Signaling Mechanisms That Mediate Nitric Oxide Production Induced by Acetylcholine Exposure and Withdrawal in Cat Atrial Myocytes

Elena N. Dedkova, Xiang Ji, Yong Gao Wang, Lothar A. Blatter, Stephen L. Lipsius

Abstract—Fluorescence microscopy and the NO-sensitive indicator 4,5-diaminofluorescein were used to determine the effects of acetylcholine (ACh) on intracellular NO (NO_i) in cat atrial myocytes. Field stimulation (1 Hz) of cells or exposure of quiescent cells to ACh (1 to 10 μmol/L) had no effect on NO_i. However, in field-stimulated cells, ACh exposure increased NO_i and ACh withdrawal elicited an additional, prominent increase in NO_i production. During ACh exposure, addition of 1 μmol/L atropine increased NO_i production similar to ACh withdrawal. ACh-induced increases in NO_i were reduced by prior exposure to 1 mmol/L extracellular Ca^{2+} (\([\mathrm{Ca}^{2+}]_\text{o}\)) and prevented by 0.5 mmol/L [Ca^{2+}]_i, 1 μmol/L verapamil, 1 μmol/L atropine, 10 μmol/L L-N(1-iminoethyl)ornithine, 10 μmol/L W-7, or incubating cells in pertussis toxin or 10 μmol/L LY294002 (inhibits phosphatidylinositol 3-kinase). Switching to 0.5 mmol/L [Ca^{2+}]_i during ACh withdrawal prevented the additional increase in NO_i. ACh exposure increased phosphorylation (Ser\(^{\text{473}}\)) of protein kinase B (Akt), and this effect was blocked by LY294002 and unaffected in low (0.5 mmol/L) [Ca^{2+}]_i. Confocal microscopy revealed that ACh exposure increased NO_i at local subsarcolemmal sites, and ACh withdrawal additionally increased NO_i by recruiting additional subsarcolemmal release sites. Disruption of caveolae by 2 mmol/L methyl-β-cyclodextrin abolished ACh-induced NO_i production. We conclude that in cat atrial myocytes, ACh stimulates NO_i from local subsarcolemmal sites, and ACh withdrawal additionally increased NO_i by recruiting additional subsarcolemmal release sites. Disruption of caveolae by 2 mmol/L methyl-β-cyclodextrin abolished ACh-induced NO_i production.

Key Words: phosphatidylinositol 3-kinase \(\bullet\) protein kinase B/Akt \(\bullet\) calmodulin \(\bullet\) calcium

Several studies indicate that NO signaling mediates the inhibitory effects of acetylcholine (ACh) on cardiac function.\(^{1-3}\) These findings, however, have been disputed\(^{1-6}\) and remain controversial.\(^{7}\) In cat atrial myocytes, ACh-induced inhibition of basal L-type Ca^{2+} current (\(I_{\text{Ca,L}}\)) is not mediated by NO signaling.\(^{8}\) However, ACh withdrawal stimulates \(I_{\text{Ca,L}}\), above control levels, i.e., rebound stimulation, and this response is mediated by NO signaling.\(^{8,9}\) The rebound stimulation of \(I_{\text{Ca,L}}\), elicited by ACh withdrawal results in stimulation of atrial contraction,\(^{8}\) atrial pacemaker activity,\(^{10}\) and the potential development of Ca^{2+}-mediated delayed afterdepolarizations and arrhythmic atrial activity.\(^{11}\) These findings are consistent with reports in multicellular atrial preparations that ACh withdrawal elicits rebound stimulation of intracellular Ca^{2+} transients and contraction.\(^{12}\) The fact that rebound stimulation is exhibited by multicellular tissue indicates that the stimulatory response to ACh withdrawal is not unique to isolated myocytes but rather is a physiological mechanism responsible for rapid recovery from cholinergic inhibition of atrial function. In both cat\(^8\) and human\(^{13}\) atrial myocytes, NO acts via cyclic GMP (cGMP)-induced inhibition of phosphodiesterase type III activity to increase endogenous cAMP levels. By this mechanism, NO signaling mediates the stimulation of \(I_{\text{Ca,L}}\), elicited by ACh withdrawal.\(^{8}\) These findings are consistent with studies in chick heart cells, in which ACh withdrawal stimulates cAMP above control levels.\(^{14}\) Others laboratories have reported that ACh withdrawal stimulates \(I_{\text{Ca,L}}\) in Purkinje fibers\(^{15}\) and ventricular myocytes prestimulated by \(\beta\)-adrenergic agonists.\(^{16,17}\) However, in contrast to atrial myocytes, the stimulatory effect of ACh withdrawal in ventricular myocytes is not mediated via NO or cGMP signaling\(^{16,18}\) but rather is attributable to stimulation of adenylate cyclase by the \(\beta\gamma\) subunit of G protein.\(^{16}\)

In the present study, we used fluorescence microscopy and the NO-sensitive indicator DAF-2 to directly determine the effects of ACh on intracellular NO (NO_i) production in cat atrial myocytes. We also sought to determine whether stim-
ulation of muscarinic receptors increases NO production via G proteins coupled to phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (Akt) signaling, similar to NO production elicited by β2-adrenergic receptor (AR) stimulation. The present results indicate that ACh exposure and withdrawal increase NO production. ACh-induced increases in NO require both muscarinic receptor–mediated G/PI-3K/Akt signaling and voltage-activated Ca2+ influx for stimulation of calmodulin (CaM)-dependent endothelial NO synthase (eNOS) activity. Moreover, ACh withdrawal increases NO production above that induced during ACh exposure, consistent with the role of NO signaling in rebound stimulation of \( I_{\text{Ca,L}} \).

**Materials and Methods**

Adult cats of either sex were anesthetized with sodium pentobarbital (50 mg/kg IP). Once anesthetized, the heart was rapidly excised and mounted on a Langendorff perfusion apparatus. Atrial myocytes were dispersed by enzymatic (collagenase; type II, Worthington Biochemical) digestion, as previously reported. Measurements of NO were obtained by incubating cells with the fluorescent NO-sensitive dye 4,5-diaminofluorescein (DAF-2), as previously described (for additional details, see the online data supplement, available at http://www.circresaha.org). Experiments were performed at room temperature. Changes in cellular DAF-2 fluorescence intensities (F) were normalized to the level of fluorescence obtained at room temperature. Changes in [NO]i are expressed as 

\[ \frac{F}{F_0} \]

where \( F_0 \) is the fluorescence intensity in the absence of stimulation. The fluorescence intensity remains constant even if NO levels decrease. In a few experiments, perforated patch recording methods were used to measure electrical activity of atrial myocytes, as previously described.

Two-dimensional (2D) and fast one-dimensional (linescan) imaging was performed using a confocal scanning unit (LSM 410, Zeiss) attached to an inverted microscope (Axiovert 100; Zeiss) fitted with a 40 oil-immersion objective lens (Plan-Neofluar, numerical aperture = 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 ion laser, and emitted fluorescence was collected at wavelength >515 nm. For linescan imaging, the specimen was scanned repetitively at 5-ms intervals. All linescan images were recorded at a central focal plane and oriented along the longitudinal axis of the cell within the subsarcolemmal region. Increases in NO recorded by linescan were quantified by measuring the frequency and amplitude of NO peaks. NO peaks were defined as those changes in NOi that reached 50% above baseline (F/Fo).

**Results**

Figure 1A shows a typical recording of NOi production obtained from a field-stimulated (+FS, 1 Hz) atrial myocyte and a second quiescent (−FS) atrial myocyte exposed to 1 μmol/L ACh. In the field-stimulated cell, ACh exposure elicited an increase in NOi, and withdrawal of ACh elicited an additional, prominent increase in NOi. A similar response was observed when NOi was obtained by incubating cells with the fluorescent NO-dye (DAF-2), as described above. DAF-2 fluorescence was excited with a 488-nm line of an argon ion laser, and emitted fluorescence was collected at wavelength >515 nm. For linescan imaging, the specimen was scanned repetitively at 5-ms intervals. All linescan images were recorded at a central focal plane and oriented along the longitudinal axis of the cell within the subsarcolemmal region. Increases in NO recorded by linescan were quantified by measuring the frequency and amplitude of NO peaks. NO peaks were defined as those changes in NOi that reached 50% above baseline (F/Fo).

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The present study, 100 μmol/L spermine/NO (Sper/NO) prominently increased NOi. B, Pretreatment with 10 μmol/L L-NIO inhibited ACh-induced increases in NOi. In the same cell, 100 μmol/L Sper/NO increased NOi. C, Incubation in 2 mmol/L methyl-β-cyclodextrin abolished ACh-induced increases in NOi. D, Graph shows mean±SE, summarizing the effects of ACh exposure (ACh) and withdrawal (ACh/w) on NOi production in the presence of L-NIO (gray bar; n=10) and methyl-β-cyclodextrin (dextrin; open bars; n=3) compared with control (black bars; n=16). *P<0.05 compared with control.
exposure, addition of 1 μmol/L atropine elicited an increase in NO production similar to that elicited by ACh withdrawal (data not shown; n=3). The effects of ACh exposure and withdrawal on NO production are summarized in Figure 1D and indicate that ACh exposure elicits a relatively modest increase in NO production and that ACh withdrawal elicits additional, prominent increases in NO above those elicited during ACh exposure. Moreover, the receptor-mediated effects of ACh to increase NOi require electrical stimulation of the cell, presumably to increase Ca2+ influx (see below). Therefore, all additional experiments were performed on myocytes field-stimulated at 1 Hz. Figure 1B shows the effects of ACh recorded from another atrial myocyte in the presence of 10 μmol/L L-NIO, a specific inhibitor of eNOS activity. As summarized in Figure 1D, compared with control responses, inhibition of eNOS essentially abolished the effects of ACh exposure and withdrawal to increase NOi (n=10). In the same cell, 100 μmol/L Sper/NO prominently increased NOi. In cardiac cells, eNOS is localized to caveolae. Methyl-β-cyclodextrin (cyclodextrin) solubilizes cholesterol and thereby disrupts caveolae formation. ACh was tested on atrial myocytes incubated (1 hour at 37°C) in 2 mmol/L cyclodextrin. As shown in Figure 1C and summarized in Figure 1D, cyclodextrin abolished the increases in NOi induced by ACh exposure and withdrawal (n=3). Together, these findings indicate that ACh increases NOi by stimulating eNOS activity localized to caveolae. The fact that ACh-induced increases in NO require that cells be electrically stimulated suggests that voltage-activated Ca2+ influx is an essential signaling element, consistent with the Ca2+-CaM dependence of eNOS activity. We therefore tested the effects of ACh to increase NOi when the extracellular Ca2+ concentration ([Ca2+]o) was reduced to either 1 or 0.5 mmol/L before ACh exposure. As shown in Figure 2A and summarized in Figure 2D, compared with control (2 mmol/L), reducing [Ca2+]o to 1 mmol/L significantly increased NOi production elicited during ACh exposure and withdrawal (n=10), and 0.5 mmol/L [Ca2+]o essentially abolished the increase in NOi elicited during ACh exposure and withdrawal (n=5). Similar results were obtained by exposure to 1 μmol/L verapamil, an L-type Ca2+ channel antagonist (data not shown; n=3). In separate experiments, bathing cells in 0.5 mmol/L [Ca2+]o, or verapamil did not prevent electrical excitation elicited by field stimulation (n=3). To determine the role of CaM, ACh was tested on atrial myocytes pretreated with 10 μmol/L W-7, a potent CaM inhibitor. As shown in Figure 2B and summarized in Figure 2D, compared with control, W-7 abolished increases in NOi induced by ACh exposure and withdrawal (n=5). In the same cell, 100 μmol/L Sper/NO prominently increased NOi. To determine whether Ca2+ influx specifically contributes to the additional increase in NOi elicited by ACh withdrawal, we switched to 0.5 mmol/L [Ca2+]o, specifically during ACh withdrawal. As shown in Figure 2C and summarized in Figure 2D, ACh exposure in normal (2 mmol/L) [Ca2+]o elicited a typical increase in NOi. However, ACh withdrawal in low [Ca2+]o, failed to significantly increase NO above that elicited by ACh exposure (n=4). In the four cells tested, NO levels during ACh exposure in normal [Ca2+]o (1.041±0.009 F/Fo) and during ACh withdrawal in 0.5 mmol/L [Ca2+]o (1.053±0.011 F/Fo) were not different. As summarized in Figure 2D, NO during ACh withdrawal was significantly smaller in 0.5 mmol/L [Ca2+]o (hatched bar) compared with control ACh withdrawal (black bar). These findings indicate that the ability of ACh exposure and withdrawal to increase NOi depends on Ca2+ influx, presumably to activate CaM-dependent eNOS.

Previous findings in cat atrial myocytes indicate that the effect of ACh withdrawal to stimulate IcaL is mediated via muscarinic receptors coupled to Gq protein signaling. Moreover, β2-AR stimulation acts via Gq protein and PI-3K signaling to increase NOi. We therefore sought to determine whether muscarinic receptors act via a similar Gq protein/PI-3K signaling pathway to increase NOi. As shown in Figure...
compared with control responses, pretreatment with the muscarinic receptor antagonist (1 μmol/L) atropine blocked NO production induced by ACh exposure and withdrawal (n=6). In Figure 3B, incubating cells in pertussis toxin (PTX) (3.5 μg/mL; 3 hours, 36°C) to inhibit G protein signaling also abolished increases in NO induced by ACh exposure and withdrawal (n=6). In the same PTX-treated cell (Figure 3B), 100 μmol/L Sper/NO prominently increased NO. To examine the role of PI-3K signaling, cells were incubated in 10 μmol/L LY294002, an inhibitor of PI-3K signaling, for 30 minutes before being tested with ACh. Previous work has shown that either LY294002 or wortmannin, another inhibitor of PI-3K signaling, inhibits β2-AR stimulation of NO release.28 As shown in Figure 3C, inhibition of PI-3K signaling by LY294002 also inhibited NO production elicited by ACh exposure and withdrawal (n=7). Once again, in the same cell treated with LY294002, 100 μmol/L Sper/NO prominently increased NO. The results are summarized in the graph in Figure 3D and indicate that ACh acts on muscarinic receptors coupled via G proteins and PI-3K signaling to stimulate NO production. Apparently, muscarinic and β2-ARs act via the same G protein/PI-3K signaling pathway to stimulate NO production.

In endothelial cells, PI-3K signaling phosphorylates protein kinase B (Akt), which in turn activates eNOS.29,30 We therefore used immunoblots to determine the effects of ACh on Akt phosphorylation. As shown in Figure 4, compared with control, 10 μmol/L ACh significantly increased phos-
phorylation of Akt. Pretreatment with 10 μmol/L LY294002 prevented ACh-induced phosphorylation of Akt (n=5). These results are consistent with the present findings that inhibition of PI-3K signaling (LY294002) inhibits ACh-induced NO production (Figure 3C). It is also important to note that in the experiments designed to measure Akt phosphorylation, cells were quiescent. That is, under these conditions, voltage-activated Ca$^{2+}$ influx is not operating and therefore the cells are not capable of ACh-induced NO production (see Figure 1A). This indicates that ACh-induced stimulation of PI-3K/Akt signaling occurs independently of voltage-activated Ca$^{2+}$ influx and is not capable of stimulating NO production. To gain additional insight into the Ca$^{2+}$ dependence of ACh-induced phosphorylation of Akt, we tested the effects of ACh in control (2 mmol/L) and low (0.5 mmol/L) [Ca$^{2+}$], (see online Figure 2, available at http://www.circresaha.org). The results of these experiments show that in low [Ca$^{2+}$], ACh still increased Akt phosphorylation. We therefore conclude that although Ca$^{2+}$ is necessary for ACh-induced NO production, presumably to activate CaM-dependent eNOS, ACh-induced activation of Akt signaling is Ca$^{2+}$-independent.

We next used high-resolution confocal imaging to examine spatial patterns of NO release induced by ACh exposure and withdrawal. Figure 5A shows typical 2D surface plots of atrial cells during control, ACh exposure, and ACh withdrawal. ACh exposure (red trace) increased NO at local subsarcolemmal sites and is not capable per se of stimulating NO production, presumably to activate CaM-dependent eNOS, ACh-induced activation of Akt signaling is Ca$^{2+}$-independent.
elicited additional increases in NO, by enhancing NO release at some sites previously stimulated during ACh exposure (for example, site 1) and by recruiting additional subsarcolemmal release sites (for example, sites 2, 3, and 4). Also, baseline NO levels were increased additionally. Incubation of cells in 2 mmol/L cyclodextrin abolished all effects of ACh to increase NO (data not shown). Similar results were obtained in a total of five cells. The graph in Figure 5D summarizes the increases in amplitude (in relation to baseline) and frequency of NO release events elicited by ACh exposure (red bars) and ACh withdrawal (blue bars) (n=5). ACh exposure increased both the amplitude and frequency of NO release events, and these parameters were significantly greater during ACh withdrawal compared with ACh exposure.

**Discussion**

Previous studies from our laboratory have shown that in cat atrial myocytes, ACh exposure inhibits I_{Ca,L} and ACh withdrawal stimulates I_{Ca,L} above control levels, ie, rebound stimulation. Pharmacological analyses indicated that although NO signaling does not participate in ACh-induced inhibition of I_{Ca,L}, the rebound stimulation of I_{Ca,L} elicited by ACh withdrawal is mediated by NO signaling. The present study directly demonstrates for the first time that in atrial myocytes, ACh exposure increases NO production and ACh withdrawal elicits an additional, prominent increase in NO, above that achieved during ACh exposure. The actions of ACh require both stimulation of muscarinic receptor–mediated G proteins, PI-3K/Akt signaling and voltage-activated Ca^{2+} influx to elicit local, subsarcolemmal increases in NO, production.

The present results indicate that muscarinic receptor stimulation by ACh is unable to stimulate NO production in quiescent atrial myocytes. β-AR stimulation also requires electrical stimulation of atrial myocytes to increase NO production. Given the Ca^{2+}-CaM dependence of eNOS activity, these findings suggest that voltage-activated Ca^{2+} influx is essential for receptor-mediated stimulation of NO. Indeed, in electrically stimulated atrial myocytes, ACh-induced increases in NO are decreased (1 mmol/L) or abolished (0.5 mmol/L) by reducing [Ca^{2+}]_{i}, inhibition of L-type Ca^{2+} channels (verapamil), or inhibition of CaM activity (W-7). In addition, lowering [Ca^{2+}]_{i} specifically during ACh withdrawal prevented the additional, prominent increase in NO. In endothelial cells, removal of extracellular Ca^{2+} or exposure to CaM antagonists also abolishes agonist-induced NO formation. Although electrical stimulation is required for receptor-mediated NO production, it is not sufficient per se to elicit NO production. In other words, in atrial myocytes, basal voltage-activated Ca^{2+} influx and presumably intracellular Ca^{2+} release induced by Ca^{2+} influx are not capable of stimulating eNOS activity. This is in contrast to findings in rat ventricular myocytes in which basal Ca^{2+} influx elicited by electrical stimulation is sufficient to increase nitrite levels. We have obtained similar results in cat ventricular myocytes, where electrical stimulation alone is sufficient to increase NO production (unpublished observations). In electrically stimulated atrial myocytes, even marked increases in Ca^{2+} influx via I_{Ca,L} and presumably intracellular Ca^{2+} release induced by β-AR stimulation fail to increase NO. On the other hand, β-AR stimulation elicits a similar increase in I_{Ca,L} and does increase NO. Together, these findings indicate that, in atrial myocytes, voltage-activated Ca^{2+} influx and intracellular Ca^{2+} release per se are not sufficient to stimulate NO production. However, Ca^{2+} influx is essential for NO production stimulated by specific receptor-mediated signaling. The contribution of intracellular Ca^{2+} release to muscarinic receptor–mediated NO production remains to be determined.

In the present study, ACh acts via muscarinic receptors coupled to G proteins and PI-3K/Akt signaling to activate CaM-dependent NO production. Our previous studies have shown that in cat atrial myocytes, stimulation of I_{Ca,L} elicited by ACh withdrawal also is mediated via muscarinic receptors coupled to G proteins and activation of CaM-dependent NO signaling. In a variety of cell systems, PI-3K signaling leads to phosphorylation and activation of Akt signaling. In both endothelial and cardiac cells, PI-3K/Akt signaling phosphorylates and activates eNOS to produce NO. The present experiments indicate that ACh is not capable of eliciting NO production in quiescent atrial cells because of the requirement for voltage-activated Ca^{2+} influx. However, the immunoblot experiments show that ACh is able to stimulate PI-3K/Akt signaling in quiescent cells (Figure 4A). These findings suggest that muscarinic receptor–mediated stimulation of PI-3K/Akt signaling is not capable per se of stimulating NO production and that stimulation of this signaling pathway occurs independently of voltage-activated Ca^{2+} influx. The latter finding is supported by the fact that lowering [Ca^{2+}]_{i}, to a level (0.5 mmol/L) that prevents ACh-induced NO production failed to prevent ACh-induced Akt phosphorylation (see online Figure 2). This is consistent with reports that Akt activation is Ca^{2+}-independent. We therefore conclude that receptor-mediated PI-3K/Akt signaling plus voltage-activated Ca^{2+} influx are both required for stimulation of NO production. Our previous experiments indicate that in cat atrial myocytes, β-AR stimulation also requires both PI-3K signaling and voltage-activated Ca^{2+} influx to elicit NO production. This dual signaling mechanism can account for the relatively small NO production elicited during ACh exposure and the more prominent increase in NO, elicited by ACh withdrawal. Thus, ACh exposure stimulates PI-3K/Akt signaling at the same time that it decreases Ca^{2+} influx via I_{Ca,L}, thereby allowing only a modest increase in NO production. However, once PI-3K/Akt signaling is stimulated by ACh exposure, rapid removal of ACh from its receptor allows rapid recovery of Ca^{2+} influx, resulting in the additional, prominent stimulation of NO production. Moreover, because ACh withdrawal results in the recovery of adenylate cyclase/cAMP signaling, increases in NO stimulate cAMP-mediated increases in Ca^{2+} influx via I_{Ca,L}, which in turn contribute to additional Ca^{2+}-dependent increases in NO. Our interpretation is that NO production is modulated by Ca^{2+} influx is supported by experiments in which we recorded intracellular [Ca^{2+}]_{i} and NO simultaneously (see online Figure 1). In endothelial cells, receptor-mediated signaling by bradykinin can act independently of PI-3K/Akt signaling to enhance the binding of CaM to eNOS and thereby enhance the Ca^{2+}. 


sensitivity of eNOS activity. This mechanism results in high-output Ca^{2+}-dependent NO production. It seems unlikely, however, that a similar mechanism plays a primary role in atrial myocytes given the present finding that ACh-induced increases in NOi are entirely dependent on PI-3K/Akt signaling.

The contribution of NO signaling to muscarinic receptor-mediated inhibition of cardiac function differs among different species, tissues, and reports from different laboratories. Although the present results show that ACh exposure modestly increases NO production, NO signaling does not contribute to ACh-induced inhibition of $I_{Ca,L}$. In fact, in cat atrial myocytes, NO signaling stimulates $I_{Ca,L}$ via cAMP-dependent protein kinase A signaling and therefore would not be expected to contribute to the inhibitory effects of ACh. Moreover, the fact that ACh inhibits basal adenylate cyclase/cAMP activity would preclude any significant effects of NO signaling on cAMP-mediated regulation of $I_{Ca,L}$. In addition, the level of NO production during ACh exposure may be below the threshold required for activation of cGMP-mediated signaling and modulation of channel function.

The present results indicate that ACh increases NO, primarily at the cell periphery and disruption of caveolae formation by cyclohexatin abolishes ACh-induced increases in NO. These findings are consistent with reports that in cardiac cells eNOS is localized to caveolae through binding to the scaffolding protein caveolin-3. The binding of caveolin holds eNOS in an inactive conformation. Increases in Ca^{2+} concentration activate CaM binding to eNOS, thereby disrupting the inhibitory eNOS-caveolin complex and activating eNOS activity. Moreover, stimulation of M2 muscarinic receptors are thought to translocate to caveolae once stimulated by agonist. Moreover, stimulation of M2 muscarinic receptors causes a reversible translocation of eNOS from caveolae and may partition the enzyme into both noncaveolar plasma membrane and more hydrophilic regions of the cell. Because cat atrial myocytes lack T-tubules, the peripheral sarcenomembrane is the only site of voltage-activated Ca^{2+} influx and the region most abundant in caveolae. The finding that ACh withdrawal stimulates additional NO release sites suggests that different release sites exhibit different thresholds for stimulation of NO production. The preferential release of NO from subsarcenomembrane sites also is consistent with the local regulation of sarcenomembrane function exerted by NO signaling.

Clearly, the prominent increase in NOi elicited by ACh withdrawal strongly supports our previous findings that rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal is mediated by NO signaling. In vivo, the actions of ACh at the muscarinic receptor are terminated almost instantaneously by cholinesterase activity. We therefore propose that the NO signaling mechanisms reported here play an important role in ensuring rapid recovery of both chronotropic and inotropic activities after cholinergic inhibition of atrial function. In fact, we have reported that NO signaling elicited by ACh withdrawal may contribute to the nonadrenergic component of postvagal tachycardia and the potential development of Ca^{2+}-mediated atrial dysrhythmias induced by withdrawal of parasympathetic nerve activity.

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Methods:

Details of intracellular NO measurements using DAF-2: In the experiments designed to measure NO\textsubscript{i}, solutions contained 100 μM L-arginine. L-arginine was omitted when L-NIO was used to block endothelial NO synthase (eNOS). Average NO\textsubscript{i} measurements were obtained within a 10 s period of a defined time point. Changes in NO\textsubscript{i} induced by ACh exposure were measured at the end of each ACh exposure and those elicited by ACh withdrawal were measured after 3 min. of ACh withdrawal in relation to baseline values before ACh exposure. Single cells were field stimulated at 1 Hz by 3 ms duration suprathreshold rectangular voltage pulses delivered through a pair of extracellular platinum electrodes. Control experiments in unstimulated cells indicated minimal autoflourescence that was constant and did not change with ACh exposure or withdrawal.

Details of immunoblot procedures: After a 5-fold dilution, cells were centrifuged (100 g for 10 min at 4\textdegree{}C) and resuspended in lysis buffer containing (in mM) HEPES 50, pH 7.4; NaCl 150; MgCl\textsubscript{2} 1.5; EGTA 1.0; Na\textsubscript{3}VO\textsubscript{4} 1.0; Na pyrophosphate 10; NaF 100; and 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. Samples were centrifuged at 16,000 g at 4\textdegree{}C for 10 min and the protein contents of the supernatants were determined by BCA method. Equal amounts of protein (50-100 μg) were separated by SDS-PAGE and Western blotting. Separated proteins were probed with specific polyclonal antibodies to phosho-Akt (Ser\textsuperscript{473}) (Cell Signaling). Protein bands were visualized using ECL detection reagents (Amersham Biosciences) and analyzed using NIH ImageJ.

Simultaneous measurement of [Ca\textsuperscript{2+}]\textsubscript{i} and NO\textsubscript{i}: 

Simultaneous measurements of $[Ca^{2+}]_i$ and NO were performed as previously described. Atrial myocytes were incubated with 5 μM fura-2/AM and 5 μM DAF-2 DA for 10 minutes at room temperature and washed for 10 minutes to allow sufficient time for de-esterification of the indicators. Fluorescence was excited by alternately illuminating the cells at 360 nm ($F_{360}$) and 380 nm ($F_{380}$) for fura-2 measurements and 480 nm ($F_{480}$) for DAF-2 measurements through a rotating filter wheel. Emitted cellular fluorescence was recorded at 540 nm. Changes in $[Ca^{2+}]_i$ were expressed as changes of the ratio $R = F_{360}/F_{380}$ and represent the changes in systolic level of $[Ca^{2+}]_i$. Changes in cellular DAF-2 fluorescence intensities (F) in each experiment were normalized to the level of fluorescence recorded prior to stimulation ($F_0$). Changes in intracellular [NO] are expressed as F/F₀, and therefore represent percent increases above basal levels.

**Measurements of $[Ca^{2+}]_i$ transients:**

$[Ca^{2+}]_i$ transients were measured using the fluorescent $Ca^{2+}$ indicator indo-1 (ratiometric, spatially averaged measurements). Myocytes were loaded with $Ca^{2+}$ indicator by exposure to 5 μM acetoxymethyl ester of indo-1 (indo-1 AM, Molecular Probes, Eugene, OR, USA) in 1 ml Tyrode solution containing 0.001 g/ml of a Pluronic F-127 for 10 min at room temperature. Subsequently, cells were washed for 10 min to allow for de-esterification of the indicator. A coverslip with the attached cells was mounted on the stage of an inverted microscope. Indo-1 fluorescence was excited at 357 nm and emitted fluorescence signals were recorded simultaneously at 405 nm ($F_{405}$) and 485 nm ($F_{485}$). Changes of $[Ca^{2+}]_i$ are expressed as changes in the ratio $R = F_{405}/F_{485}$.

**Results and Discussion**
We have proposed that the extent of NO\textsubscript{i} production elicited during ACh exposure and withdrawal is modulated by Ca\textsuperscript{2+} influx. More specifically, during ACh exposure Ca\textsuperscript{2+} influx is decreased and during ACh withdrawal Ca\textsuperscript{2+} influx is increased. To support this proposal we measured the effects of ACh exposure and withdrawal on intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) and NO\textsubscript{i} ([NO\textsubscript{i}]) simultaneously in single atrial myocytes. In these experiments, intracellular Ca\textsuperscript{2+} was measured at approximately the peak of the systolic Ca\textsuperscript{2+} transient. To complement these data, separate experiments measured the effects of ACh exposure and withdrawal on the entire Ca\textsuperscript{2+} transient. As shown in Figure 1A (supplement), exposure to 1 \textmu M ACh decreased intracellular Ca\textsuperscript{2+} and increased NO\textsubscript{i}. Note that the intracellular Ca\textsuperscript{2+} decreased earlier and more rapidly than the increase in NO\textsubscript{i} production. On the other hand, ACh withdrawal increased both intracellular Ca\textsuperscript{2+} and NO\textsubscript{i} above control levels. Also note that the initial increase in intracellular Ca\textsuperscript{2+} occurred earlier and more rapidly than the increase in NO\textsubscript{i}. The fact that changes in intracellular Ca\textsuperscript{2+} precede changes in NO\textsubscript{i} production supports our interpretation that intracellular Ca\textsuperscript{2+} modulates NO\textsubscript{i} production. It is also worth noting that the effects of ACh to decrease intracellular Ca\textsuperscript{2+} remained constant, indicating that receptor desensitization was not a factor.

This is consistent with previous findings in cat atrial myocytes that ACh-induced inhibition of I\textsubscript{Ca,L} also does not fade with time.\textsuperscript{2} Included in this figure are insets showing the effects of ACh on Ca\textsuperscript{2+} transients recorded from another atrial cell. ACh exposure and withdrawal significantly decreased and increased above control levels, respectively, peak Ca\textsuperscript{2+} transient amplitudes. Increases in peak systolic intracellular Ca\textsuperscript{2+} elicited by ACh withdrawal reached steady-state after approximately 2-3 minutes. The graph in Figure 1B shows that ACh exposure (empty bars) significantly decreased intracellular Ca\textsuperscript{2+} and modestly increased NO\textsubscript{i}, and ACh withdrawal (filled bars) significantly increased both intracellular Ca\textsuperscript{2+} and NO\textsubscript{i} compared to control (100%).
(n=5; P<0.05). The graph in Figure 1C shows that ACh exposure (empty bar) and ACh withdrawal (filled bar) significantly decreased and increased, respectively, Ca\(^{2+}\) transient amplitudes compared with control (n=7; p<0.05). These findings support the conclusion that ACh-induced changes in Ca\(^{2+}\) influx modulate ACh-induced NO\(_i\) production.

In the present study, exposure to low (0.5 mM) [Ca\(^{2+}\)]\(_o\) prevents ACh-induced NO\(_i\) production, presumably by preventing Ca\(^{2+}\)-dependent eNOS activity. However, this raises the question of whether low [Ca\(^{2+}\)]\(_o\) also may prevent ACh-induced NO\(_i\) production by inhibiting ACh-induced Akt phosphorylation. As shown in Figure 2 and summarized in the graph, the effect of ACh exposure on Akt phosphorylation was tested in control 2 mM [Ca\(^{2+}\)]\(_o\) and in low 0.5 mM [Ca\(^{2+}\)]\(_o\) (n=6). Under control (C) conditions, ACh exposure increased Akt phosphorylation. Exposure to low [Ca\(^{2+}\)]\(_o\) increased basal Akt phosphorylation, probably by decreasing Ca\(^{2+}\)-dependent phosphatase activities. In low [Ca\(^{2+}\)]\(_o\) ACh exposure still significantly increased Akt phosphorylation. These results support our conclusion that ACh-induced signaling via PI-(3)K/Akt is Ca\(^{2+}\)-independent, and that Ca\(^{2+}\) influx modulates ACh-induced NO\(_i\) production primarily through Ca\(^{2+}\)-dependent eNOS activity.

References


**Legends**

**Figure 1** – The effects of ACh on intracellular Ca\(^{2+}\) \([Ca^{2+}]_i\) and NO\(_i\) \([NO]_i\) measured simultaneously in an atrial myocyte. Also shown are the effects of ACh on Ca\(^{2+}\) transients recorded from another atrial myocyte. A, ACh exposure decreased intracellular Ca\(^{2+}\) and increased NO\(_i\). The decrease in intracellular Ca\(^{2+}\) preceded the increase in NO\(_i\). ACh withdrawal increased intracellular Ca\(^{2+}\) and NO\(_i\) above control. The increase in intracellular Ca\(^{2+}\) preceded the increase in NO\(_i\). In a separate cell, ACh exposure decreased the Ca\(^{2+}\) transient amplitude and ACh withdrawal increased the Ca\(^{2+}\) transient amplitude compared with control. B, ACh exposure (ACh; empty bars) significantly decreased intracellular Ca\(^{2+}\) and modestly increased NO\(_i\). ACh withdrawal (ACh/w; filled bars) significantly increased both intracellular Ca\(^{2+}\) and NO\(_i\) above control (n=5). C, ACh exposure (ACh; empty bar) significantly decreased Ca\(^{2+}\) transient amplitude and ACh withdrawal (ACh/w; filled bar) significantly increased Ca\(^{2+}\) transient amplitude compared with control (n=7). Cells were field stimulated at 1 Hz. \(* = P<0.05\) compared with control.

**Figure 2** - Effect of low 0.5 mM \([Ca^{2+}]_o\) on ACh-induced Akt phosphorylation. The upper panel shows typical bands obtained by phospho-Akt (p-Akt) specific antibodies and lower panel shows
Akt loading controls. Under control (C) conditions, ACh (10 μM) increased Akt phosphorylation. Exposure to 0.5 mM [Ca^{2+}]_{o} increased basal Akt phosphorylation (C). In 0.5 mM [Ca^{2+}]_{o}, ACh still increased Akt phosphorylation. The graph shows mean values ± SE normalized to control (1.0) (n=6). * = P<0.05 compared with respective controls.

![Graph A](image)

**Figure 1 -**
Figure 2 -

Akt Phosphorylation (fold)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>ACh</th>
</tr>
</thead>
<tbody>
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<td>2 mM [Ca$^{2+}$]$_0$</td>
<td>1.0</td>
<td>2.0 $^*$</td>
</tr>
<tr>
<td>0.5 mM [Ca$^{2+}$]$_0$</td>
<td>1.0</td>
<td>3.0 $^*$</td>
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