NAADP Controls Cross-talk between Distinct Ca\textsuperscript{2+} Stores in the Heart*

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In cardiac muscle the sarcoplasmatic reticulum (SR) plays a key role in the control of contraction, releasing Ca\textsuperscript{2+} in response to Ca\textsuperscript{2+} influx across the sarcolemma via voltage-gated Ca\textsuperscript{2+} channels. Here we report evidence for an additional distinct Ca\textsuperscript{2+} store and for actions of nicotinic acid adenine dinucleotide phosphate (NAADP) to mobilize Ca\textsuperscript{2+} from this store, leading in turn to enhanced Ca\textsuperscript{2+} loading of the SR. Photorelease of NAADP increased Ca\textsuperscript{2+} transients accompanying stimulated action potentials in ventricular myocytes. The effects were prevented by bafilomycin A (an H\textsuperscript{+}-ATPase inhibitor acting on acidic Ca\textsuperscript{2+} stores), by desensitizing concentrations of NAADP, and by ryanodine and thapsigargin to suppress SR function. Bafilomycin A also suppressed staining of acidic stores with Lysotracker Red without affecting SR integrity. Cytosolic application of NAADP by means of its membrane permeant acetoxymethyl ester increased myocyte contraction and the frequency and amplitude of Ca\textsuperscript{2+} sparks, and these effects were inhibited by bafilomycin A. Effects of NAADP were associated with an increase in SR Ca\textsuperscript{2+} load and appeared to be regulated by \(\beta\)-adrenoreceptor stimulation. The observations are consistent with a novel role for NAADP in cardiac muscle mediated by Ca\textsuperscript{2+} release from bafilomycin-sensitive acidic stores, which in turn enhances SR Ca\textsuperscript{2+} release by increasing SR Ca\textsuperscript{2+} load.

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Ca\textsuperscript{2+} plays a pivotal role in excitation-contraction coupling in cardiac muscle and is a multifunctional regulator of diverse cellular functions (1). During the cardiac action potential, cytosolic Ca\textsuperscript{2+} concentration rises as a consequence of Ca\textsuperscript{2+} flux across the sarcolemma via voltage-gated L-type Ca\textsuperscript{2+} channels, and by subsequent Ca\textsuperscript{2+} release from the SR via ryanodine receptors (RyRs) (2). Clearance of elevated cytosolic Ca\textsuperscript{2+} concentration involves at least four pathways: the Ca\textsuperscript{2+}-ATPase on SR membranes (SERCA), the sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, the sarcolemmal Ca\textsuperscript{2+}-ATPase, and the mitochondrial Ca\textsuperscript{2+} uniporter. Discrete changes in the regulation of intracellular Ca\textsuperscript{2+} concentration can result in congestive heart failure or arrhythmia, though possible mechanisms remain controversial, and hence it remains important to understand the detailed mechanisms that control Ca\textsuperscript{2+} signaling in the heart.

Nicotinic acid adenine dinucleotide phosphate (NAADP)\textsuperscript{2} is a newly discovered Ca\textsuperscript{2+} messenger with unique properties that has been extensively investigated in various tissues and cell lines (3–8). Cardiac tissue expresses high affinity binding sites for NAADP, and NAADP stimulates Ca\textsuperscript{2+} efflux from microsomal fractions derived from rat heart (9). NAADP is an endogenous molecule, with levels in mouse heart reported to be 0.4 nmol/mg protein (10). Accumulating data have confirmed that NAADP has an ability to release Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores, but whether NAADP releases Ca\textsuperscript{2+} from endoplasmic reticulum (ER) or non-ER remains unclear (11). The most intriguing behavior of this molecule is, perhaps, the specific self-induced inactivation of its Ca\textsuperscript{2+} releasing mechanism that is not seen to the same extent with any other intracellular messenger (12–14). Examples of this behavior are provided by the ability of subthreshold concentrations of NAADP to inactivate NAADP-induced Ca\textsuperscript{2+} release in invertebrates and plants (12, 13, 15), and by the ability of high concentrations of NAADP to cause profound self-desensitization in intact mammalian cells (16).

Here we report the first observations of NAADP actions in intact cardiac ventricular myocytes. Two methods were used in the majority of experiments to raise cytosolic levels of NAADP: photorelease of NAADP from a caged compound and loading of NAADP into the cytosol by rapid switch of the extracellular solution to one containing the membrane-permeant acetoxymethyl ester of NAADP (allowing rapid access of NAADP-AM to the cytosol and subsequent liberation of NAADP following action of intracellular esterases). NAADP applied by these methods in cardiac myocytes caused elevation of peak Ca\textsuperscript{2+} transient amplitude, enhancement of cell contraction and increases in the frequency and amplitude of Ca\textsuperscript{2+} sparks. Effects of NAADP were prevented by bafilomycin A1 (an H\textsuperscript{+}-ATPase inhibitor that acts on acidic Ca\textsuperscript{2+} stores), by desensitizing concentrations of NAADP and by ryanodine and thapsigargin (to suppress SR function). The observations are consistent with NAADP-induced release of Ca\textsuperscript{2+} from acidic Ca\textsuperscript{2+} stores followed by uptake of additional Ca\textsuperscript{2+} by the SR, leading in turn to an enhanced Ca\textsuperscript{2+} transient. Actions of NAADP were also influenced by \(\beta\)-adrenoreceptor stimulation in ways that are consistent with regulation of

\textsuperscript{2}The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide phosphate; SR/ER, sarcoplasmic/endoplasmic reticulum.
NAADP levels by this receptor pathway, providing support for a physiological role for NAADP in the heart.

EXPERIMENTAL PROCEDURES

Cell Isolation—Guinea pig ventricular myocytes were isolated using methods described previously. Extracellular solution for superfusion of isolated cells contained (mM): NaCl 118.5, KCl 4.2, NaHCO₃ 14.5, Na₂HPO₄ 1.18, MgSO₄·6H₂O 1.18, CaCl₂ 2.5, glucose 11.1 (BDH Chemicals Ltd, Poole, UK), gassed with 95% O₂, 5% CO₂ to maintain a pH of 7.4. All experiments were carried out at 36 °C.

Electrophysiology—Electrophysiological experiments were performed using an Axoclamp 2B microelectrode system in voltage or current clamp mode as appropriate (Axon Instruments). Action potentials were simulated by 2 ms current pulse injections (magnitude 3–5 nA) in bridge current clamp mode. Recordings were made in either the whole cell or permeabilized patch (200 µg/ml amphotericin B; Sigma-Aldrich) configurations using glass microelectrodes. The electrodes were pulled from 1.5 mm (external diameter), 1.17 mm (internal diameter) thin-walled, filamented borosilicate glass capillary tubing (GC150TF-15, Harvard Apparatus Ltd, Kent) using a vertical electrode puller (Narishige PE-2, Japan); electrode resistances were in the range 1.5–4 MΩ when filled with patch pipette solution containing (mM): KCl 140, NaCl 5, MgCl₂ 2, ATP 1, and HEPES 5 (pH 7.2). L-type Ca²⁺ currents were activated by step depolarizations from a holding potential of −40 mV to potentials between −30 mV and +50 mV for 200 ms at a frequency of 0.3 Hz. Ca²⁺ current was measured as the difference between peak current and current at the end of the pulse, using the Clampfit 9.0 (pClamp, Axon Instruments) software package.

Measurement of Ca²⁺ Transients—The loading of ventricular myocytes with fluo-4 was achieved by either inclusion of the dye in the patch pipette solution (75 µM) or incubation for 15 min with the acetoxymethyl ester of fluo-4 (fluo-4 AM, 5 µM). Coverslips were mounted in a static chamber on a confocal microscope system that consisted of a Leica TCS NT scanning confocal microscope. Bodipy-FL-X-ryanodine (excitation: 488 nm, emission: 513 nm) and LysoTracker Red (excitation: 568 nm, emission: 570 nm) were used to switch to the drug-containing solution. The same perfusion system was used for experiments using isoproterenol.

NAADP-AM Synthesis—Synthesis and characterization of NAADP-AM was described.³

Imaging of Internal Organelles—Cells were loaded with 0.1 µM Bodipy-FL-X-ryanodine, 0.1 µM Bodipy-TR-X-thapsigargin, or 50 nM LysoTracker Red (stock solutions dissolved in Me₂SO) for 20 min at room temperature. Labeled cells were visualized (after removing excess dye in the bath) using a Zeiss LSM510 laser scanning confocal microscope (Zeiss). Excitation light was provided by an argon laser for Bodipy-FL-X-ryanodine (excitation: 505 nm, emission: 513 nm), and by a HeNe laser for both Bodipy-TR-X-thapsigargin (excitation: 488 nm, emission: 513 nm) and LysoTracker Red (excitation: 568 nm, emission: 590 nm).

Measurement of NAADP Levels in the Guinea Pig Cardiac Tissue—Experiments were carried out on guinea pig tissue extracts. Guinea pigs were sacrificed by stunning and cervical dislocation and the heart removed and placed on a standard Langendorff apparatus. Control hearts were perfused for 10 min with a Ca²⁺-containing solution, composition in mM: NaCl 137, KCl 5, NaHCO₃ 12, CaCl₂ 1.8, glucose 5, sodium pyruvate 1, NaH₂PO₄ 0.4, MgCl₂ 1, NaOH 1, EGTA 0.1, pH 7.4, gassed with 95% O₂/5% CO₂. Isoproterenol-treated hearts were initially perfused for 5 min with physiological salt solution (as

³ R. Parkesh, A. M. Lewis, P. K. Aley, A. Arredouani, S. Rossi, R. Tavares, S. R. Vasudevan, D. Rosen, A. Galione, J. Dowden, and G. C. Churchill, submitted manuscript.
**RESULTS**

**Actions of NAADP Photoreleased from a Caged Analogue on Whole Cell Ca\(^{2+}\) Transients**—In the first series of experiments presented here, NAADP was photoreleased from a caged analogue to test whether or not NAADP could exert any effect on whole cell Ca\(^{2+}\) transients accompanying action potentials in cardiac ventricular myocytes. Guinea pig ventricular myocytes were loaded with fluo-4 together with various concentrations of caged-NAADP through a cell-attached patch electrode. Cells were then electrically stimulated at 0.5 Hz to fire action potentials. The rises in Ca\(^{2+}\) accompanying the action potential were compared before and after photorelease of NAADP (with a single 500-ms pulse of UV light) using laser scanning confocal microscopy. Fig. 1A shows line-scan images of Ca\(^{2+}\) transients in a representative cell before (as a control) and 60 s after photorelease of NAADP from caged-NAADP (5 \(\mu\)M in the patch pipette). Fig. 1B presents Ca\(^{2+}\) transient profiles calculated as the integrated fluorescence along the scanned line at each time point in Fig. 1A. It may be seen from these Figs that photorelease of NAADP resulted in an enhancement in the magnitude of the Ca\(^{2+}\) transient. The increases in the Ca\(^{2+}\) transient induced by photolysis of caged-NAADP showed progressive changes with time and were greater at 60 s than at 20 s after photolysis (Fig. 1C). Photolysis of caged-NAADP (5 \(\mu\)M in the patch pipette) caused a significant increase in the Ca\(^{2+}\) transient amplitude 60 s after photolysis (Fig. 1B); the mean increase in 8 cells was 41 ± 10\% (\(n = 8, p < 0.05\)). Under these conditions, photorelease of NAADP also increased background Ca\(^{2+}\) fluorescence (data not shown). When the concentration of caged-NAADP was reduced from 5 to 1.5 \(\mu\)M, there were smaller but still significant increases in the amplitudes of the Ca\(^{2+}\) transients following photolysis of caged-NAADP (Fig. 1D, 19 ± 5\% at 1.5 \(\mu\)M, \(n = 4, p < 0.05\)). Significant changes in Ca\(^{2+}\) transient magnesium were not detected at a concentration of 0.5 \(\mu\)M caged-NAADP (Fig. 1D). The specific effect of photoreleased NAADP was confirmed by comparison with effects of photolysis of the same concentrations of caged-phosphate (Fig. 1D). Photoreleased phosphate failed to increase whole cell Ca\(^{2+}\) transients at all concentrations tested.

**Effects of NAADP Applied via a Patch Pipette on Myocyte Contraction**—In another series of experiments, guinea pig ventricular myocytes were stimulated to fire action potentials at a frequency of 1 Hz and NAADP was introduced to the cytosol at various concentrations by diffusion of NAADP from the patch...
pipette solution. Control contractions were measured in the permeabilized patch configuration before physical rupture of the patch by suction that allowed NAADP access to the cytosol. NAADP applied in this way failed to cause any significant consistent changes in contraction amplitude when applied at a range of concentrations from 50 nM to 100 μM. There was, however, a significant reduction in contraction magnitude in the presence of NAADP at concentrations higher than 500 μM. 1 mM NAADP caused a significant decrease in contraction of 26 ± 5% 3 min after gaining access to the cytosol following physical rupture of the patch (n = 10, p < 0.05). This reduction may reflect the profound self-inactivation mechanism that has been previously reported in a variety of mammalian systems exposed to higher concentrations of NAADP (16–19). Evidence that this action of high concentrations of NAADP is not simply a nonspecific toxic effect is presented in a later section. Because the discovery of the self-inactivation mechanism of NAADP in the sea urchin egg (12, 13), this property of NAADP has been used as the best tool to abolish NAADP signals in several systems such as mouse pancreatic acinar cells (14). The self-inactivation mechanism provides a possible reason as to why no increase in contraction was detected in experiments in which low concentrations of NAADP were applied via the patch pipette: diffusion may be too slow to allow sufficiently speedy access of low concentrations of NAADP to the cytosol to detect an increased contraction, and intermediate concentrations may provoke competing activating and inactivating mechanisms. The self-inactivation mechanism was further tested and put to good use in the next series of experiments.

**Effects of Photolysis of Caged NAADP on Whole Cell Ca\(^{2+}\) Transients in the Presence of High Concentrations of NAADP That Elicit the Self-inactivation Mechanism**—The effects of high concentrations of NAADP were investigated in myocytes in which NAADP was photoreleased from a caged analogue. Both NAADP (100 μM or 1 mM) and caged-NAADP (5 μM) were introduced into the cytosol through the patch pipette, allowing 5 min for diffusion. Whole cell Ca\(^{2+}\) transients (accompanying action potentials) were evoked by stimuli at 0.5 Hz, as in the experiments with caged NAADP alone. In the presence of 100 μM NAADP, the increase in the peak Ca\(^{2+}\) transient following photolysis of caged NAADP was significantly reduced from 41 ± 10% (Fig. 2A, bar 1, n = 8, p < 0.05) to 12 ± 4% (Fig. 2A, bar 2, n = 5, p < 0.05). The effects of 1 mM NAADP were even more substantial since there was no increase in the whole cell Ca\(^{2+}\) transient following photorelease of NAADP when this concentration of NAADP was applied to the cell via the patch electrode (Fig. 2, A and B, 0.3 ± 6%, n = 6, p > 0.05) (bar 3). The data clearly demonstrate a self-inactivating mechanism for high concentrations of NAADP in cardiac myocytes.

**Influence of Bafilomycin A on the Enhancement of Whole Cell Ca\(^{2+}\) Transients by Photoreleased NAADP**—It has been reported that NAADP may release Ca\(^{2+}\) from an acidic compartment in several mammalian cells (20–24). In such systems, a vacuolar proton pump inhibitor, bafilomycin A has been used to collapse the H\(^+\) gradient across the organelle membrane and this subsequently results in an inhibition of Ca\(^{2+}\) uptake (25, 26). As a consequence, bafilomycin A has been applied as a useful experimental tool to test for the involvement of acidic stores in the control of Ca\(^{2+}\) signals in a variety of cell types (20–24, 27, 28).

The effects of bafilomycin A on whole cell Ca\(^{2+}\) transients were therefore investigated in guinea pig ventricular myocytes. Cells were loaded with fluo-4 AM and field stimulated to produce whole cell Ca\(^{2+}\) transients. When such cells were exposed to bafilomycin A (1 μM for 3 min), peak whole cell Ca\(^{2+}\) transients showed a 21 ± 4% reduction compared with control levels (n = 6, p < 0.05). In cells that were loaded with fluo-4 AM and superfused with an identical solution lacking bafilomycin A, there was no significant change in peak Ca\(^{2+}\) transient magnitude over the time course of the experiments. In a second series of experiments, the effects of bafilomycin A were investigated in cells loaded with fluo-4 and 5 μM caged NAADP, and stimulated via the patch pipette to elicit Ca\(^{2+}\) transients. Exposure to 1 μM bafilomycin A again caused a significant reduction in the magnitude of the whole cell Ca\(^{2+}\) transient (red trace in Fig. 3B, 17 ± 5%, n = 6, p < 0.05) that was similar to that seen in the field stimulated cells. Subsequent photorelease of NAADP from caged-NAADP in cells exposed to bafilomycin A...
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A. whole cell Ca\(^{2+}\) transients recorded from a representative cell. The black trace shows the Ca\(^{2+}\) transient under control conditions. The red trace represents the Ca\(^{2+}\) transient 3 min after application of bafilomycin (1 \(\mu\)M) showing 17 \(\pm\) 5\% reduction from controls (n = 6, p < 0.05). The blue trace shows Ca\(^{2+}\) transients observed 60 s after photorelease of NAADP (5 \(\mu\)M in the patch pipette) in the presence of bafilomycin A; under these conditions, there was no significant increase the Ca\(^{2+}\) transient (6 \(\pm\) 2\%, n = 6, p > 0.05). B, whole cell Ca\(^{2+}\) transients recorded from a representative cell. The black trace shows the Ca\(^{2+}\) transient under control conditions. Application of a mixture of ryanodine (2 \(\mu\)M) and thapsigargin (2 \(\mu\)M) significantly reduced Ca\(^{2+}\) transient magnitude (red trace). Subsequent photorelease of NAADP (5 \(\mu\)M caged NAADP; blue trace) in the presence of ryanodine and thapsigargin failed to increase the Ca\(^{2+}\) transient. C, mean data from several cells. In the presence of (2) bafilomycin A (1 \(\mu\)M) or (3) a mixture of ryanodine (2 \(\mu\)M) and thapsigargin (2 \(\mu\)M), photorelease of NAADP (5 \(\mu\)M in the patch pipette) was without significant effect on Ca\(^{2+}\) transient amplitude. D, photorelease of NAADP (5 \(\mu\)M in the patch pipette) was without significant effect on L-type Ca\(^{2+}\) currents activated by step depolarizations from –40 mV to potentials in the range –30 to +50 mV.

Effects of Bafilomycin A and Self-inactivating Concentrations of NAADP on Cell Contraction Are Not Additive—In further experiments, the effects of bafilomycin A on myocyte contraction were investigated. In cells stimulated to fire action potentials at 1 Hz, exposure of guinea pig ventricular myocytes to bafilomycin A (1 \(\mu\)M for 3 min) reduced contraction amplitude by 20 \(\pm\) 4\% (n = 8, p < 0.05). In cells that had been pre-exposed to bafilomycin A, application of 1 mM NAADP from a patch pipette failed to cause further reduction of contraction (compared with control levels, a decrease of 21 \(\pm\) 2\% 3 min after application of both 1 mM NAADP and 1 \(\mu\)M bafilomycin A, n = 6, p < 0.05), in contrast to the effects of this concentration of NAADP in the absence of bafilomycin A reported above. The lack of further effect of 1 mM NAADP in bafilomycin A-treated cells demonstrates that the actions of this concentration of NAADP on contraction do not result from a nonspecific toxic effect.

Involvement of the SR in the Actions of NAADP—Next, a possible involvement of the SR in the actions of NAADP were investigated by testing the effects of NAADP on whole cell Ca\(^{2+}\) transients in the presence of agents that inhibit SR function. Ryanodine and thapsigargin were used to suppress SR function by respectively blocking the release of Ca\(^{2+}\) from the SR via ryanodine receptors and preventing Ca\(^{2+}\) uptake by the Ca\(^{2+}\)-ATPase. When cells were incubated with 2 \(\mu\)M ryanodine together with 2 \(\mu\)M thapsigargin (Ry/Thaps) for 5 min, the peak whole cell Ca\(^{2+}\) transient was reduced to 38 \(\pm\) 9\% of control (Fig. 3B, red trace, n = 4, p < 0.05). In such cells, subsequent photorelease of NAADP from caged-NAADP (5 \(\mu\)M in the patch pipette) failed to cause any significant change in the amplitudes of whole cell Ca\(^{2+}\) transients (measured 60 s after photolysis, blue trace in Fig. 3B and C, 3 \(\pm\) 3\%, n = 4, p > 0.05).

To examine how NAADP interacts with SR Ca\(^{2+}\) handling, the Ca\(^{2+}\) content of the SR was measured before and after photorelease of NAADP. The SR Ca\(^{2+}\) content was evaluated by
solution to guinea pig ventricular myocytes (field stimulated at 1 Hz to fire action potentials and hence contract). A rapid switch system was used for solution exchange and cell contraction was monitored with an endoscope detection system. NAADP-AM caused a consistent increase in myocyte contraction that reached a peak in about 20 s (Fig. 4A, center panel; increase of 28 ± 5% at 20 s, n = 8, p < 0.05). Time controls showed that rapid switch to a solution without NAADP-AM did not cause any significant changes over the same period (Fig. 4A, left panel; n = 6, p > 0.05). In cells that had been pretreated with bafilomycin A (1 μM), application of NAADP-AM (60 nM) in the continued presence of bafilomycin A failed to cause any increase in myocyte contraction over the time period studied (Fig. 4A, right panel; -1 ± 1% at 20 s, n = 6, p > 0.05).

**Effects of NAADP-AM on Ca\(^{2+}\) sparks in rat ventricular myocytes**—Ca\(^{2+}\) sparks were recorded in rat ventricular myocytes in preference to guinea pig cells (see Ref. 30), because under our experimental conditions, guinea pig ventricular myocytes fail to exhibit Ca\(^{2+}\) sparks. 20 s after application of 60 nM NAADP-AM by rapid switch, both the amplitude and frequency of Ca\(^{2+}\) sparks were significantly increased (Fig. 4B; Ca\(^{2+}\) spark frequency: from 2.67 ± 1.64 to 4.52 ± 1.97 sparks/s/100 μm; amplitude: increase above control of 37.0 ± 6.3%, n = 6 p < 0.05). The background Ca\(^{2+}\) level, as indicated by the baseline fluorescence, was also increased by 24.5 ± 5.4% (n = 6, p < 0.05). The effect of NAADP on Ca\(^{2+}\) spark amplitude is particularly interesting since this observation is thought to provide additional evidence that the Ca\(^{2+}\) load of the SR is increased under these conditions. The effects of NAADP-AM on the frequency and amplitude of Ca\(^{2+}\) sparks, and on background Ca\(^{2+}\) levels were all suppressed by bafilomycin A: in cells pretreated with bafilomycin A (1 μM) for 3 min, rapid application of 60 nM NAADP-AM in the continued presence of bafilomycin A failed to cause any significant effect in a spark amplitude, frequency and background Ca\(^{2+}\) level (measured 20 s after application of NAADP-AM, Fig. 4B; Ca\(^{2+}\) spark frequency: from 3.52 ± 1.03 to 3.87 ± 0.94 sparks/s/100 μm, amplitude: −1.2 ± 8.4%, background Ca\(^{2+}\) level: 4 ± 2.4%, n = 7, p > 0.05).

**Specific Effect of Bafilomycin A on Acidic Compartments**—The specificity of the action of bafilomycin A on NAADP-sensitive stores was tested by labeling targeted organelles with...
LysoTracker Red, a weak base that selectively accumulates and labels acidic compartments. Bodipy-FL-ryanodine and Bodipy-TR-thapsigargin were used as markers for the SR. The upper panels of Fig. 5 show confocal images of guinea pig ventricular myocytes labeled with Bodipy-FL-ryanodine, Bodipy-TR-thapsigargin, and LysoTracker Red. The LysoTracker Red labeling of the acidic compartments (Fig. 5A) showed a punctate staining pattern throughout the cell that was clearly different from that seen with SR staining using either Bodipy-FL-ryanodine (Fig. 5B) or Bodipy-TR-thapsigargin (Fig. 5C). Upon application of bafilomycin A (1 μM for 20 min), a dramatic reduction in LysoTracker Red staining was observed (Fig. 5A, lower panel). In contrast, the Bodipy-FL-ryanodine or Bodipy-TR-thapsigargin labeling were unaffected by bafilomycin A (Fig. 5, B and C, lower panels). The data demonstrate that bafilomycin A acts specifically on the acidic store labeled by LysoTracker Red without affecting the integrity of the SR.

Role of NAADP during β-Adrenoceptor Stimulation—Finally, we considered the physiological relevance of NAADP in cardiac myocytes. It has been suggested that ADP-ribosyl cyclase may be responsible for catalyzing NAADP synthesis, and previously it has been reported that the activity of ADP-ribosyl cyclase may be up-regulated by β-adrenoceptor stimulation (31).

The effects of bafilomycin A and of high self-inactivating concentrations of NAADP on myocyte contraction were also investigated during β-adrenoceptor stimulation. Contraction were measured from the video image of cells using an edge detection technique. Treatment of single guinea pig ventricular myocytes with 2 nM isoproterenol caused significant increases in the amplitude of contraction (Fig. 6F, bars 1 and 2, 45 ± 8% increase over that in the absence of isoproterenol, n = 6, p < 0.05). In these experiments, 1 mM NAADP was included in the patch pipette solution but could not enter the cytosol until the cell membrane under the patch pipette was ruptured (though the permeabilized patch conditions allowed electrical stimulation). When the membrane under the patch pipette was physically ruptured to allow access of NAADP to the cytosol during the continued activation of β-adrenoceptors by isoproterenol, 1 mM NAADP caused a very marked reduction in the magnitude of contraction (40 ± 6% decrease of the amplitude of contraction; Fig. 6F, bars 3 and 4, n = 6, p < 0.05). This reduction of contraction was substantially larger than the reduction caused by 1 mM NAADP in the absence of 2 nM isoproterenol (Fig. 6D, F, bar 2) 26 ± 5%, n = 10, p < 0.05) (Fig. 6F, bars 1–4). These observations are again consistent with an effect of β-adrenoceptor stimulation to increase endogenous levels of NAADP: the self-inactivating concentration of NAADP (1 mM) would be expected to have a greater effect on the actions of endogenous NAADP when levels are raised following β-adrenoceptor stimulation.
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In this study, we have provided the first observations concerning NAADP actions in intact cardiac myocytes. The most direct observations are provided by experiments in which NAADP was photoreleased from a caged analogue. It is clear that NAADP applied to the cytosol in this way increased the amplitude of whole cell Ca\(^{2+}\) transients. It is interesting that the effects were accompanied by a small but significant increase in the resting level of cytosolic Ca\(^{2+}\) and that the effects were slow to develop, taking tens of seconds. The effects were not artifacts of photorelease since they showed a dependence on the concentration of caged NAADP and were not seen following photorelease of the free compound (such as Ca\(^{2+}\) probes and Ca\(^{2+}\) chelators) remarkably quickly, particularly at physiological temperature.

The observations with bafilomycin A are particularly important. This substance prevented all effects of NAADP observed, whether these involved whole cell Ca\(^{2+}\) transients or myocyte contractions. Furthermore, bafilomycin A prevented the actions of both photoreleased NAADP and NAADP-AM, two differing methods of application of NAADP to the cytosol. Because bafilomycin A is known to inhibit Ca\(^{2+}\) uptake into acidic organelles by collapse of the proton gradient generated by the H\(^+\)-ATPase, it has been used extensively as an experimental tool in the field of NAADP signaling to support an involvement of acidic stores (20–24, 27, 28). It appears that in cardiac myocytes, as in many other mammalian cell types, NAADP Signaling in the Heart

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The observations with bafilomycin A are particularly important. This substance prevented all effects of NAADP observed, whether these involved whole cell Ca\(^{2+}\) transients or myocyte contractions. Furthermore, bafilomycin A prevented the actions of both photoreleased NAADP and NAADP-AM, two differing methods of application of NAADP to the cytosol. Because bafilomycin A is known to inhibit Ca\(^{2+}\) uptake into acidic organelles by collapse of the proton gradient generated by the H\(^+\)-ATPase, it has been used extensively as an experimental tool in the field of NAADP signaling to support an involvement of acidic stores (20–24, 27, 28). It appears that in cardiac myocytes, as in many other mammalian cell types,
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bafilomycin-sensitive acidic stores may again be involved in the actions of NAADP. In this context it is particularly important that the SR remained structurally intact (as indicated by Bodipy-FL-ryanodine and Bodipy-TR-thapsigargin labeling) during exposure to 1 μM bafilomycin A, while under the same conditions the LysoTracker Red staining was greatly reduced, as expected if bafilomycin A collapses the proton gradients in the acidic organelles where LysoTracker Red is accumulated.

The proposal that actions of NAADP in cardiac myocytes are associated with an increase in the Ca\(^{2+}\) load of the SR is supported by two independent observations. The first is that, following photorelease of NAADP the magnitude of the Ca\(^{2+}\) transient in response to rapid application of caffeine was significantly greater than that prior to photorelease. Measurement of the Ca\(^{2+}\) liberated from the SR in response to caffeine (acting as an agonist at the RyR2s) is a well established technique for assessing SR Ca\(^{2+}\) load, and our data provide strong evidence for an enhancement in SR Ca\(^{2+}\) load following NAADP application. The second observation is that NAADP-AM increased the amplitude as well as the frequency of Ca\(^{2+}\) sparks. The increase in Ca\(^{2+}\) spark amplitude is again important evidence for an increase in SR Ca\(^{2+}\) load.

The enzyme responsible for synthesis of NAADP is thought to be ADP ribosyl cyclase working by base exchange with NADP as the substrate. Interestingly, the same enzyme catalyzes the synthesis of cADP-ribose, using NAD as the substrate. As with the observations reported here for NAADP, photorelease of cADP-ribose from a caged analogue increases whole cell Ca\(^{2+}\) transients and increases the frequency of Ca\(^{2+}\) sparks, but in the case of cADP-ribose (at least for short times of exposure of ~3 min) there is no accompanying increase in the Ca\(^{2+}\) load of the SR (because the Ca\(^{2+}\) signal accompanying emptying of the SR by caffeine is unchanged, and the amplitude of Ca\(^{2+}\) sparks is not increased) (30). There is therefore a very interesting contrast between the actions of cADP-ribose and NAADP, even though both can increase Ca\(^{2+}\) transients and spark frequency, and both could potentially be synthesized by action of the same enzyme.

The effects of NAADP on whole cell Ca\(^{2+}\) transients were suppressed by application of ryanodine and thapsigargin, providing strong evidence that a functioning SR is required for the effect. However, the observations with bafilomycin A show that disruption of the acidic store without an effect on the integrity of the SR is sufficient to prevent NAADP actions. A hypothesis that combines these observations is that NAADP leads to Ca\(^{2+}\) release from the bafilomycin-sensitive acidic store and this released Ca\(^{2+}\) is in turn taken up by the SR, giving an increased Ca\(^{2+}\) load of the SR (Fig. 7). A functional SR is therefore required for the effect, but the primary action of NAADP is to release Ca\(^{2+}\) from the acidic store. Such a mechanism would therefore represent an interaction between two different Ca\(^{2+}\) pools, with NAADP effectively enlarging the SR Ca\(^{2+}\) pool by mobilizing Ca\(^{2+}\) from acidic organelles.

An additional point of interest is that bafilomycin A and NAADP (photoreleased, or applied as NAADP-AM) have opposite effects on Ca\(^{2+}\) transients, despite the fact that both are believed to discharge the acidic Ca\(^{2+}\) stores. This apparent paradox may arise from differences in timing of Ca\(^{2+}\) release from the lysosome-related stores and, perhaps, from differences in the precise site of release of Ca\(^{2+}\) in relation to uptake sites on the SR. NAADP is expected to cause rapid local release of Ca\(^{2+}\), permitting a sufficiently large rise in Ca\(^{2+}\) close to the SR uptake sites to overcome local Ca\(^ {2+}\) buffering mechanisms and provoke the SR Ca\(^{2+}\)-ATPase to take up this extra Ca\(^{2+}\), thereby enhancing SR Ca\(^{2+}\) load. Because mechanisms to maintain Ca\(^{2+}\) levels in the acidic stores would still be operation, NAADP would be able to provide a maintained release of Ca\(^{2+}\) from these stores that could be taken up by the SR. In contrast, bafilomycin A (under these conditions) may cause a much slower leak of Ca\(^{2+}\) from the lysosome-related compartments, with the result that Ca\(^{2+}\) in the vicinity of the SR uptake sites does not rise sufficiently to cause additional Ca\(^{2+}\) uptake. Ca\(^ {2+}\) buffering mechanisms may play an important role in preventing a significant rise in cytosolic free Ca\(^{2+}\) concentration in the presence of such a slow leak of Ca\(^{2+}\) from the acidic stores.

In addition, the site of Ca\(^ {2+}\) release provoked by NAADP and bafilomycin A may differ. In the case of NAADP, release sites may be strategically placed in close apposition to the SR Ca\(^{2+}\) uptake. In contrast, the leak sites for Ca\(^{2+}\) following exposure to bafilomycin A may be more diffusely located on the acidic store membrane, so that Ca\(^{2+}\) buffering and mechanisms for removal of Ca\(^ {2+}\) across the sarclemma may operate to limit the amount of Ca\(^{2+}\) that the SR can take up.

The above arguments provide an explanation for the opposite actions of NAADP and bafilomycin A observed in our experiments. We propose that bafilomycin can depress Ca\(^{2+}\) transients by slow depletion of Ca\(^{2+}\) from the acidic stores, which in turn would prevent an action of NAADP to release Ca\(^ {2+}\) from this store. If, as we suggest, there are endogenous levels of NAADP that continuously provoke Ca\(^ {2+}\) release from the acidic stores, it is not surprising that bafilomycin can prevent these actions and therefore reduce Ca\(^ {2+}\) transients. Consistent with these arguments is the observation that self-inacti-
vating concentrations of NAADP (see below) have a similar effect on cell contraction to that of bafilomycin.

The self-inactivation property of bafilomycin, in which high concentrations of NAADP inhibit its actions on the whole cell Ca^{2+} transient and the magnitude of contraction, was seen in cardiac myocytes. This is consistent with previous reports in other mammalian cells (16–19). This self-inactivating property has been useful in that high concentrations of NAADP can be used as a specific antagonist to the effects of lower concentrations of this molecule. This was illustrated by the reduction of the effects of photoreleased NAADP by 100 μM NAADP and by the prevention of these effects by 1 mM NAADP. It seems unlikely that the effects of 1 mM NAADP result from a simple nonspecific toxic action since there was no further effect of this concentration of NAADP in cells pretreated with bafilomycin A.

The reduction in contraction amplitude of ~20% seen with either bafilomycin A or NAADP might be taken to indicate that there is an ongoing influence of a NAADP-dependent pathway supported by endogenous NAADP acting via the bafilomycin-sensitive Ca^{2+} store. Further support for this proposal is provided by evidence for endogenous levels of NAADP in cardiac tissue provided here and elsewhere (9).

Our observations using Langendorff perfused intact hearts show that the endogenous NAADP level can be elevated upon application of the β-adrenoreceptor agonist isoproterenol. This would not be surprising if NAADP were synthesized in cardiac muscle by the action of ADP ribosyl cyclase, because Higashida et al. (31, 33) have reported that sympathetic stimulation may up-regulate the activity of this enzyme that is also responsible for synthesis of cADPR. Functional evidence to support elevated levels of NAADP during β-adrenoreceptor stimulation is provided by our observations: while there was a significant increase in peak whole cell Ca^{2+} transient magnitude with photorelease of NAADP in the presence of isoproterenol, this increase was significantly reduced compared with the effects of photoreleased NAADP in the absence of β-adrenoreceptor stimulation (Fig. 6, B–E). In addition, during β-adrenoreceptor stimulation, high concentrations (1 mM) of patch-applied NAADP caused a decrease in the amplitude of contraction in ventricular myocytes that was significantly larger than that seen in the absence of β-adrenoreceptor stimulation (Fig. 6F). This is consistent with the proposal that β-adrenoreceptor stimulation regulates endogenous levels of NAADP, such that there is effectively more NAADP present, so that there is a smaller increment of NAADP when exogenous NAADP is added, yet a greater effect of an antagonising influence. These observations therefore support the hypothesis that NAADP is an endogenous regulator of cardiac function with a physiological role.

In summary, our experiments provide the first description of the regulation by NAADP of cardiac excitation-contraction coupling. NAADP causes an increase in the whole cell Ca^{2+} transient by enhancing the Ca^{2+} loading of the SR. The ability of NAADP to release Ca^{2+} from a bafilomycin A-sensitive acidic Ca^{2+} store, together with evidence that NAADP production is regulated by β-adrenoreceptor stimulation, strongly indicate that NAADP may control an important novel mechanism underlying cardiac inotropy.

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