002 (10 μM) and ryanodine (10 μM) each significantly inhibited PE-induced stimulation of $I_{Ca,L}$. Incubation of cells in PTX or exposure to chelerythrine (4 μM) failed to affect PE-induced stimulation of $I_{Ca,L}$. The numbers in parentheses indicate the number of cells tested in each experiment. $^*P < 0.05$.

Measurements of $I_{Ca,L}$, NO_i and Ca^{2+} sparks involving one cell or two groups of cells were analysed using either paired or unpaired Student's t test for significance at $P < 0.05$. Data from Western blots and Ca^{2+} sparks involving multiple values were evaluated using ANOVA and Student-Newman-Keuls test for significance at $P < 0.05$.

Results

Figure 1*A* shows the typical effect of 10 μM phenylephrine (PE) to increase L-type Ca^{2+} current ($I_{Ca,L}$) in an atrial myocyte. PE had no effect on holding current at −40 mV and reversibly increased peak $I_{Ca,L}$ by 52%. In a total of 40 cells, 10 μM PE increased $I_{Ca,L}$ by $51 \pm 3\%$ ($P < 0.001$). Figure 1*B* shows peak $I_{Ca,L}$ amplitude recorded from another cell plotted against time throughout a typical experiment. PE-induced stimulation of $I_{Ca,L}$ amplitude reach steady-state in approximately 1 min, remained constant during exposure to PE and reversed to control upon withdrawal of PE. In Fig. 1*C* the current–voltage (I–V) relationships show that 10 μM PE reversibly increased peak $I_{Ca,L}$ from −30 to +40 mV and elicited a −10 mV shift in the voltage at which maximum $I_{Ca,L}$ was activated compared to control, without affecting the reversal potential ($n = 6$). Figure 1*D* shows that PE elicited a dose-dependent stimulation of $I_{Ca,L}$ with an EC_{50} of 13.6 μM.

In the following experiments we used a variety of specific pharmacological agents to analyse the signalling mechanisms responsible for PE-induced stimulation of $I_{Ca,L}$. All experiments were performed by comparing the effects of 10 μM PE in the absence (control) and presence (test) of each drug in cells isolated from the same hearts. $I_{Ca,L}$ was activated by clamp steps from −40 to 0 mV for 200 ms. Figure 2 summarizes the results. For clarity, all control responses to 10 μM PE ($51 \pm 3\%$, $n = 40$) are grouped together (open bar). Pre-treatment of cells with 1 μM prazocin, a specific α-AR blocking agent, abolished the effects of PE to stimulate $I_{Ca,L}$ (control, $43 \pm 8\%$ *versus* prazosin, $3 \pm 2\%$; $P < 0.05$). Propranolol (0.05 μM) failed to affect PE-induced stimulation of $I_{Ca,L}$ (control, $34 \pm 3\%$ *versus* propranolol, $37 \pm 3\%$, $n = 4$). To determine whether the effects of PE were mediated via PTX-insensitive G-protein, cells were incubated in PTX (see Methods). As summarized in Fig. 2, PTX failed to prevent PE-induced stimulation of $I_{Ca,L}$ and in fact somewhat enhanced the effects of PE (control, $59 \pm 5\%$ *versus* PTX, $66 \pm 6\%$), although the increase did not reach statistical significance. In both human (Kirstein *et al.* 1995) and cat (Wang *et al.* 1998) atrial myocytes NO stimulates $I_{Ca,L}$. Because NO is produced by Ca^{2+}/calmodulin (CaM)-dependent eNOS, we examined PE-induced stimulation of $I_{Ca,L}$ in the absence and presence of L-NIO, a specific eNOS inhibitor (Rees *et al.*

1990) and W-7, a specific CaM inhibitor (Hidaka *et al.* 1981). As summarized in Fig. 2, compared with control PE-induced stimulation of $I_{Ca,L}$ was significantly inhibited by 10 μM L-NIO (control, 42 ± 4% *versus* L-NIO, 11 ± 3%; $P < 0.05$) and by 10 μM W-7 (control, 51 ± 10% *versus* W-7, 9 ± 1%; $P < 0.05$). In atrial myocytes NO acts via cGMP-induced inhibition of phosphodiesterase (PDE) type III to stimulate endogenous cAMP-dependent PKA signalling (Kirstein *et al.* 1995; Wang *et al.* 1998). In the present study, PE-induced stimulation of $I_{Ca,L}$ was essentially abolished by 10 μM ODQ (control, 40 ± 5% *versus* 7 ± 2%; $P < 0.05$), a specific inhibitor of soluble guanylate cyclase (Brunner *et al.* 1996) and by 2 μM H-89 (control, 46 ± 4% *versus* H-89, 7 ± 4%; $P < 0.05$), an inhibitor of cAMP-dependent PKA activity (Chijiwa *et al.* 1990). Additional experiments showed that 2 μM H-89 also prevented the effects of PE to shift the voltage of maximum $I_{Ca,L}$ activation to more negative values ($n = 4$; not shown), indicating that this effect of PE is mediated by cAMP-dependent PKA signalling. In endothelial (Dimmeler *et al.* 1999) and cardiac (Vila Petroff *et al.* 2001; Wang *et al.* 2002) cells, NO release is mediated via phosphoinositol-3′ kinase (PI-3K) signalling. In the present study, preincubation (30 min) of atrial myocytes with 10 μM LY294002, a specific inhibitor of PI-3K (Vlahos *et al.* 1994), inhibited PE-induced stimulation of $I_{Ca,L}$ (control, 55 ± 11% *versus* 10 ± 3%; $P < 0.05$). Because eNOS is Ca^{2+} dependent we determined the role of intracellular Ca^{2+} release by exposing cells to 10 μM ryanodine. This concentration of ryanodine locks the SR Ca^{2+} release channel in the open position, and thereby depletes SR Ca^{2+} content, preventing SR Ca^{2+} release (Fill & Copello, 2002). Ryanodine alone did not inhibit basal $I_{Ca,L}$ amplitude. However, ryanodine pretreatment abolished PE-induced stimulation of $I_{Ca,L}$ (control, 39 ± 5% *versus* ryanodine, 4 ± 4%; $P < 0.05$). Similar results were obtained with 5 μM thapsigargin, an agent that depletes SR Ca^{2+} content by inhibiting SR Ca^{2+} uptake (control, 40 ± 8% *versus* thapsigargin, 12 ± 5%; $P < 0.05$, $n = 7$). Finally, because PE is known to activate PKC activity we tested the effects of 4 μM chelerythrine, a non-selective PKC inhibitor (Herbert *et al.* 1990). Chelerythrine failed to prevent PE-induced stimulation of $I_{Ca,L}$ (control, 52 ± 8% *versus* chelerythrine, 51 ± 6%). None of the drugs used in the experiments summarized in Fig. 2 had any significant direct effects on basal $I_{Ca,L}$

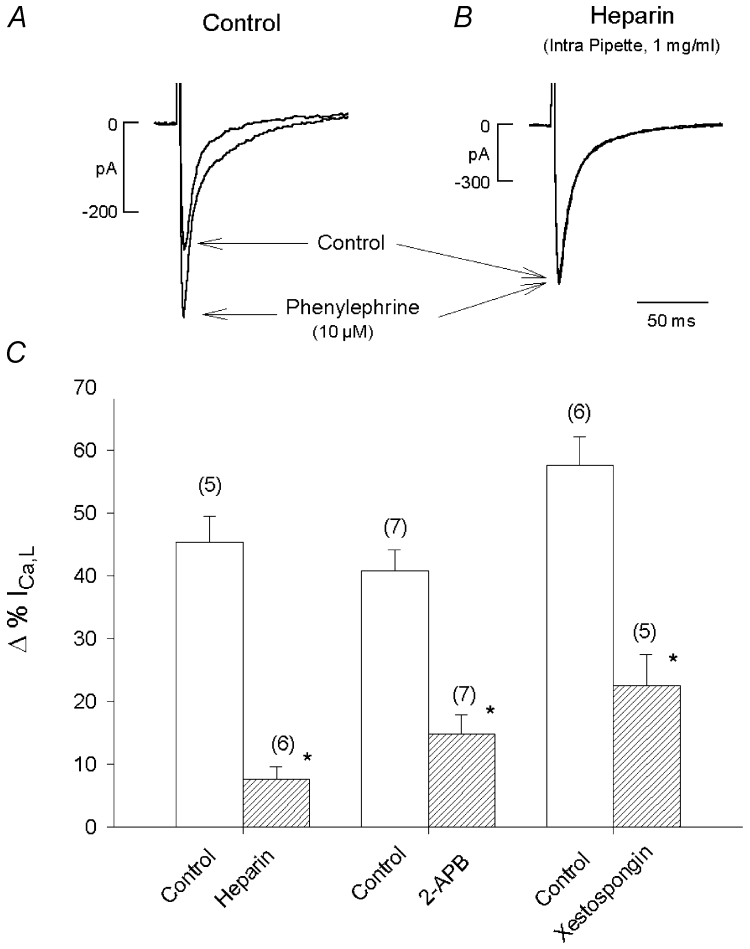

Figure 3. Inhibition of IP_3 signalling inhibits PE-induced stimulation of $I_{Ca,L}$
A, original records showing the effects of PE to stimulate $I_{Ca,L}$ recorded with a ruptured patch method. PE elicited a typical increase in $I_{Ca,L}$ amplitude. *B*, another atrial myocyte in which $I_{Ca,L}$ was recorded during intracellular dialysis of heparin (1 mg ml^{-1}) contained within the pipette solution. PE failed to increase $I_{Ca,L}$ amplitude. In two additional series of experiments, PE was tested in the absence and presence of 2 μM 2-APB or 10 μM xestospongin C (incubation 2 h) using a perforated patch recording method. *C*, summary of the inhibitory effects of heparin, 2 μM 2-APB or 10 μM xestospongin C. Each IP_3 receptor blocking agent significantly inhibited PE-induced stimulation of $I_{Ca,L}$. Numbers in parentheses indicate the number of cells tested in each experiment. *$P < 0.05$.

amplitude. Together, these findings indicate that PE acts via α_1-ARs coupled to PTX-insensitive G-protein and PI-3K signalling to activate Ca^{2+}/CaM-dependent eNOS activity. NO acts via cGMP-mediated inhibition of PDE type III and subsequent stimulation of endogenous cAMP-dependent PKA signalling to stimulate $I_{Ca,L}$, as previously reported (Wang *et al.* 1998). PE does not act via PKC to stimulate $I_{Ca,L}$ in cat atrial myocytes.

The fact that ryanodine and thapsigargin prevented PE-induced stimulation of $I_{Ca,L}$ indicates that Ca^{2+} release from the SR is essential, presumably to stimulate Ca^{2+}-dependent eNOS activity. In addition, PE activates IP_3 signalling and IP_3 receptor (IP_3R) stimulation enhances SR Ca^{2+} release in atrial myocytes (Lipp *et al.* 2000; Mackenzie *et al.* 2002; Zima & Blatter, 2004). We therefore examined the role of IP_3 signalling by testing the effects of PE to stimulate $I_{Ca,L}$ in the absence and presence of three different putative IP_3R blockers: heparin, 2-APB (Maruyama *et al.* 1997) and xestospongin C (Gafni *et al.* 1997). Figure 3*A* and *B* shows original traces of $I_{Ca,L}$ recorded from two different atrial myocytes using a ruptured patch method. In control (Fig. 3*A*), 10 μM PE elicited a typical increase in $I_{Ca,L}$. In Fig. 3*B*, the recording pipette contained 1 mg ml^{-1} heparin, a potent IP_3R blocker. With intracellular dialysis of heparin, PE failed to stimulate $I_{Ca,L}$. As summarized in the bar graph (Fig. 3*C*) PE-induced stimulation of $I_{Ca,L}$ was essentially abolished by heparin (control, 45 ± 4% *versus* heparin, 8 ± 2%; $P < 0.05$). Additional experiments used a perforated (nystatin) patch method to determine the effects of 2 μM 2-APB and 10 μM xestospongin, which are both membrane permeant. Both acute exposure to 2-APB (control, 41 ± 3% *versus* 2-APB, 15 ± 3%; $P < 0.05$) and incubating cells in xestospongin C (2 h) (control, 58 ± 5% *versus* xestospongin C, 23 ± 5%; $P < 0.05$) significantly inhibited PE-induced stimulation of $I_{Ca,L}$. Together, these findings suggest that IP_3-dependent signalling participates in PE-induced stimulation of $I_{Ca,L}$.

The present results suggest that PE acts via NO signalling to stimulate $I_{Ca,L}$. We therefore used fluorescence microscopy and the NO-sensitive indicator DAF-2 to directly measure PE-induced NO_i production. Figure 4*A* shows that PE was unable to release NO_i in quiescent cells, i.e. not field stimulated. In the same cell, exposure to the NO donor spermine/NO (300 μM) significantly increased NO_i. Similar results were obtained in a total of three cells. Figure 4*B* shows that in another atrial myocyte field stimulated at 1 Hz, PE increased NO_i release. The

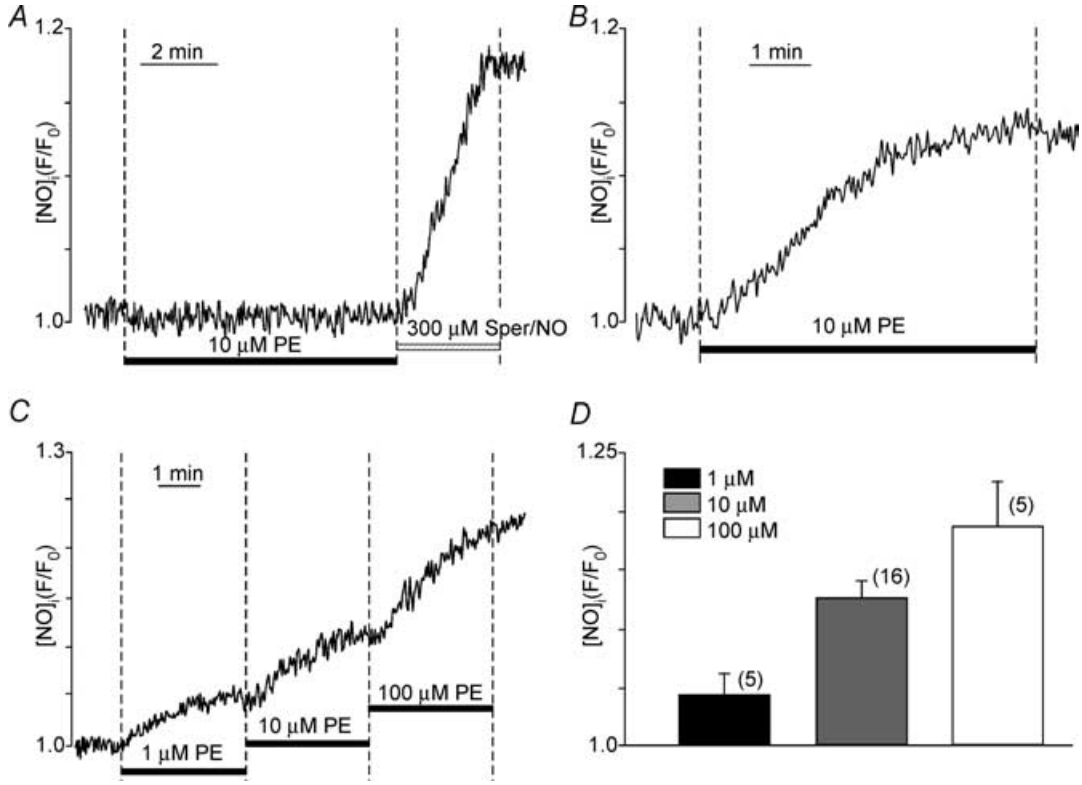

Figure 4. Effects of PE to stimulate NO_i release in atrial myocytes
A; PE (10 μM) is unable to stimulate NO_i release in a quiescent atrial myocyte. Exposure to 300 μM spermine/NO (an NO donor) increases NO_i. *B*; PE (10 μM) stimulates NO_i release in an atrial myocyte field stimulated at 1 Hz. *C*; PE (1, 10, 100 μM) elicits a dose-dependent increase in NO_i release. *D*; summary of dose-dependent PE-induced stimulation of NO_i. The numbers in parentheses indicate the number of cells tested in each experiment.

obligatory requirement of field stimulation suggests the importance of Ca^{2+} influx and/or intracellular Ca^{2+} release for receptor-mediated NO_i production. Figure 4*C* shows that PE elicited a dose-dependent (1, 10, 100 μM) increase in NO_i release in atrial myocytes field stimulated at 1 Hz. Figure 4*C* summarizes the dose-dependent PE-induced stimulation of NO_i release.

Figure 5 summarizes the results of experiments in which we studied the signalling mechanisms underlying PE-induced NO_i production by using many of the same pharmacological agents that we used to study PE-induced stimulation of $I_{Ca,L}$. Again, control and test cells were obtained from the same hearts and control values are grouped together for clarity. Compared with control responses, 10 μM PE-induced NO_i release was abolished by the specific α-AR antagonist prazocin (1 μM) and unaffected by incubating cells in PTX. Non-selective inhibition of β-ARs by 0.1 μM propranolol did not affect PE-induced NO_i release ($n = 3$; data not shown). As expected, PE-induced NO_i production was abolished by inhibition of eNOS with 10 μM L-NIO and by inhibition of CaM by 10 μM W-7. Additionally, inhibition of PI-3K signalling by 10 μM LY294002 significantly inhibited PE-induced NO_i production. In the presence of either L-NIO or LY294002, 100 μM spermine/NO elicited a typical increase in DAF-2 fluorescence, indicating that neither L-NIO nor LY294002 blocked PE-induced stimulation of NO_i by somehow interfering with DAF-2 fluorescence (data not shown). These findings indicate that PE acts via α_1-ARs coupled to PI-3K signalling to stimulate Ca^{2+}/CaM-dependent eNOS and the production of NO_i. Moreover, the fact that interventions which inhibit PE-induced NO_i release also inhibit PE-induced stimulation of $I_{Ca,L}$ strongly suggests that NO_i release is responsible for stimulation of $I_{Ca,L}$. This is consistent with reports that exogenous NO stimulates $I_{Ca,L}$ in both human (Kirstein *et al.* 1995) and cat (Wang *et al.* 1998) atrial myocytes.

Does NO-induced stimulation of $I_{Ca,L}$ (and subsequent CICR) elicited by PE contribute to further stimulation of NO_i production? The present work as well as our previous studies (Wang *et al.* 1998) indicates that NO stimulates $I_{Ca,L}$ via cGMP/cAMP-dependent PKA activity. We therefore determined the effects of PE to stimulate NO_i when NO-mediated stimulation of $I_{Ca,L}$ is blocked by 2 μM H-89. As summarized in Fig. 5, inhibition of cAMP-dependent PKA significantly attenuated (−51.8% of control) but did not prevent PE-induced stimulation of NO_i. These findings suggest that PE elicits the release of NO_i, which acts via cAMP/PKA signalling to stimulate $I_{Ca,L}$ (and CICR), which in turn stimulates additional Ca^{2+}-dependent eNOS/NO_i production. As described below, the PE-induced release of NO_i which is independent of cAMP/PKA signalling, is mediated via IP_3-dependent Ca^{2+} signalling.

In endothelial cells, PI-3K acts via phosphorylation of Akt (protein kinase B) to activate eNOS activity (Fulton *et al.* 1999). We therefore determined whether PE phosphorylates Akt via a PI-3K-dependent mechanism

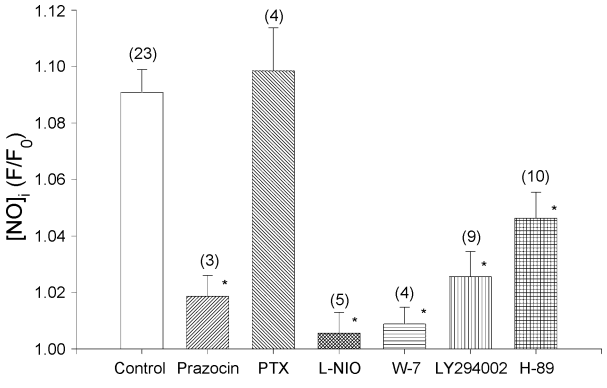

Figure 5. Pharmacological analysis of the signalling mechanisms responsible for PE-induced stimulation of NO_i release
Each experiment was performed by testing PE in the absence (control) and presence (test) of each drug on myocytes isolated from the same hearts. The control values from each experiment ($n = 23$) are grouped together for clarity. Compared with control (open bar) prazocin (1 μM), L-NIO (10 μM), W-7 (10 μM) and LY294002 (10 μM) each significantly inhibited PE-induced stimulation of NO_i release. H-89 (2 μM) blocked approximately 50% of PE-induced NO_i production. Incubation of cells in PTX had no effect on PE-induced NO_i production. The numbers in parentheses indicate the number of cells tested in each experiment. *$P < 0.05$.

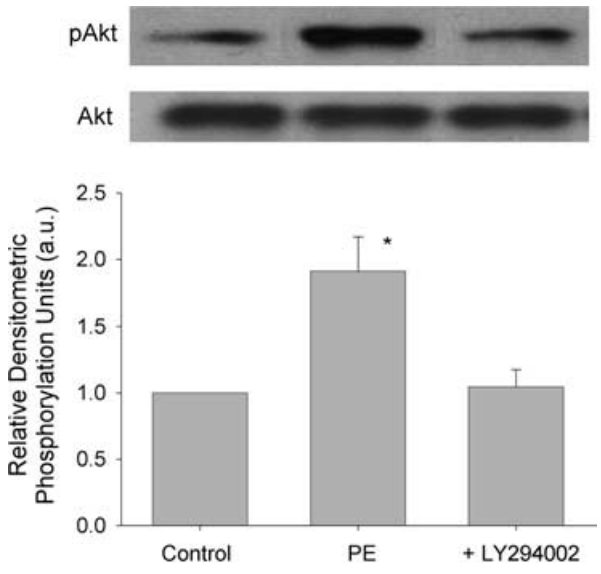

Figure 6. PE acts via PI-3K-dependent signalling to phosphorylate Akt
The Western blots show phosphorylated (pAkt; Ser^{473}) (upper) and total (lower) Akt in the absence and presence of 10 μM LY294002. Compared with control 10 μM PE significantly increased phosphorylation of Akt. Prior exposure to 10 μM LY294002 blocked PE-induced Akt phosphorylation. The graph summarizes the normalized data obtained in 9 separate experiments. *$P < 0.05$.

in atrial myocytes. The Western blots in Fig. 6 show that 10 μM PE significantly increased phosphorylation of Akt (Ser^{473}) 1.7 ± 0.1-fold compared with control (normalized to 1) and that 10 μM LY294002 blocked PE-induced phosphorylation of Akt. The graph shows mean ± S.E.M. values obtained in nine experiments. These results, together with those presented in Fig. 5, suggest that PE-induced activation of PI-3K/Akt signalling is required for stimulation of NO_i production.

The present findings indicate that PE-induced stimulation of $I_{Ca,L}$ is dependent on NO_i release and IP_3 signalling. Therefore, we next determined whether PE-induced NO_i release is dependent on IP_3 signalling. Figure 7*A* shows recordings from three different atrial myocytes where compared with control responses, PE (10 μM)-induced stimulation of NO_i was significantly inhibited by 10 μM xestospongin C and abolished by 2 μM 2-APB. In another atrial myocyte (Fig. 7*B*), prior exposure to 10 μM ryanodine also abolished PE-induced NO_i release. Similar experiments using 5 μM thapsigargin to deplete SR Ca^{2+} stores inhibited PE-induced NO_i production by 80% (control, 1.091 ± 0.008 *versus* thapsigargin, 1.018 ± 0.007; $P < 0.001$, $n = 6$). Figure 7*D* summarizes the effects of 2-APB, xestospongin C, and ryanodine compared with control responses (filled bar) to10 μM PE. Together, these results suggest that PE-induced NO_i release is dependent on IP_3-mediated Ca^{2+} signalling. Moreover, because similar interventions blocked PE-induced stimulation of $I_{Ca,L}$ they further support the idea that PE acts via NO_i signalling to stimulate $I_{Ca,L}$. It may be argued that 2-APB (Bootman *et al.* 2002) as well as xestospongin C or heparin exerts non-specific effects. However, in permeabilized cat atrial myocytes heparin or 2-APB prevented direct IP_3-induced increases in basal intracellular $[Ca^{2+}]$ and Ca^{2+} spark frequency (Zima & Blatter, 2004).

In Fig. 8 we used confocal fluorescence microscopy to directly visualize the spatial pattern of NO_i release induced by PE. In Fig. 8*A*, compared with control, after 3 min of exposure to 10 μM PE, NO_i was increased primarily along the cell periphery. After 5 min of PE exposure additional NO_i release sites along the periphery are evident, as are smaller increases of NO_i within the cell interior. This spatial pattern of NO_i release

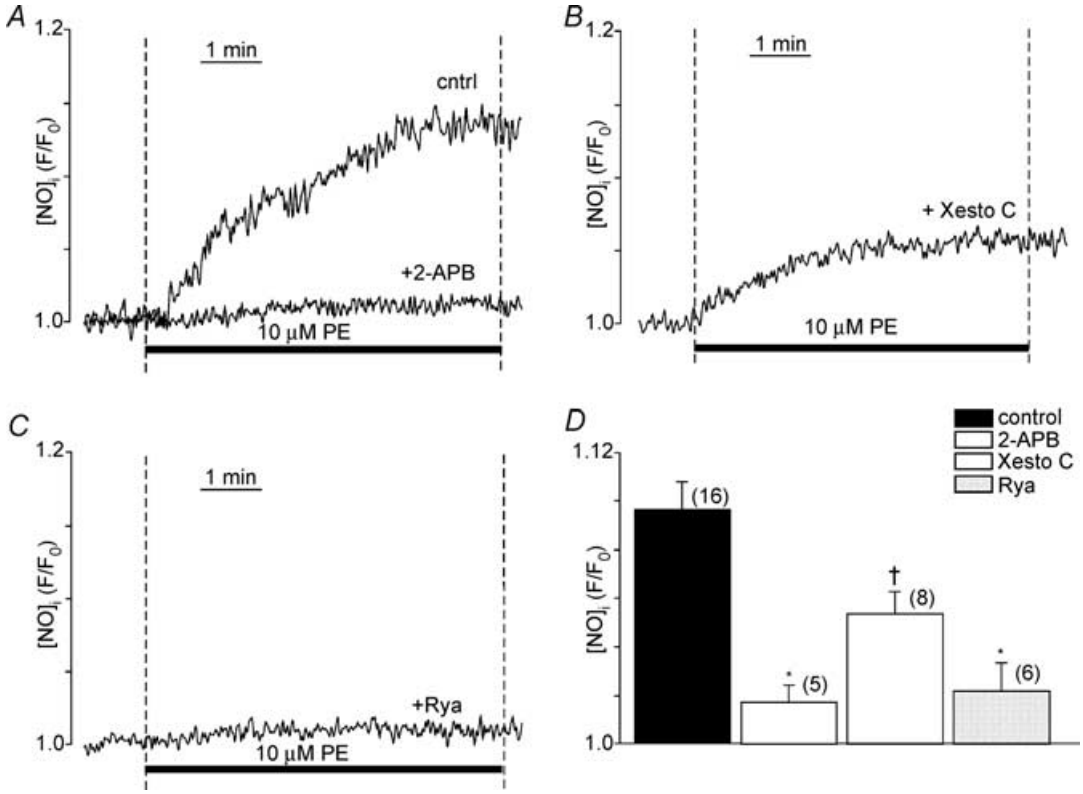

Figure 7. Inhibition of IP_3 receptor signalling or SR Ca^{2+} release inhibits PE-induced stimulation of NO_i
A and *B*, compared to control (cntrl) PE (10 μM)-induced stimulation of NO_i release was blocked by 2 μM 2-APB (*A*) and markedly inhibited by 2 h incubation in 10 μM xestospongin C (+ Xesto C) (*B*). *C*, prior exposure to 10 μM ryanodine blocked PE-induced stimulation of NO_i release. *D*, summary of the effects of 2-APB, xestospongin C, and ryanodine compared to control (filled bar) effects of PE to increase NO_i. The numbers in parentheses indicate that number of cells tested in each experiment. *$P < 0.01$; †$P < 0.05$.

was evident in a total of five atrial myocytes. In cardiac cells, eNOS is localized within caveolae (Feron *et al.* 1996). We therefore determined whether PE acts to release NO_i from caveolae by testing PE in cells previously incubated (1 h) in 2 mM methyl-β-cyclodextrin (cyclodextrin), an agent that disrupts caveolae formation (Smart & Anderson, 2002). As shown in Fig. 8*B*, PE failed to increase NO_i release in cyclodextrin-treated cells. Similar results were obtained in five atrial myocytes. These findings indicate that PE acts via IP_3-mediated Ca^{2+} release to stimulate NO_i production from subsarcolemmal regions.

The previous findings raise the question of whether PE increases subsarcolemmal SR Ca^{2+} release that is mediated via IP_3 signalling. We therefore used confocal laser scanning microscopy to determine whether PE increases local subsarcolemmal SR Ca^{2+} release, i.e. Ca^{2+} sparks, and whether this response is sensitive to inhibition of IP_3R signalling. A repetitively scanned line was positioned parallel with the longitudinal axis of quiescent atrial myocytes within the subsarcolemmal space. Because the cells are quiescent, PE is unable to release NO_i (see Fig. 4*A*). Therefore any increase in subsarcolemmal SR Ca^{2+} release cannot be due to NO-mediated stimulation of Ca^{2+} influx via stochastic opening of $I_{Ca,L}$ channels. In Fig. 9 each panel shows confocal line scan images of spontaneous subsarcolemmal Ca^{2+} spark activity and the traces below each panel show local subcellular changes in $[Ca^{2+}]_i$ within the subsarcolemmal space that correspond with the positions of the arrows at the left margin of each panel. Compared with control (Fig. 9*Aa*), exposure to 10 μM PE (2 min) (Fig. 9*Ab*) increased subsarcolemmal Ca^{2+} spark activity and raised baseline $[Ca^{2+}]_i$. In another atrial myocyte, compared with control (Fig. 9*Ba*), exposure to 2 μM 2-APB (Fig. 9*Bb*) had little effect on Ca^{2+} spark activity and prevented PE-induced increases in Ca^{2+} sparks (Fig. 9*Bc*). The graphs (Fig. 9*C* and *D*) summarize the results normalized to control. PE significantly increased Ca^{2+} spark frequency (170%) (Fig. 9*C*) and 2-APB prevented PE-induced stimulation of Ca^{2+} sparks (Fig. 9*D*). Although PE raised baseline $[Ca^{2+}]_i$ in some cells, the change did not reach statistical significance. These findings indicate that in cat atrial myocytes, PE increases subsarcolemmal SR Ca^{2+} release mediated by IP_3-dependent signalling.

Together, the present findings suggest that IP_3-dependent SR Ca^{2+} signalling mediates activation of eNOS contained within caveolae. Figure 10*A* shows an electron micrograph of a cat atrial myocyte sectioned longitudinally. The cell was sectioned just below the sarcolemma and reveals sarcomere units (Z line) and an

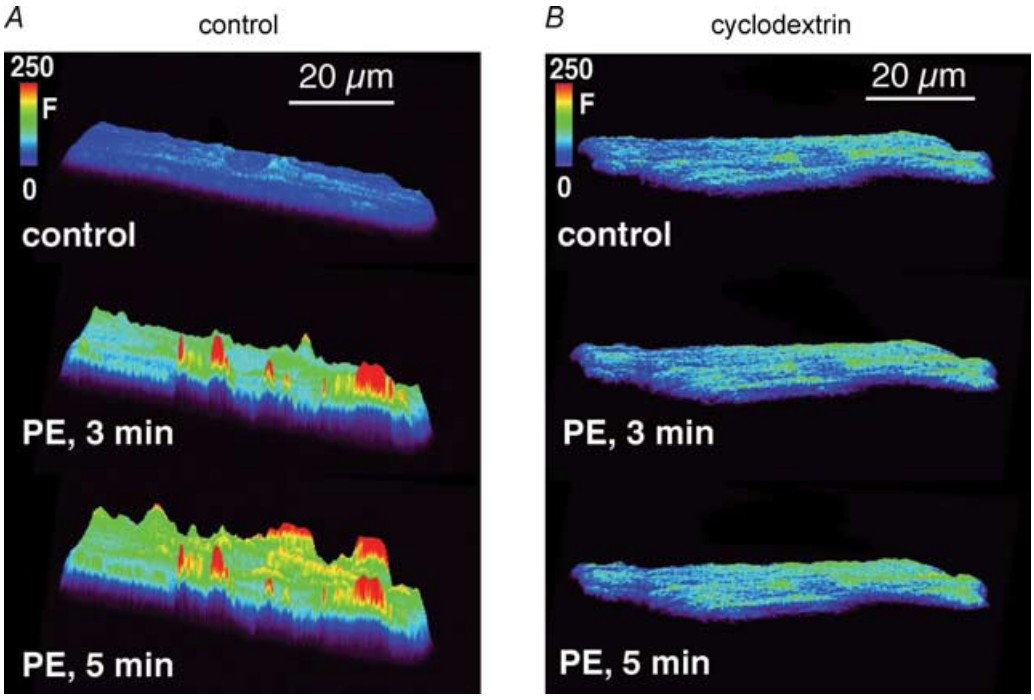

Figure 8. 2-Dimensional surface plots obtained from atrial myocytes showing the spatial patterns of NO_i production induced by 10 μM PE

A, compared with control, after 3 min of exposure to PE, NO_i production increased at discrete sites primarily along the cell periphery. At 5 min of PE exposure NO_i production along the cell periphery was further increased at additional sites along the cell periphery with smaller increases of NO_i within the cell interior. *B*, another atrial myocyte was incubated (1 h) in 2 mM methyl-β-cyclodextrin (cyclodextrin) to disrupt caveolae formation. Exposure to 10 μM PE failed to increase NO_i.

extensive network of longitudinal sarcoplasmic reticulum (lsr) throughout the cell interior that is connected to numerous electron-dense terminal SR cisternae located at intervals within the subsarcolemmal space. These terminal cisternae are the peripheral SR Ca^{2+} release sites. The subsarcolemmal space also contains numerous caveolae. The inset shown in Fig. 10*A* is magnified in Fig. 10*B* and shows that closely interspersed between each terminal SR (tsr) Ca^{2+} release site are grape-like clusters of caveolae (Cav), the sites of eNOS and NO_i release. This ultrastructural architecture is compatible with localized NO_i release along the cell periphery that is mediated by local SR Ca^{2+} release.

Discussion

The present study shows, for the first time, that PE stimulates NO_i release in atrial myocytes. PE-induced release of NO_i required that atrial cells be electrically stimulated. This is similar to our previous findings in which NO_i release induced by β_2-AR (Wang *et al.* 2002; Dedkova *et al.* 2002) or muscarinic (Dedkova *et al.* 2003) receptor stimulation also required that atrial myocytes be electrically stimulated. In beating neonatal or quiescent adult rat ventricular myocytes PE has no effect on NO_i release (Kanai *et al.* 1997). As demonstrated previously (Dedkova *et al.* 2003), cat atrial cells require voltage-activated Ca^{2+} influx for receptor-mediated stimulation of NO_i production. However, as reported here and in our previous studies (Dedkova *et al.* 2002; Dedkova *et al.* 2003), basal Ca^{2+} influx and/or SR Ca^{2+} release elicited by electrical stimulation alone (without receptor stimulation) is not sufficient to stimulate NO_i release. This is in contrast to ventricular myocytes in which electrical stimulation is sufficient to release NO_i (Kaye *et al.* 1996; Dedkova *et al.* 2004). In the present study, ryanodine or thapsigargin, agents that interfere with SR Ca^{2+} release, inhibited PE-induced stimulation of both NO_i release and

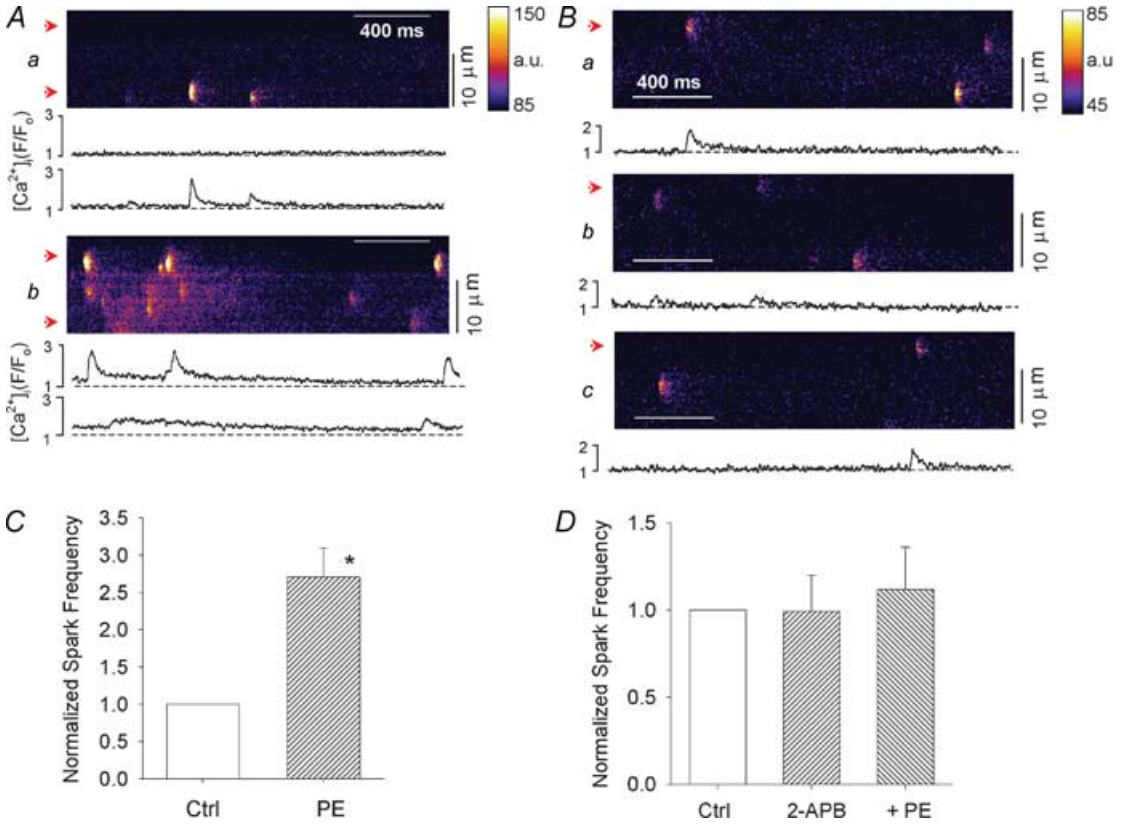

Figure 9. Confocal laser linescan images of local SR Ca^{2+} release, i.e. Ca^{2+} sparks, recorded from quiescent atrial myocytes

Each panel shows confocal line scan images of spontaneous subsarcolemmal Ca^{2+} spark activity. The traces below each panel show local subcellular changes in $[Ca^{2+}]_i$ within the subsarcolemmal space that correspond with the arrows at the left margin of each panel. *A*, compared with control (*a*), PE (10 μM) increased Ca^{2+} spark activity (*b*). *B*, compared with control (*a*), 2 μM 2-APB had little effect on Ca^{2+} spark activity (*b*) but blocked PE-induced increases in Ca^{2+} sparks (*c*). *C*, normalized Ca^{2+} spark frequency (sparks s^{-1} (100 μm)$^{-1}$); compared with control (Ctrl) PE significantly increased Ca^{2+} spark frequency (170%; $n = 6$). *D*, compared with control (Crtl), 2-APB blocked the effect of PE to increase Ca^{2+} spark frequency ($n = 9$). $^*P < 0.05$.

$I_{Ca,L}$. Because neither drug inhibits basal $I_{Ca,L}$ amplitude, i.e. Ca^{2+} influx, the present findings indicate that SR Ca^{2+} release and not Ca^{2+} influx *per se* is the critical Ca^{2+} source for PE-induced activation of Ca^{2+}-dependent eNOS activity.

In atrial myocytes, α_1-AR stimulation mediates IP_3 production and IP_3 enhances SR Ca^{2+} release (Nosek *et al.* 1986; Fabiato, 1986; Zima & Blatter, 2004). The present results indicate, for the first time, that PE-induced stimulation of NO_i and subsequent stimulation of $I_{Ca,L}$ are dependent on IP_3 signalling. Moreover, PE stimulates local subsarcolemmal SR Ca^{2+} release, i.e. Ca^{2+} sparks, through IP_3-dependent signalling. These findings are consistent with reports that in atrial myocytes IP_3 stimulates SR Ca^{2+} release and may enhance Ca^{2+}-induced Ca^{2+} release (CICR) (Lipp *et al.* 2000; Mackenzie *et al.* 2002; Zima & Blatter, 2004) and that IP_3Rs are colocalized with subsarcolemmal SR Ca^{2+} release channels, i.e. ryanodine receptors (Lipp *et al.* 2000; Mackenzie *et al.* 2002). On the other hand, stimulation of rat atrial myocytes with endothelin or exposure to membrane-permeant IP_3 increased SR Ca^{2+} release more in central than peripheral regions of the cell (Mackenzie *et al.* 2004).

In cardiac cells, eNOS (Feron *et al.* 1996) as well as α_1-ARs, G_q and PLC are colocalized with caveolin-3 within caveolae (Fujita *et al.* 2001). As shown in the present experiments, PE activated local release of NO_i from subsarcolemmal sites and this local NO_i release was prevented by disruption of caveolae formation. These findings are similar to our previous study in which ACh exposure and withdrawal increase NO_i release from subsarcolemmal caveolae sites in atrial myocytes (Dedkova *et al.* 2003). Moreover, our electron micrographs clearly show that the ultrastructure of cat atrial myocytes exhibit peripheral SR Ca^{2+} release sites located in close proximity to abundant subsarcolemmal caveolae. Together, the present findings suggest that IP_3-dependent Ca^{2+} signalling mediates NO_i release from subsarcolemmal caveolae sites.

Several of the present findings, however, make it unlikely that Ca^{2+} released via IP_3Rs directly stimulates eNOS/NO_i production. First, in cat atrial myocytes IP_3R-dependent Ca^{2+} release events are 75–80% smaller in amplitude and their rise time is approximately 2-fold longer than average Ca^{2+} spark events (Zima & Blatter, 2004). Moreover, in quiescent atrial cells PE stimulates both PI-3K/Akt signalling and local IP_3-dependent SR Ca^{2+} release, yet fails to stimulate NO_i production. In addition, PE-induced NO_i release requires voltage-activated Ca^{2+} influx and SR Ca^{2+} release. Finally, a significant portion of PE-induced NO_i production is stimulated by cAMP/PKA signalling, indicative of a critical role for CICR. It therefore seems likely that IP_3-dependent Ca^{2+} signalling acts indirectly by enhancing release of a localized SR Ca^{2+} pool that is stimulated by extracellular Ca^{2+} influx via $I_{Ca,L}$ (CICR) and targeted for Ca^{2+}-dependent eNOS activity in subsarcolemmal caveolae sites. Speculation leads to the possibility that the voltage-activated Ca^{2+} influx channel also may be localized to caveolae

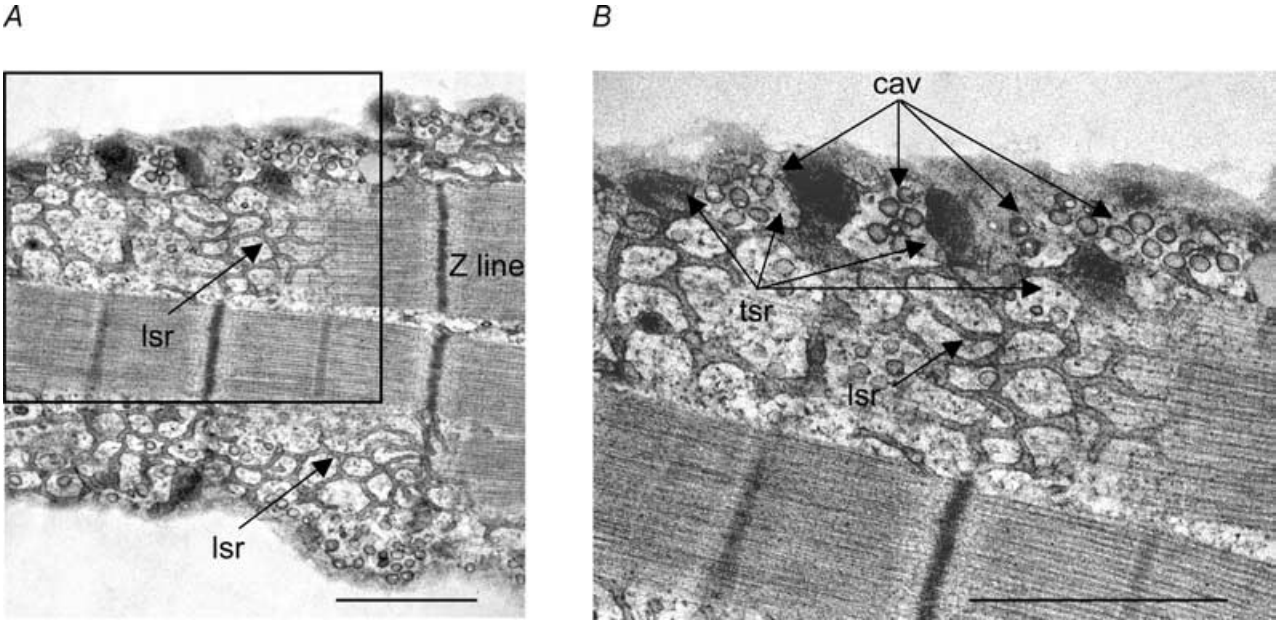

Figure 10. Electron micrographs of an atrial myocyte cut parallel with the longitudinal axis of the cell
A, micrograph shows sarcomere units (Z lines) and an extensive network of longitudinal sarcoplasmic reticulum (lsr) throughout the cell interior. *B*, higher magnification of inset in *A* shows that the longitudinal sarcoplasmic reticulum is connected to electron dense regions at the cell periphery which are terminal SR (tsr) Ca^{2+} release sites. Between each terminal SR Ca^{2+} release site are grape-like clusters of caveolae (Cav). Calibration bars = 1.0 μm.

closely apposed to ryanodine receptors (Löhn *et al.* 2000).

In the present study, PE-induced stimulation of both $I_{Ca,L}$ and NO_i release were PTX-insensitive, consistent with coupling of α_1-ARs to G_q signalling. In fact, PE-induced stimulation of $I_{Ca,L}$ was somewhat enhanced in cells incubated in PTX, suggesting that G_i may, to some extent, modulate α_1-AR stimulation. PKC signalling is not involved in PE-induced stimulation of either $I_{Ca,L}$ or NO_i release in cat atrial myocytes. This is in contrast to findings in guinea pig (Woo & Lee, 1999) and rat (Zhang *et al.* 1998) ventricular myocytes in which PE increases $I_{Ca,L}$ via PKC signalling. Moreover, in the present study PE elicited only stimulation of $I_{Ca,L}$, in contrast to rat ventricular myocytes where PE elicited a biphasic effect: an initial inhibition followed by a sustained stimulation of $I_{Ca,L}$ (Zhang *et al.* 1998).

PE-induced stimulation of $I_{Ca,L}$ and NO_i release were each inhibited by LY294002 and therefore dependent on PI-3K signalling. PI-3K signalling leads to phosphorylation of Akt (Brazil & Hemmings, 2001) which in turn activates eNOS (Fulton *et al.* 1999) and NO_i production. Indeed, the present findings show that PE increases Akt phosphorylation through a PI-3K-dependent mechanism. It is important to note that the Western blot experiments were performed on quiescent atrial myocytes treated with PE, i.e. in the absence of voltage-activated Ca^{2+} influx or release. This is consistent with reports that stimulation of Akt is Ca^{2+} independent (Conus *et al.* 1998). Therefore, the present results indicate that α_1-AR stimulation of eNOS activity, i.e. NO_i production, requires activation of both Ca^{2+}-independent PI-3K/Akt signalling and Ca^{2+}-dependent CaM signalling pathways. This dual signalling mechanism is similar to that responsible for muscarinic receptor stimulation of NO_i release (Dedkova *et al.* 2003). In cat atrial myocytes both β_2-ARs (Wang *et al.* 2002) and muscarinic receptors (Dedkova *et al.* 2003) act via PTX-sensitive G_i to release of NO_i via PI-3K-dependent signalling. The present findings indicate that G_q also mediates activation of PI-3K signalling in atrial myocytes. This is consistent with reports in a variety of cell systems that receptor-coupled G_q activates PI-3K signalling (Murga *et al.* 1998; Graness *et al.* 2005; Xie *et al.* 2005).

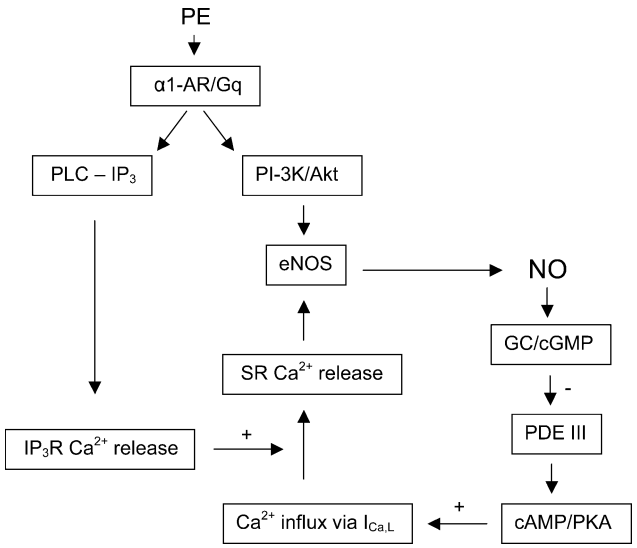

Figure 11. Proposed signalling mechanisms underlying PE-induced stimulation of $I_{Ca,L}$ via NO signalling
PE acts via α_1-ARs coupled to G_q, presumably localized within caveolae, to activate both PLC – IP_3 and PI-3K/Akt signalling. IP_3 stimulates SR Ca^{2+} release via IP_3Rs. IP_3-mediated Ca^{2+} signalling enhances local SR Ca^{2+} release triggered by Ca^{2+} influx via $I_{Ca,L}$. In conjunction with receptor-mediated stimulation of PI-3K/Akt signalling, SR Ca^{2+} release activates CaM-dependent eNOS to stimulate NO_i production. NO acts via cGMP-induced inhibition of PDE III to stimulate endogenous cAMP-dependent PKA activity. cAMP/PKA stimulates Ca^{2+} influx via $I_{Ca,L}$ which in turn enhances Ca^{2+}-mediated stimulation of eNOS and NO_i production. Steady-state stimulation of NO_i production may be determined by the dose-dependent receptor-mediated stimulation of PI-3K/Akt/eNOS signalling.

The present study also shows that PE-induced stimulation of $I_{Ca,L}$ was blocked by inhibition of guanylate cyclase or inhibition of cAMP-dependent PKA activity. This is consistent with NO acting via cGMP-inhibited PDE type III to raise endogenous cAMP-dependent PKA activity (Kirstein *et al.* 1995; Wang *et al.* 1998). The negative shift in maximum $I_{Ca,L}$ activation voltage elicited by PE also is explained by cAMP/PKA signalling. In addition, PE-induced NO_i release was decreased by approximately 50% by inhibition of cAMP-dependent PKA (H-89) while it was abolished by inhibition of IP_3R signalling (2-APB). These findings suggest that PE acts initially via IP_3-dependent Ca^{2+} signalling to indirectly stimulate NO_i production, and NO in turn acts via cAMP/PKA signalling to stimulate $I_{Ca,L}$ and CICR to further increase NO_i production. Because NO signalling acts locally to regulate ion channel function (Dittrich *et al.* 2001; Wang *et al.* 2002; Dedkova *et al.* 2002) the effects of α_1-AR stimulation may not correlate with changes in cellular cAMP levels (Schumann *et al.* 1975; Brodde *et al.* 1978; Bogoyevitch *et al.* 1993).

In rat atrial muscle, PE increases Ca^{2+} uptake possibly in part through secondary changes in Na^+/Ca^{2+} exchange (Jahnel *et al.* 1991, 1992*b*, 1994). A proposed mechanism involves PE-induced depolarization and increases in TTX-sensitive Na^+ influx (Jahnel *et al.* 1991, 1992*b*). It may therefore be argued that Ca^+ influx via Na^+/Ca^{2+} exchange contributes to PE-induced stimulation of Ca^{2+}-dependent NO_i production. However, any contribution by Na^+/Ca^{2+} exchange is unlikely or at best very small. In the present study, the voltage clamp method used to analyse $I_{Ca,L}$ precludes any increase in TTX-sensitive Na^+ influx induced by PE. Moreover, the contribution of Ca^+ influx via Na^+/Ca^{2+} exchange is a small fraction of total Ca^{2+} influx (Weber *et al.* 2002). The fact that PE-induced NO_i

release is significantly stimulated by cAMP/PKA signalling also makes it unlikely that Ca^{2+} influx via Na^+/Ca^{2+} exchange is a primary mechanism. Finally, PE-induced NO_i production is strongly dependent on SR Ca^{2+} release. However, Ca^{2+} influx via the exchanger is a very inefficient trigger for stimulating SR Ca^+ release (Sipido *et al.* 1997).

We therefore propose the scheme shown in Fig. 11 to explain the effects of PE to stimulate $I_{Ca,L}$ in cat atrial myocytes. PE acts via α_1-ARs coupled to G_q to stimulate two signalling pathways: PI-3K/Akt and PLC-induced IP_3 signalling. In conjunction with stimulation of PI-3K/Akt signalling, IP_3-mediated Ca^{2+} release via IP_3Rs enhances CICR from a localized SR Ca^{2+} pool that targets stimulation of Ca^{2+}/CaM-dependent eNOS contained within caveolae. The resulting increase in NO_i release acts via GC/cGMP-induced inhibition of PDE III to enhance endogenous cAMP/PKA stimulation of $I_{Ca,L}$. Stimulation of Ca^{2+} influx via $I_{Ca,L}$ stimulates CICR to further stimulate eNOS/NO_i production. This positive feedback reaches steady-state presumably by the dose-dependent stimulation of PI-3K/Akt signalling. There also may be negative feedback mechanisms by various phosphatases not considered in the present experiments.

Finally, numerous reports which have studied parasympathetic nerve-induced stimulation of NO_i release *in vivo* use PE as a pressor to elicit baroreflex responses. The assumption is that PE does not directly release NO_i. The present study now indicates that these types of studies need to consider the possible direct effects of PE to induce NO_i release from atrial muscle.

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