Nicotinic Acid Adenine Dinucleotide Phosphate Mediates Ca\textsuperscript{2+} Signals and Contraction in Arterial Smooth Muscle via a Two-Pool Mechanism

François-Xavier Boittin, Antony Galione, A. Mark Evans

Abstract—Previous studies of arterial smooth muscle have shown that inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and cyclic ADP-ribose mobilize Ca\textsuperscript{2+} from the sarcoplasmic reticulum. In contrast, little is known about Ca\textsuperscript{2+} mobilization by nicotinic acid adenine dinucleotide phosphate, a pyridine nucleotide derived from β-NADP\textsuperscript{+}. We show here that intracellular dialysis of nicotinic acid adenine dinucleotide phosphate (NAADP) induces spatially restricted “bursts” of Ca\textsuperscript{2+} release that initiate a global Ca\textsuperscript{2+} wave and contraction in pulmonary artery smooth muscle cells. Depletion of sarcoplasmic reticulum Ca\textsuperscript{2+} stores with thapsigargin and inhibition of ryanodine receptors with ryanodine, respectively, block the global Ca\textsuperscript{2+} waves by NAADP. Under these conditions, however, localized Ca\textsuperscript{2+} bursts are still observed. In contrast, xestospongin C, an IP\textsubscript{3} receptor antagonist, had no effect on Ca\textsuperscript{2+} signals by NAADP. We propose that NAADP mobilizes Ca\textsuperscript{2+} via a 2-pool mechanism, and that initial Ca\textsuperscript{2+} bursts are amplified by subsequent sarcoplasmic reticulum Ca\textsuperscript{2+} release via ryanodine receptors but not via IP\textsubscript{3} receptors. (Circ Res. 2002;91:1168-1175.)

Key Words: NAADP ■ calcium ■ smooth muscle ■ sarcoplasmic reticulum ■ ryanodine receptors

It is generally accepted that Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) can be triggered by the ubiquitous Ca\textsuperscript{2+} mobilizing messenger IP\textsubscript{3}, via the activation of one or more of the known inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor (IP\textsubscript{3}R) subtypes.\textsuperscript{1,2} However, a consideration of the wide variety of cellular processes regulated by changes in intracellular Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}]i, from cell differentiation and gene expression to muscle contraction, underscores the need for a versatile Ca\textsuperscript{2+} signaling system that would necessarily require multiple Ca\textsuperscript{2+} mobilizing second messengers. It is not surprising, therefore, that recent studies have demonstrated that intracellular Ca\textsuperscript{2+} release may also be triggered by Ca\textsuperscript{2+} mobilizing pyridine nucleotides, namely cyclic ADP-ribose (cADPR)\textsuperscript{3,4} and nicotinic acid adenine dinucleotide phosphate (NAADP).\textsuperscript{5,5}

The enzymes for the synthesis and metabolism of NAADP and cADPR are present in mammalian cells,\textsuperscript{3,6} and both messengers have been shown to release Ca\textsuperscript{2+} from microsomes derived from a variety of cell types including vascular smooth muscle.\textsuperscript{6–12}

It is generally accepted that cADPR mobilizes Ca\textsuperscript{2+} from SR/endoplasmic reticulum stores by activating ryanodine receptors (RyRs).\textsuperscript{13–17} As yet, however, the Ca\textsuperscript{2+} release pathway through which NAADP mobilizes Ca\textsuperscript{2+} remains poorly characterized.

Recent studies have suggested that NAADP triggers Ca\textsuperscript{2+} release via a mechanism that is fundamentally different from those controlled by IP\textsubscript{3} or by cADPR, and that this may involve the activation of putative NAADP receptors.\textsuperscript{5,18–20} Indeed, investigations in sea urchin eggs suggest that NAADP mobilizes Ca\textsuperscript{2+} from a store that is pharmacologically and physically distinct from those accessed by IP\textsubscript{3} and cADPR, respectively.\textsuperscript{5,21,22} Despite the proposed physical segregation of the Ca\textsuperscript{2+} stores, however, all three Ca\textsuperscript{2+} release pathways may act in concert to initiate complex Ca\textsuperscript{2+} signals. Thus, NAADP can trigger Ca\textsuperscript{2+} release that is amplified by subsequent Ca\textsuperscript{2+} release via RyRs and via IP\textsubscript{3}Rs.\textsuperscript{12,19,22,23} Likewise, IP\textsubscript{3} can trigger Ca\textsuperscript{2+} release via IP\textsubscript{3}Rs that is amplified by subsequent Ca\textsuperscript{2+} release via RyRs.\textsuperscript{24,25}

To date, however, there have been no investigations of Ca\textsuperscript{2+} mobilization by NAADP in intact vascular smooth muscle cells. We show here that NAADP is a powerful Ca\textsuperscript{2+} mobilizing second messenger in pulmonary artery smooth muscle cells, and propose that NAADP mobilizes Ca\textsuperscript{2+} via a 2-pool mechanism.

Materials and Methods

Cell Isolation

Single smooth muscle cells were isolated from second-order branches of the pulmonary artery of male Wistar rats (150 to 200 g). Briefly, an artery was dissected out and placed in a low-Ca\textsuperscript{2+} solution...
of the following composition (in mmol/L): 124 NaCl, 5 KCl, 1 MgCl₂, 0.5 NaH₂PO₄, 0.5 KH₂PO₄, 15 NaHCO₃, 0.16 CaCl₂, 0.5 EDTA, 10 glucose, 10 taurine, and 10 HEPES (pH 7.4). After 20 minutes, the artery was placed in the same solution containing 0.5 mg/mL papain (Fluka) and 1 mg/mL BSA (Sigma) for 1 hour at room temperature. Then, 0.25 mg/mL 1,4-DTT (Sigma) was added to the solution, followed by a further 30-minute incubation at room temperature. The tissue was then washed three times in fresh low-Ca²⁺ solution without enzymes, and single smooth muscle cells were isolated by gentle trituration with a fire-polished Pasteur pipette. Samples of the cell suspension were placed as required onto a glass coverslip in the experimental chamber and allowed to settle for 10 minutes.

## Ca²⁺ Imaging

Cells were washed with physiological salt solution of the following composition (in mmol/L): 130 NaCl, 5.2 KCl, 1 MgCl₂, 1.7 CaCl₂, 10 glucose, and 10 HEPES (pH 7.45). Cells were then incubated for 30 minutes in the same solution with 5 μmol/L fura-2-acetoxymethyl ester (fura-2), washed, and allowed to equilibrate for 20 minutes. The experimental chamber was then placed on a Leica DMRB inverted microscope. Changes in [Ca²⁺], were monitored by assessing fura-2 fluorescence ratio within a given area. Emitted fluorescence was monitored using a Hamamatsu 4880 image-intensifying charge-coupled device camera and recorded and analyzed using Openlab imaging software (Improvision) on an Apple Macintosh G4 personal computer. Fluorescence intensity was measured at 0.25 to 5 Hz, with background subtraction being carried out online. Changes in fura-2 fluorescence were reported as the F340/F380 ratio and as the estimated [Ca²⁺].

## Intracellular Dialysis of NAADP and IP₃

Ca²⁺ mobilizing second messengers were applied intracellularly in the whole-cell configuration of the patch-clamp technique and in current-clamp mode (i=0), as described previously. The pipette solution contained the following (in mmol/L): 140 KCl, 10 HEPES, 1 MgCl₂, and 0.005 fura-2 (free acid) (pH 7.4). Throughout each experiment the seal resistance was ≥3 GΩ, whereas the series resistance and pipette resistance were ≤10 MΩ and 2 to 3 MΩ, respectively, as measured using an Axopatch 200B amplifier (Axon Instruments).

## Experiments in the Absence of Extracellular Ca²⁺

To limit the impact on intracellular Ca²⁺ stores, cells were superfused with Ca²⁺-free physiological salt solution containing 1 mmol/L BAPTA using a flow pipe positioned close to the cell, as described previously. By this method complete exchange of the extracellular solution is ensured within 5 seconds, as determined previously from the shift in the potassium equilibrium potential. Superfusion with Ca²⁺-free solution began after first establishing the cell-attached patch configuration and was continued for 30 seconds before entering the whole-cell configuration and throughout the subsequent period of intracellular dialysis. All experiments were carried out at room temperature (22°C).

## Data Analysis

Data are expressed as mean±SEM for n experiments. Statistical significance was determined using a Student t test.

## Drugs

All compounds were from Sigma, except papain (Fluka) and fura-2 (Molecular Probes). Ryanodine and thapsigargin were dissolved in DMSO. The minimum dilution of DMSO was 1:1000, which had no effect on the smooth muscle cells. All other stock solutions were in H₂O.

## Results

### NAADP Induces Global Ca²⁺ Oscillations in Pulmonary Artery Smooth Muscle Cells

The role of NAADP as a Ca²⁺-mobilizing second messenger in pulmonary artery smooth muscle cells was investigated by applying fixed concentrations intracellularly by dialysis from a patch pipette, in the whole-cell configuration and under current-clamp conditions (i=0). At 10 nmol/L, NAADP induced a global increase in the fura-2 fluorescence ratio (F340/F380), from 0.7±0.1 to 1.9±0.1, in isolated pulmonary artery smooth muscle cells. This occurred within 180 seconds of entering the whole-cell configuration (n=17; Figure 1A). In paired cells, intracellular dialysis of NAADP-free pipette solution had little or no effect over time periods (up to 10 minutes) that far exceeded those within which intracellular dialysis of NAADP triggered an increase in F340/F380 fluorescence ratio (n=17; Figures 1A and 1D).

Moreover, NAADP (10 nmol/L) failed to induce an increase in the F340/F380 fluorescence ratio when applied intracellularly in combination with the fast calcium chelator BAPTA (1 mmol/L; n=4; Figures 1B and 1D). In marked contrast, superfusion with Ca²⁺-free extracellular solution containing BAPTA (1 mmol/L) had little or no effect on Ca²⁺ signals induced by intracellular perfusion of NAADP (10 nmol/L). In the absence of extracellular Ca²⁺, NAADP increased the fura-2 fluorescence ratio from 0.54±0.1 to 1.84±0.2 (n=6; Figures 1C and 1D). NAADP, therefore, triggers an increase in cytoplasmic free Ca²⁺ concentration in pulmonary artery smooth muscle cells by mobilizing intracellular stores.

In 80% of cells studied, the global Ca²⁺ wave triggered a pronounced contraction of the smooth muscle cell (Figure 1A). Furthermore, in 3 of 17 cells, NAADP (10 nmol/L) induced global, regenerative Ca²⁺ waves. These oscillations in [Ca²⁺], occurred with a frequency of 1.1±0.1 minute⁻¹ (see Figure 2A). The events leading up to the initiation of a global Ca²⁺ wave appeared complex, in that there was an initial increase in intracellular Ca²⁺ at the perimeter of the cell (Figures 1A and 1B). This initial “Ca²⁺ burst” was observed in 10 of 17 cells and appeared as (1) a uniform increase in fura-2 fluorescence ratio around the entire perimeter of the cell (Figure 1A) or (2) a spatially restricted “focal” Ca²⁺ burst covering between 2 and 10 μm of the perimeter of the cell (Figure 3C). The Ca²⁺ burst initiated by NAADP either declined back to basal levels without initiating a global Ca²⁺ wave, or preceded and then triggered a global Ca²⁺ wave (Figures 1A and 3C). Significantly, the global Ca²⁺ wave, but not the Ca²⁺ burst, induced contraction.

Our findings suggest that a global Ca²⁺ wave is only initiated by NAADP when the initial increase in cytoplasmic Ca²⁺ concentration breaches a given threshold (Figure 1A). Further analysis revealed that a global Ca²⁺ wave was initiated when the fura-2 fluorescence ratio within a given “region of interest” at the perimeter of the cell rose from 0.6±0.1 to 0.9±0.1 (n=10). This corresponded to a local increase in [Ca²⁺], from ≈100 to ≈400 nmol/L, as quantified by interpolation with a standard in vitro Ca²⁺ calibration curve.
Concentration Dependence of NAADP-Induced Ca\(^{2+}\) Signals

Low concentrations of NAADP (\(\approx 2\) nmol/L) failed to initiate a global Ca\(^{2+}\) wave or contraction (not shown). The threshold concentration at which NAADP induced a global Ca\(^{2+}\) wave was 10 nmol/L (Figure 2A; \(n=17\)). As the concentration of NAADP was increased between 10 nmol/L and 10 \(\mu\)mol/L, no increase in the magnitude or frequency of the Ca\(^{2+}\) waves was observed (not shown). Thus, between 10 nmol/L and 10 \(\mu\)mol/L, NAADP initiates a global Ca\(^{2+}\) wave in an all-or-none manner. Surprisingly, however, intracellular dialysis of 100 \(\mu\)mol/L NAADP was less effective. In 3 of 6 cells paired with those infused with 10 nmol/L NAADP, 100 \(\mu\)mol/L NAADP had little or no effect on the [Ca\(^{2+}\)], (Figure 2B). In the remaining 3 cells global Ca\(^{2+}\) signals of varying amplitude were observed. On average, 100 \(\mu\)mol/L NAADP increased the fura-2 fluorescence ratio to a peak of 41±19\% (\(n=6\)), compared with an increase to a peak of 192±20\% in cells dialyzed with 10 nmol/L NAADP (\(n=4\), Figure 2C). The inability of 100 \(\mu\)mol/L NAADP to consistently induce a global Ca\(^{2+}\) wave in these cells did not, however, result from the presence of dysfunctional SR stores, because the increase in fura-2 fluorescence ratio induced by 2.5 mmol/L caffeine remained unaffected (Figures 2B and 2C).

Global Ca\(^{2+}\) Waves by NAADP Require the Release of Ca\(^{2+}\) From Ryanodine-Sensitive SR Stores

To determine the role of ryanodine-sensitive SR stores in the generation of Ca\(^{2+}\) waves by NAADP, we studied the effect of blocking RyR function with ryanodine and of depleting SR Ca\(^{2+}\) stores with thapsigargin, respectively. When cells were preincubated (15 minutes) with thapsigargin (1 mmol/L), 10 nmol/L NAADP failed to induce a global Ca\(^{2+}\) wave and contraction (\(n=4\); Figure 3B, bottom panel). However, low-magnitude and spatially restricted Ca\(^{2+}\) bursts were still observed (Figure 3B). On average the maximum increase in the F340/F380 ratio measured only 27±3\% (\(n=8\), Figure 3D). After preincubation (20 minutes) of cells with 20 \(\mu\)mol/L ryanodine, intracellular application of NAADP (10

---

**Figure 1.** NAADP induces global Ca\(^{2+}\) waves and contraction in isolated pulmonary artery smooth muscle cells. A, Top, Series of pseudocolor images of fura-2 fluorescence ratio (F340/F380) recorded in a pulmonary artery smooth muscle cell during the intracellular dialysis of 10 nmol/L NAADP. Bottom, Corresponding record of fura-2 fluorescence ratio against time and record of fura-2 fluorescence ratio in a different cell during intracellular dialysis of NAADP-free solution (control), respectively. In this and all subsequent figures, vertical lines at the start of the record indicate the point at which intracellular dialysis began. B, Record of fura-2 fluorescence ratio against time during intracellular dialysis of 10 nmol/L NAADP with 1 mmol/L BAPTA. C, Record of fura-2 fluorescence ratio against time during intracellular dialysis of 10 nmol/L NAADP in the absence of extracellular Ca\(^{2+}\) and in the presence of extracellular BAPTA (1 mmol/L). D, Bar chart showing maximal percentage change (mean±SEM) in fura-2 fluorescence ratio measured in pulmonary artery smooth muscle cells during intracellular dialysis of NAADP-free pipette solution (control), 10 nmol/L NAADP, and 10 nmol/L NAADP in the presence of 1 mmol/L intracellular and extracellular BAPTA, respectively. For this and all subsequent figures, intracellular dialysis was carried out under current-clamp conditions (\(I=0\)). Images were acquired at 3-second intervals.
mmol/L) again failed to induce a global Ca\textsuperscript{2+} wave, and no cell contraction was observed (n=9, Figure 3C). Once more, however, low-magnitude and spatially restricted Ca\textsuperscript{2+} bursts were observed (Figure 3C). On average the maximum increase in the F340/F380 ratio under these conditions measured only 27±3% (n=8, Figure 3D). In marked contrast, no Ca\textsuperscript{2+} bursts were observed in cells exposed to intracellular dialysis of NAADP-free pipette solution after preincubation with thapsigargin (n=3) and ryanodine (n=3), respectively.

**Global Ca\textsuperscript{2+} Waves by NAADP Do Not Require the Activation of IP\textsubscript{3}Rs**

In pulmonary artery smooth muscle cells, intracellular dialysis of 1 \( \mu \text{mol/L} \) IP\textsubscript{3} triggered a global Ca\textsuperscript{2+} wave (n=5, Figure 4A). The Ca\textsuperscript{2+} wave was triggered \( \approx \) 180 seconds after entering the whole-cell configuration, as was the case with NAADP, as indicated by an increase in the fura-2 fluorescence ratio from 0.6±0.01 to a peak of 2.5±0.1 (n=5). The global Ca\textsuperscript{2+} wave by IP\textsubscript{3} remained in the presence of ryanodine and peaked at a level not significantly different from that of control (Figure 4B). Thus, after preincubation (20 minutes) of cells with 20 \( \mu \text{mol/L} \) ryanodine, intracellular dialysis of 1 \( \mu \text{mol/L} \) IP\textsubscript{3} increased the fura-2 fluorescence ratio from 0.8±0.1 to a peak of 1.8±0.2 (n=5).

Preincubation (15 minutes) of cells with the IP\textsubscript{3} antagonist xestospongin C (0.1 \( \mu \text{mol/L} \)) abolished the increase in F340/F380 ratio induced by 1 \( \mu \text{mol/L} \) IP\textsubscript{3} (n=5, Figures 4A and 4B). In marked contrast, Ca\textsuperscript{2+} waves by 10 nmol/L NAADP remained unaffected in paired cells preincubated (15 minutes) with 0.1 \( \mu \text{mol/L} \) xestospongin C (Figure 4C). The peak increase in F340/F380 ratio induced by NAADP was 208±42% (n=5) in the absence and 211±54% in the presence of xestospongin C (n=5; Figure 4D).

**Discussion**

Recent studies have suggested that NAADP triggers Ca\textsuperscript{2+} release via a mechanism that is fundamentally different from those controlled by IP\textsubscript{3} or by cADPR, and that this may involve the activation of putative NAADP receptors.\textsuperscript{8,11,19,20} Indeed, it has been proposed that NAADP mobilizes Ca\textsuperscript{2+} from a store that is pharmacologically and physically distinct from those accessed by IP\textsubscript{3} and cADPR, respectively.\textsuperscript{5,21,22} Furthermore, all three Ca\textsuperscript{2+} release pathways may act in concert to initiate complex Ca\textsuperscript{2+} signals. Thus, NAADP can trigger Ca\textsuperscript{2+} release that is amplified by subsequent Ca\textsuperscript{2+} release via RyRs and via IP\textsubscript{3}Rs.\textsuperscript{12,19,22} We have, therefore, investigated the role of NAADP as a Ca\textsuperscript{2+}-mobilizing second messenger in pulmonary artery smooth muscle.

**NAADP Induces Global Ca\textsuperscript{2+} Oscillations in Pulmonary Artery Smooth Muscle Cells**

Intracellular dialysis of NAADP into acutely isolated pulmonary artery smooth muscle cells induced global, regenerative Ca\textsuperscript{2+} waves that were abolished when NAADP was coinfused with the fast Ca\textsuperscript{2+} chelator BAPTA. In contrast, superfusion of cells with Ca\textsuperscript{2+}-free extracellular solution containing BAPTA had little or no effect on Ca\textsuperscript{2+} signals by NAADP. Thus, intracellular dialysis of NAADP mobilizes Ca\textsuperscript{2+} from intracellular stores in pulmonary artery smooth muscle cells, as would be expected given previous investigations.\textsuperscript{5,8,11,19–22}

The Ca\textsuperscript{2+} signals induced by NAADP were, however, complex. Spatially restricted Ca\textsuperscript{2+} bursts were observed before the initiation of a global Ca\textsuperscript{2+} wave. The Ca\textsuperscript{2+} bursts took two clearly identifiable forms. In one subset of cells, a uniform increase in Ca\textsuperscript{2+} around the entire perimeter of the cell was observed, indicative of an increase in Ca\textsuperscript{2+} proximal to the plasma membrane as has been reported previously in
sea urchin eggs. In the second subset of cells, a focal increase in Ca$^{2+}$ was triggered at the perimeter of the cells, between 2 and 10 μm in diameter. The Ca$^{2+}$ burst either decayed or preceded and then triggered a global Ca$^{2+}$ wave and contraction in the smooth muscle cell. Significantly, only the global Ca$^{2+}$ wave and not the initial Ca$^{2+}$ burst triggered contraction. These data suggested that the initial Ca$^{2+}$ burst had to breach a threshold before it was able to initiate a global Ca$^{2+}$ wave, consistent with the proposal that NAADP induces global waves via a 2-pool mechanism by priming IP$_3$- and cADPR-sensitive SR stores.

**NAADP Triggers Ca$^{2+}$ Release From a Discrete Intracellular Store That Is Amplified by Subsequent Ca$^{2+}$ Release From Thapsigargin-Sensitive SR Stores**

Previous investigations have demonstrated that NAADP mobilizes Ca$^{2+}$ from a thapsigargin-insensitive store. It was surprising, therefore, that prior depletion of SR Ca$^{2+}$ stores with thapsigargin resulted in marked attenuation of NAADP-induced Ca$^{2+}$ signals in pulmonary artery smooth muscle cells. Importantly, however, thapsigargin did not block the initiation of the spatially localized Ca$^{2+}$ bursts by NAADP. Thus, NAADP initially mobilizes Ca$^{2+}$ from a discrete, thapsigargin-insensitive store, which then triggers a global Ca$^{2+}$ wave by subsequent Ca$^{2+}$ release from the SR. Further support for this proposal may be derived from the fact that high concentrations of NAADP can induce self-inactivation without affecting Ca$^{2+}$ release from the SR by RyR activation.

It is interesting to note, however, that depletion of SR Ca$^{2+}$ stores with thapsigargin resulted in such marked attenuation of Ca$^{2+}$ signals by NAADP, such that cell contraction was no longer observed. This suggests that Ca$^{2+}$ signals by NAADP are more heavily dependent on the SR in pulmonary artery...
smooth muscle than is the case in, for example, pancreatic acinar cells.19

Ca2+ Signals by NAADP Require Subsequent Ca2+ Release via RyRs but not via IP3Rs
Consistent with previous investigations,12,19,22,23 we found that inhibition of RyRs with ryanodine resulted in marked attenuation of Ca2+ signals by NAADP. Under these conditions, only spatially restricted Ca2+ bursts were observed, as was the case after depletion of SR stores with thapsigargin. The ability of ryanodine to block global Ca2+ waves by NAADP did not, however, result from slow depletion of SR Ca2+ stores, as demonstrated by the finding that ryanodine had little effect on Ca2+ signals triggered by IP3. Thus, global Ca2+ waves by NAADP, but not local Ca2+ bursts, require subsequent SR Ca2+ release via RyRs.

This raises the possibility that Ca2+ signals by NAADP in pulmonary artery smooth muscle may be modulated by cADPR, which may release SR Ca2+ via RyRs and/or sensitize RyR to Ca2+-induced Ca2+ release,13 as has been shown to be the case in pancreatic acinar cells.28

In marked contrast to previous reports,12,19,22,23 we found no evidence to support a role for IP3Rs in the generation of NAADP-induced Ca2+ waves in pulmonary artery smooth muscle cells. Thus, the IP3R antagonist xestospongin C blocked Ca2+ signals induced by IP3, but not those induced by NAADP. It would appear, therefore, that NAADP first induces local Ca2+ bursts via a mechanism independent of RyRs, IP3Rs, and SR Ca2+ stores, which then initiates a global Ca2+ wave and contraction in pulmonary artery smooth muscle cells by further Ca2+ release from SR stores via RyRs, but not via IP3Rs.

Clearly, our finding that initial Ca2+ bursts by NAADP do not induce further Ca2+ release via IP3Rs is contrary to previous reports.12,19,22,23 This discrepancy may be explained if the initiation of a global Ca2+ wave relies on Ca2+-induced Ca2+ release and if IP3Rs are expressed in a Ca2+-insensitive form29 in pulmonary artery smooth muscle. Alternatively, in pulmonary artery smooth muscle, NAADP receptors may colocalize with SR “compartments” with a high density of RyRs, and not with SR compartments where IP3Rs are concentrated.30

It is also interesting to note that recent investigations in veinous smooth muscle have shown that IP3 induces a global Ca2+ wave by first activating IP3Rs, which in turn trigger further Ca2+ release via RyRs.24,25,31 This is clearly not
consistent with our finding that ryanodine has little effect on the magnitude of global signals by IP3 in arterial smooth muscle. These contrary findings may result from differences in the spatial localization of RyRs and IP3Rs in arterial versus venous smooth muscle. Thus, IP3Rs may colocalize with clusters of RyRs in venous smooth muscle, thereby allowing for the threshold for Ca2+-induced Ca2+ release via RyRs to be breach with ease. In contrast, IP3Rs in pulmonary artery smooth muscle cells may be localized in sections of the SR that are spatially segregated from sections of the SR within which RyRs are clustered, leading to spatially segregated Ca2+ signals via these two families of SR Ca2+ release channel.

Concentration Dependence of Ca2+ Signals by NAADP

Previous studies in pancreatic acinar cells, T lymphocytes, and sea urchin eggs have demonstrated that high concentrations of NAADP prove ineffective as a trigger of intracellular Ca2+ release.19,23,32–34 It has been proposed, therefore, that NAADP receptors may self-inactivate in the presence of high concentrations of NAADP. Our findings provide some support for this proposal, because global Ca2+ signals by NAADP were consistently observed between 10 nmol/L and 10 μmol/L, whereas 100 μmol/L NAADP proved to be less effective. Thus, 100 μmol/L NAADP failed to induce a Ca2+ signal in 3 of 6 cells and induced a global Ca2+ signal somewhat smaller than control in the other 3 cells. The failure of 100 μmol/L NAADP to consistently induce Ca2+ signals in pulmonary artery smooth muscle cells was not, however, due to the presence of dysfunctional SR stores, because SR Ca2+ release triggered by caffeine remained unaffected after self-inactivation of NAADP signaling by intracellular dialysis of 100 μmol/L NAADP. The fact that high concentrations of NAADP conferred a variable degree of self-inactivation in pulmonary artery smooth muscle cells suggests that the susceptibility to self-inactivation is less than has been reported in other preparations.19,23,32–34 Ca2+ mobilization by NAADP may therefore be more robust in pulmonary artery smooth muscle cells than in other cell types.19,23,32–34 raising the possibility that the characteristics of the Ca2+ release process triggered by NAADP may be tailored to suit a particular function in a given cell type. Unfortunately, the robust nature of NAADP signaling in pulmonary artery smooth muscle cells mitigates against the use of self-inactivation as a tool for identifying vasoconstrictors that mediate Ca2+ signals via NAADP, as described in studies of secretagogue signaling in pancreatic acinar cells.19

Physiological Significance of Ca2+ Signaling by NAADP

Consideration of the wide variety of cellular processes regulated by changes in [Ca2+], in vascular smooth muscle, from cell differentiation and gene expression to muscle contraction and relaxation,1,2,35 underscores the need for a versatile Ca2+ signaling system. Such versatility with respect to Ca2+ signaling would necessarily require equally versatile mechanisms of Ca2+ mobilization. When taken together with the fact that the enzymes for the synthesis and metabolism of NAADP are present in vascular smooth muscle,6,36 the findings of the present investigation suggest that this requirement may be fulfilled, in part, by NAADP. Thus, relatively low concentrations of NAADP mobilize Ca2+ from intracellular stores in arterial smooth muscle cells and do so by a mechanism distinct from that of IP3. The pronounced smooth muscle cell contraction triggered by the associated Ca2+ wave suggests that NAADP likely mediates intracellular Ca2+ release and, in part, contraction triggered by certain vasoconstrictors. However, development of selective NAADP antagonists is required before this can be proven.

Recent investigations have demonstrated that different Ca2+ signals may regulate differential gene expression37,38 and transcription,39 respectively, in preparations including native vascular smooth muscle. It seems likely, therefore, that the required code for differential gene expression and transcription could be determined, at least in part, by the discrete mechanisms through which IP3 and NAADP trigger Ca2+ release and by the spatiotemporal characteristics of the resultant Ca2+ signal. Thus, differential activation of these signaling pathways by vasoactive agents may trigger smooth muscle contraction and promote, over prolonged periods of stimulation, associated, differential gene expression required to maintain normal physiology, or promote the development of pathophysiological processes associated with, for example, hypertension and atherogenesis.

In conclusion, we have demonstrated that NAADP acts as a potent Ca2+ mobilizing second messenger in arterial smooth muscle. Our data support previous proposals that NAADP induces Ca2+ signals via a 2-pool mechanism,22,23 which requires initial Ca2+ release from a thapsigargin-insensitive Ca2+ store.6,9 We propose, therefore, that NAADP induces Ca2+ bursts from a thapsigargin-insensitive store, which are then amplified by subsequent SR Ca2+ release via RyRs, but not via IP3Rs. Given that certain agonists mediate Ca2+ signals via NAADP in pancreatic acinar cells whereas others mediate Ca2+ signals via IP3, it seems likely that certain vasoconstrictors may mediate Ca2+ signals and contraction in arterial smooth muscle by NAADP, whereas others rely on IP3. Further investigations of Ca2+ signaling by NAADP in arterial smooth muscle will, therefore, advance our understanding of the fundamental mechanisms that regulate arterial function in health and disease.

Acknowledgments

This work was funded by a Wellcome Trust Project Grant (reference No. 056423).

References


Nicotinic Acid Adenine Dinucleotide Phosphate Mediates $\text{Ca}^{2+}$ Signals and Contraction in Arterial Smooth Muscle via a Two-Pool Mechanism
François-Xavier Boittin, Antony Galione and A. Mark Evans

_Circ Res._ 2002;91:1168-1175; originally published online November 14, 2002; doi: 10.1161/01.RES.0000047507.22487.85

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/91/12/1168

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2002/12/02/91.12.1168.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Appendix 1 Intracellular dialysis of 10 nM NAADP in the presence of 2 mM ATP induced an initial Ca$^{2+}$ burst at the perimeter of a pulmonary artery smooth muscle cell, which in turn triggered a global Ca$^{2+}$ wave and contraction. Upper panel shows a series of pseudocolour images which represent the change in Fura-2 fluorescence ratio in the cell. Lower panel shows a record of the Fura-2 fluorescence ratio against time. Intracellular dialysis began at the start of the record, as indicated by the horizontal lines. The red numbers indicate the time point at which the images shown in the upper panel were acquired.