Acidic NAADP-sensitive Calcium Stores in the Endothelium

AGONIST-SPECIFIC RECRUITMENT AND ROLE IN REGULATING BLOOD PRESSURE

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Accumulating evidence implicates nicotinic acid adenine dinucleotide phosphate (NAADP) in the control of Ca2+-dependent functions. Little, however, is known concerning its role in the vascular endothelium, a major regulator of blood pressure. Here, we show that NAADP acetoxymethyl ester (NAADP-AM), a cell-permeant NAADP analog, increases cytosolic Ca2+ concentration in aortic endothelial cells. We demonstrate that these signals and those evoked by acetylcholine are blocked by disrupting acidic organelles with bafilomycin A1. In contrast, Ca2+ signals in response to thrombin are only partially inhibited by bafilomycin A1 treatment, and those to ATP were insensitive, suggesting that recruitment of acidic stores is agonist-specific. We further show that NAADP-evoked Ca2+ signals hyperpolarize endothelial cells and generate NO. Additionally, we demonstrate that NAADP dilates aortic rings in an endothelium- and NO-dependent manner. Finally, we show that intravenous administration of NAADP-AM into anesthetized rats decreases mean arterial pressure. Our data extend the actions of NAADP to the endothelium both in vitro and in vivo, pointing to a previously unrecognized role for this messenger in controlling blood pressure.

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent and widespread Ca2+-mobilizing messenger first described in sea urchin eggs (1, 2). Although its mechanism of action is subject to debate, much evidence indicates that NAADP mobilizes Ca2+ from acidic, lysosome-like organelles (3) through activation of novel Ca2+-permeable channels (1, 4). These channels have recently been identified as members of the two-pore channel family (5–9). Importantly, mobilization of so-called “acidic Ca2+ stores” (10) by NAADP is often linked to mobilization of the well established endoplasmic reticulum Ca2+ stores through the process of Ca2+-induced Ca2+ release (11). NAADP is thus thought to act as trigger during agonist-evoked Ca2+ signaling (2). The functional positioning of NAADP-sensitive Ca2+ stores upstream of those targeted by the messengers, inositol 1,4,5-trisphosphate (IP3) and cyclic ADP-ribose, raises the possibility that agonist-evoked Ca2+ signals previously ascribed to endoplasmic reticulum Ca2+ release might also involve NAADP and acidic organelles.

Using isolated cells or tissues, NAADP-induced Ca2+ signaling has been implicated in the regulation of several physiological functions such as egg fertilization (12, 13), T lymphocyte activation (14), muscle contraction (15), and neuronal differentiation (16). The role of NAADP in vivo, however, is not known, although a recent study demonstrated that a newly described NAADP antagonist could prevent T cell motility in an animal model of multiple sclerosis (17).

In the cardiovascular system, a role for NAADP has been demonstrated in agonist-evoked Ca2+ signaling in pulmonary (15, 18) and coronary arteries (19, 20), cardiac myocytes (21), and the renal microcirculation (22). Direct measurements of NAADP have confirmed its messenger role in response to endothelin-1 (15) and β-adrenoreceptor agonists (21). What role NAADP plays in the vascular endothelium has yet to be explored. A variety of extracellular cues such as acetylcholine mediate changes in cytosolic Ca2+ concentration within endothelial cells (23). These signals drive production of vasodilators such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor. The endothelium is thus critical for regulating vascular tone and blood pressure.

Like many other cell types, changes in cytosolic Ca2+ levels within the endothelium are generally ascribed to mobilization of endoplasmic reticulum Ca2+ stores through activation of IP3 or ryanodine receptors (23). Given emerging evidence implicating NAADP in Ca2+ signaling pathways attributed to more traditional messengers (24, 25), we examined the role of NAADP in the endothelium. By combining Ca2+ voltage, and NO imaging of single cultured endothelial cells with measurement of contractility ex vivo and blood pressure in vivo, we show that NAADP-sensitive Ca2+ stores are present in the endothelium, that they are recruited in an agonist-specific manner, and that their mobilization is sufficient to regulate contractility and blood pressure.

5-methylamino-2′,7′-difluorofluorescein) diacetate; DiBAC4(3), bis-(1,3-dibutylbarbituric acid) trimethine oxonol; HAECl, human aortic endothelial cell; HbSS, Hanks’ balanced salt solution; IP3, inositol 1,4,5-trisphosphate; l-NAME, l-Nω-nitroarginine methyl ester.
EXPERIMENTAL PROCEDURES

HAEC Culture—Human aortic endothelial cells (HAECs) were grown in M199 medium and the serum concentration reduced from 20% to 0.5% prior to experimentation.

Measurement of Ca2+—Intracellular Ca2+ concentration ([Ca2+]i) was measured by the microfluorimetric technique, as described previously (6).

Measurement of Membrane Potential—Bis-(1,3-dibutylbarbitalic acid) trimethine oxonol (DiBAC4(3), Invitrogen), a slow response voltage-sensitive dye, was used to assess relative membrane hyperpolarization, the dye concentrates in the cell membrane, leading to a decrease of fluorescence intensity, whereas depolarization results in a sequestration of the dye into cytosol and is associated with an increase in the fluorescence intensity (27).

Measurement of NO—Intracellular NO was monitored with (4-amino-5-methylamino-2′,7′-difluorofluorescein) diacetate (DAF-FM), a pH-insensitive fluorescent dye that emits increased fluorescence upon reaction with an active intermediate of NO formed during spontaneous oxidation of NO to NO2− (28, 29).

Measurement of Contractility—Mechanical activity of vascular rings from rat thoracic aorta was measured using an isometric force transducer (30).

Measurement of Blood Pressure—Arterial pressure of anesthetized rats was measured using a high-sensitivity pressure transducer.

Statistics—Statistical significance between groups was tested using one-way analysis of variance followed by Bonferroni test.

RESULTS AND DISCUSSION

To probe the presence of functional NAADP-sensitive Ca2+ channels in the endothelium, we first examined the effect of NAADP-AM, a cell-permeant analog of NAADP (21, 31), on the [Ca2+]i, in HAECs. Stimulation of cells with NAADP-AM induced a transient increase in [Ca2+]i (Fig. 1A), which was abolished by pretreatment with Ned-19, a blocker of NAADP signaling (32). The effect of NAADP-AM on [Ca2+]i was concentration-dependent (Fig. 1B). A delay of 30–90 s was noted between the moment of NAADP-AM administration and the increase in [Ca2+]i. Application of NAADP-AM also elevated [Ca2+]i, in Ca2+-free saline (Fig. 1C), indicating that NAADP mobilizes intracellular Ca2+ stores. This response was blocked by pretreatment with bafilomycin A1 (Fig. 1C), a V-type ATPase inhibitor (33), confirming the involvement of an acidic store as reported in a variety of other cell types (3, 15, 16, 19, 20). Previous studies have shown that in intact cells, NAADP evokes Ca2+ signals from acidic Ca2+ stores that are then subsequently amplified by ryanodine receptors (18), IP3 receptors (34), or both (11). We therefore tested the effects of ryanodine and xestospongin C on NAADP-AM-evoked Ca2+ signals in endothelial cells. Pretreatment with ryanodine caused a partial (33%) reduction in the evoked response, whereas xestospongin C was without effect (Fig. 1C). These data, summarized in Fig. 1D, provide the first evidence for functional NAADP-sensitive Ca2+ channels in endothelial cells that, as in pancreatic beta cells, appear largely uncoupled from Ca2+ release from the endoplasmic reticulum (35).

Having established the presence of acidic NAADP-sensitive Ca2+ stores in endothelial cells, we next probed their recruitment by Ca2+-mobilizing vasoactive agonists. To achieve this, we compared [Ca2+]i upon stimulation with acetylcholine, thrombin, and ATP in the absence and presence of bafilomycin A1 (Fig. 2). All three agonists evoked comparable cytosolic Ca2+ signals in Ca2+-free saline (Fig. 2, A–C). The responses to acetylcholine were abolished by bafilomycin A1, whereas those to thrombin were only partially blocked (Fig. 2, A–C). In stark
Acidic \( \text{Ca}^{2+} \) stores in endothelial cells are recruited in an agonist-specific manner. A–C, representative traces showing the effect of 10 \( \mu M \) acetylcholine (A), thrombin (B), and ATP (C) on [\( \text{Ca}^{2+} \)], in \( \text{Ca}^{2+} \)-free saline. Cells were untreated (first column) or preincubated with bafilomycin A1 (BAF, 1 \( \mu M \), 1 h; second column), ryanodine (Ry, 1 \( \mu M \), 10 min; third column), xestospongin C (XeC, 10 \( \mu M \), 15 min; fourth column), or Ned-19 (Ned, 1 \( \mu M \), 15 min, fifth column). D, summary data quantifying \( \Delta[\text{Ca}^{2+}] \) in response to the agonists in the absence or presence of the indicated inhibitor (\( n = 6 \) for each condition). The far right panel expresses responses of all three agonists in the presence of the inhibitors as the percentage of the control (Ctrl) responses. *, \( p < 0.05 \).

**FIGURE 2.**

contrast, the ATP responses were unaffected (Fig. 2, A–C). We also probed the contribution of endoplasmic reticulum \( \text{Ca}^{2+} \) stores to agonist-evoked \( \text{Ca}^{2+} \) signals by examining the effect of ryanodine and xestospongin C to block ryanodine and IP\(_3\) receptors, respectively. As shown in Fig. 2, A–C, the responses to acetylcholine were substantially reduced by ryanodine but unaffected by xestospongin C. In contrast, the responses to thrombin were substantially reduced by both ryanodine and xestospongin C, whereas the responses to ATP were unaffected by ryanodine but inhibited by xestospongin C. Moreover, pretreatment with Ned-19 (33) abolished acetylcholine-evoked \( \text{Ca}^{2+} \) signals, reduced the responses to thrombin, and did not significantly affect the responses to ATP (Fig. 2, A–C).

A summary of the effects of the inhibitors on the evoked \( \text{Ca}^{2+} \) signals is shown in Fig. 2D. We conclude: (i) that acetylcholine-evoked \( \text{Ca}^{2+} \) signals involve recruitment of acidic \( \text{Ca}^{2+} \) stores and ryanodine receptors with little contribution of IP\(_3\) receptors; (ii) that thrombin-evoked \( \text{Ca}^{2+} \) signals involve recruitment of acidic \( \text{Ca}^{2+} \) stores, ryanodine receptors, and IP\(_3\) receptors; and (iii) that ATP-evoked \( \text{Ca}^{2+} \) signals appear independent of acidic \( \text{Ca}^{2+} \) stores and ryanodine receptors and are instead driven primarily by IP\(_3\) receptors. Agonist-evoked \( \text{Ca}^{2+} \) signals in endothelial cells thus involve differential recruitment of intracellular \( \text{Ca}^{2+} \) pools. Our results with thrombin and ATP are consistent with data in other cell types where thrombin was shown in part to recruit acidic stores in platelets (36), and ATP was shown to act largely independently of acidic stores in hippocampal neurons and glia (37). This is the first report implicating acidic \( \text{Ca}^{2+} \) stores in acetylcholine action. Together, this raises the possibility that coupling of the specific receptors to acidic \( \text{Ca}^{2+} \) stores is malleable and likely cell type-specific.

Agonist stimulation of endothelial cells results in hyperpolarization due to the activation of \( \text{Ca}^{2+} \)-dependent K\(^+\) channels (38). Hyperpolarization increases the driving force for \( \text{Ca}^{2+} \) entry, which likely contributes to NO synthesis via activation of endothelial nitric oxide synthase. Hyperpolarization is also transmitted to smooth muscle cells, which contributes to vasodilation through attenuating the opening of voltage-sensitive \( \text{Ca}^{2+} \) channels (38). To determine whether NAADP-mediated \( \text{Ca}^{2+} \) signals could mediate electrical responses in endothelial cells, we examined the effect of NAADP-AM on membrane potential using the slow response voltage-sensitive dye DiBAC\(_4\). The resting membrane potential of HAECs was \(-47 \pm 6.2 \text{ mV} \) (\( n = 57 \)). Administration of NAADP-AM induced a hyperpolarization of \( 12 \pm 2.6 \text{ mV} \) (\( n = 19 \)) (Fig. 3A). Endothelial cells express several types of \( \text{Ca}^{2+} \)-activated K\(^+\) channels such as high and intermediate conductance \( \text{Ca}^{2+} \)-activated K\(^+\) channels, which are blocked by charybdotoxin, and small conductance \( \text{Ca}^{2+} \)-activated K\(^+\) channels, which are blocked by apamin. The effect of NAADP-AM on membrane potential was abolished by pretreatment with a combination of apamin and charybdotoxin (\( n = 12 \)) consistent with the activation of \( \text{Ca}^{2+} \)-activated K\(^+\) channels (Fig. 3A). Pretreatment with bafilomycin A1 also abolished the response (\( n = 15 \)), indicating that the effects of NAADP were due to mobilization of an acidic store (Fig. 3A). Thus, NAADP can mediate hyperpolarization in addition to depolarization as reported recently in medulla neurons (39).
To examine the role of NAADP-mediated Ca\(^{2+}\) signals in vasomotor responses, we next characterized the effects of NAADP-AM on the contractility of aortic rings, with and without endothelium. As shown in Fig. 3C, administration of NAADP-AM to aortic rings with intact endothelium precontracted with KCl produced a rapid and marked relaxation (91.6 ± 3.8\%, \(n = 6\)). This effect was similar to that evoked by acetylcholine (85.2 ± 2.7\%, \(n = 11\)), which is known to activate endothelial nitric oxide synthase in endothelial cells. Administration of L-NAME to precontracted aortic rings produced a supplemental contraction (82.8 ± 5.9\%, \(n = 5\)). Under these conditions, the relaxation to NAADP after L-NAME administration was reduced to 7.5 ± 0.6\% (\(n = 5\); Fig. 3D). These data are consistent with endothelial cells as targets for NAADP-AM. Accordingly, in denuded preparations, which were unresponsive to acetylcholine, NAADP-AM failed to evoke relaxation but instead evoked a significant contraction (37 ± 3\%, \(n = 5\)), likely reflecting an action on smooth muscle cells (Fig. 3E).

The effects of NAADP have been extensively characterized in isolated cells and tissues, but corresponding in vivo data are currently lacking. Given the ability of NAADP to regulate arterial contractility ex vivo, the final set of experiments was undertaken to examine the effects of NAADP-AM on blood pressure in anesthetized rats. The basal mean arterial pressure was 108 ± 3.7 mm Hg (\(n = 6\)). Intravenous administration of NAADP-AM induced a decrease in mean arterial pressure by 51.6 ± 5.9 mm Hg (\(n = 5\)). The effects of NAADP-AM were partially sensitive to L-NAME. Thus, in the presence of L-NAME, the mean arterial pressure before NAADP-AM administration was 132 ± 4.2 mm Hg (\(n = 5\)). Subsequent administration of NAADP-AM reduced mean arterial pressure by 13.6 ± 5.3 mm Hg (Fig. 3F). These data suggest that NAADP-AM mediates vasodilatation in vivo by both NO-dependent and NO-independent means.

To conclude, we provide the first evidence that NAADP-sensitive Ca\(^{2+}\) stores are present in endothelial cells and that they are recruited by select physiologically relevant vasoactive signals.
agonists. We identify hyperpolarization and NO production as new downstream effectors for this messenger and show that NAADP is capable of regulating contractility ex vivo, and importantly, blood pressure in vivo. Although the role of NAADP in smooth muscle cells in mediating contraction is established (15, 18–20), our data showing that NAADP in neighboring endothelial cells is coupled to opposing, relaxation pathways highlight the versatility of this messenger in regulating vascular tone. When coupled with the demonstrated agonist-specific recruitment of acidic Ca^2+ stores, this renders NAADP an attractive therapeutic target in combating endothelium dysfunction.

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