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**NAADP-mediated channel “chatter” in neurons of the rat medulla oblongata.****G. Cristina Brailoiu\*, Eugen Brailoiu\*, Raman Parkesh†, Antony Galione†, Grant C. Churchill†, Sandip Patel‡<sup>1</sup> and Nae J. Dun\***

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Running title: NAADP activates rat medulla oblongata neurons

**Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent  $\text{Ca}^{2+}$ -mobilizing messenger that stimulates  $\text{Ca}^{2+}$  release in a variety of cells. NAADP-sensitive  $\text{Ca}^{2+}$  channels are thought to reside on acidic  $\text{Ca}^{2+}$  stores and to be functionally coupled to inositol trisphosphate and/or ryanodine receptors located on the endoplasmic reticulum. Whether NAADP-sensitive  $\text{Ca}^{2+}$  channels “chatter” to other channels, however, is not clear. In the present study, we have used a cell-permeant NAADP analogue to probe NAADP-mediated responses in rat medulla oblongata neurons. NAADP acetoxymethyl ester (NAADP-AM) evoked global cytosolic  $\text{Ca}^{2+}$  signals in isolated neurons that were reduced in amplitude by removal of external  $\text{Ca}^{2+}$ , abolished by disruption of acidic compartments and substantially inhibited by blockade of ryanodine receptors. In rat medullary slices, NAADP-AM depolarized neurons from the nucleus ambiguus in the presence of intracellular EGTA but not of the faster  $\text{Ca}^{2+}$  chelator BAPTA. Depolarization was also dependent upon extracellular  $\text{Ca}^{2+}$ , acidic stores and ryanodine receptors. In voltage-clamp mode, NAADP-AM induced an inward current with a reversal potential of approximately 0 mV. Our data reveal the presence of acidic NAADP-sensitive  $\text{Ca}^{2+}$  stores in medulla neurons the mobilization of which results not only in global  $\text{Ca}^{2+}$  signals but also in local signals that activate non-selective cation channels on the cell surface resulting in depolarization. Thus, NAADP is capable of coordinating channels both within the cell interior and at the cell membrane representing a novel mechanism for excitation of central neurons.**

Keywords: NAADP, calcium, non-selective cation channels, patch-clamp.

Abbreviations used: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BAF, bafilomycin A1;  $[\text{Ca}^{2+}]_i$ , cytosolic calcium concentration; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; NAADP-AM, nicotinic acid adenine dinucleotide phosphate acetoxymethyl ester; NMDG, N-methyl-D-glucamine.

**INTRODUCTION**

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent mobilizer of intracellular  $\text{Ca}^{2+}$  stores [1, 2]. The actions of NAADP were first described in sea urchin eggs where it

targets a unique  $\text{Ca}^{2+}$  channel located on  $\text{Ca}^{2+}$  stores insensitive to inositol trisphosphate and cyclic ADP-ribose and thus, un-related to the endoplasmic reticulum [3-5]. The NAADP-sensitive  $\text{Ca}^{2+}$  channel has yet to be unequivocally defined at the molecular level [6] but the stores have been identified as acidic, lysosome-like organelles in most preparations studied [7]. Indeed, in a variety of cells, NAADP-induced  $\text{Ca}^{2+}$  release is inhibited by agents that interfere with acidic compartments, such as bafilomycin A1, a highly selective inhibitor of V-type  $\text{H}^+$ -ATPases [8]. These proteins are expressed on several acidic organelles and responsible for generation of proton gradients thought to drive  $\text{Ca}^{2+}$  uptake [7, 9-15]. One remarkable feature of the release process is that activation of NAADP receptors is invariably associated with the recruitment of inositol trisphosphate and/or ryanodine receptors via  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release [11, 16-20]. Thus, NAADP may serve to coordinate the activity of  $\text{Ca}^{2+}$ -sensitive endoplasmic reticulum based  $\text{Ca}^{2+}$  channels [2, 21-22]. That several extracellular stimuli have now been demonstrated to increase cellular NAADP levels [9, 14, 20, 23-27] establish NAADP as a bona fide  $\text{Ca}^{2+}$  mobilizing messenger critical for the generation of agonist-specific  $\text{Ca}^{2+}$  signals.

Although the mechanism by which NAADP mediates release of  $\text{Ca}^{2+}$  mobilization has been intensively investigated, much less explored are possible effects of NAADP on plasma membrane channels. In both mature starfish oocytes [28] and sea urchin eggs [23], NAADP responses initiate in the cell peripherally. In starfish, NAADP-mediated  $\text{Ca}^{2+}$  signals require functional inositol trisphosphate or ryanodine receptors for propagation, however, they are heavily reliant on removal of extracellular  $\text{Ca}^{2+}$  [19]. These data suggest that NAADP-mediated  $\text{Ca}^{2+}$  influx is the primary trigger. In the sea urchin, the first detectable NAADP-mediated event is a small, highly localized and rapid cortical "flash" that is followed by a  $\text{Ca}^{2+}$  wave that then traverses the cell entirety [23]. Removing extracellular  $\text{Ca}^{2+}$  from these cells blocks the former response only [23] again suggesting that NAADP mediates  $\text{Ca}^{2+}$  influx. A dependence for extracellular  $\text{Ca}^{2+}$  in NAADP-mediated  $\text{Ca}^{2+}$  signaling has also been noted in T lymphocytes [29] and NAADP demonstrated to activate TRPM2 channels [30] although relatively high concentrations are required to achieve the latter. In all cases, however, whether the observed influx is due to activation of a plasmalemmal NAADP-sensitive  $\text{Ca}^{2+}$  channel or mediated indirectly through prior store release is not clear.

There is increasing evidence that NAADP is an important second messenger in the nervous system [22, 31]. NAADP triggers  $\text{Ca}^{2+}$  release from brain microsomes [32] and cultured cortical neurons [11], and has been implicated in important neuronal functions, such as neurosecretion [33, 34], neurite outgrowth [11] and neuronal differentiation [12]. NAADP might also function as an extracellular messenger in several neural cell types [35]. Our previous autoradiographic analysis revealed wide and heterogeneous distribution of NAADP binding sites throughout the mammalian brain with particularly high levels noted in the medulla [36]. This region is crucial to the regulation of autonomic functions. Parasympathetic regulation of heart rate, for example, is mediated by cardiac vagal neurons located within the nucleus ambiguus [37].

In the present study, we used a newly described cell-permeant analogue of NAADP [20, 38] to probe the role of NAADP in medulla neurons using both dissociated and slice preparations. We provide evidence for the presence of functional NAADP receptors located on acidic  $\text{Ca}^{2+}$  stores and for coupling of these channels to channels on the plasma membrane. This newly described form of "chatter" [21] may provide a novel means of excitation of autonomic neurons.

## EXPERIMENTAL PROCEDURES

### NAADP-AM synthesis

NAADP (0.030 g, 0.040 mmol) was evaporated 2-3 times with 0.5 ml of diisopropylethylamine (DIEA) to form the diisopropylethylammonium salt. The salt was then dissolved in 2 ml of acetonitrile (CH<sub>3</sub>CN). To this was added DIEA (0.026 g, 0.201 mmol) and the mixture stirred at room temperature for 15 min under an argon atmosphere. Acetoxymethylene bromide (0.031, 0.201 mmol) was then slowly added to the above stirred solution and the resulting mixture stirred at room temperature for 24 h. After the completion of the reaction, the solids were filtered off and the solution evaporated to dryness to provide the acetoxymethyl derivate of NAADP as a light-yellow solid.

### Neuronal culture

Neurons from medulla oblongata were dissociated from neonatal (1-3-day-old) Sprague Dawley rats, by enzymatic digestion with papain followed by mechanical trituration, as previously described [11, 39]. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. After centrifugation at 500 x g, fractions enriched in neurons were collected and re-suspended in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing 2 mM glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin and 10 % fetal bovine serum. Cells were plated on round glass coverslips in 24-well plates. Cultures were maintained at 37° C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Localization of acidic compartments and endoplasmic reticulum in medulla oblongata neurons

Cultured neurons were incubated with LysoTracker Red (1 µM), ER-Tracker Green (1 µM), or a combination of both, for 30-60 min. In another series of experiments, neurons were incubated with LysoTracker Red (1 µM) following a 1-h pretreatment with either bafilomycin A1 (1 µM) or the vehicle DMSO (0.1 % v/v). Cells were fixed in 4% paraformaldehyde, mounted with Citifluor and examined under a confocal laser scanning microscope (Leica TCS SL) with excitation/emission wavelengths set to 488/520 nm for ER-Tracker Green and 543/620 nm for LysoTracker Red in the sequential mode.

### Measurement of cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>)

[Ca<sup>2+</sup>]<sub>i</sub> was measured by the calcium imaging technique, as previously described [11, 12]. Cultured neurons were loaded with the fluorescent Ca<sup>2+</sup> indicator fura-2 (3 µM) by incubation of the cells in Hank's balanced salt solution (HBSS) plus fura-2 AM for 45 min and HBSS alone for an additional 15-60 min to allow de-esterification of the dye. Coverslips were mounted in a 500 µl bath on the stage of a TE2000U Eclipse Nikon inverted microscope equipped with a Photometrics CoolSnap HQ CCD camera. Fura-2 fluorescence (excitation wavelength = 340 and 380 nm, emission wavelength = 520 nm) of single cells was acquired at a frequency of 0.3 Hz. Experiments were analyzed off-line using the MetaFluor software. The ratio of the fluorescence signals (340 nm/380 nm) was converted to Ca<sup>2+</sup> concentrations as described [40].

### Intracellular microinjection

Concurrent intracellular microinjection and calcium imaging were performed as previously described [41]. Femtotips II (Eppendorf) were back-filled with injection solutions. Eppendorf Transferman NK2 and Femtojet were used for semiautomatic microinjection. Injection settings were adjusted to inject < 1% of cell volume. Injections were performed with solution containing (in mM): 110 KCl, 10 NaCl, 20 HEPES (pH 7.2) with or without inositol trisphosphate (10 µM).

### Electrophysiology

Visual patch-clamp recordings were made from neurons of nucleus ambiguus in neonatal rat medullary slices. The whole-cell patch-clamp technique was similar to that described earlier [39]. The hindbrain was dissected and placed in ice-cold Krebs solution of following composition (in mM): 127 NaCl, 1.9 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, and 10 glucose, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Brainstem slices of 200 µm thickness were prepared using a vibratome. In experiments where Ca<sup>2+</sup>-free Krebs solution was used, Ca<sup>2+</sup> was omitted and EGTA (2.5 mM) was added. In some experiments, low external Na<sup>+</sup> solution containing 26 mM NaCl and 127 mM N-methyl-D-glucamine (NMDG) was used. Recordings were conducted at room temperature (20 ± 1°C) from neurons in the ventral perimeter of nucleus ambiguus, previously identified as cardiac vagal neurons [42]. Patch electrodes pulled from thin-walled borosilicate glass capillaries were filled with a solution containing (in mM): 130 K gluconate, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 4 ATP, 0.3 GTP, 10 EGTA and 10 HEPES, and had a resistance of 2-5 MΩ; the pH of the solution was adjusted to 7.3. In some experiments EGTA was replaced with BAPTA (10 mM). In other experiments, K<sup>+</sup> was replaced with Cs<sup>+</sup> in intracellular solution. Signals were recorded using an Axopatch 1C amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA) and a Digidata 1320 digitizer in voltage- or current-clamp mode, filtered at 2 KHz, displayed on a two-channel Gould chart recorder RS 3200. Experimental protocols were controlled and data acquired by a personal computer using the Clampex 8.0 software (Axon Instruments). Steady-state I-V relationships of NAADP-AM-induced currents were investigated in rat nucleus ambiguus neurons voltage-clamped to -60 mV. The I-V relationships were obtained by applying a series of 400 ms voltage command steps every 5 s from a holding potential of -60 mV to potentials varying from -120 to +60 mV, with 10 mV increments, before and during the superfusion of NAADP-AM. Currents elicited by each voltage command step in control media were subtracted from their counterparts in the presence of NAADP-AM to yield a steady-state I-V curve of NAADP-AM-induced currents.

### Chemicals

ER-Tracker Green, LysoTracker Red and fura-2 AM were from Molecular Probes (Eugene, OR); ryanodine, bafilomycin A1, xestospongin C, charybdotoxin, and apamin were from Calbiochem, EMD Biosciences, La Jolla, CA) and N-methyl-D-glucamine was from Sigma-Aldrich (St. Louis, MO).

### Statistical analysis

Paired *t*-test followed by one way ANOVA was used to evaluate significant differences between controls and NAADP-AM-treated neurons: *P*<0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

We first measured [Ca<sup>2+</sup>]<sub>i</sub> in isolated medulla oblongata neurons loaded with the fluorescent Ca<sup>2+</sup> indicator, fura-2. Basal [Ca<sup>2+</sup>]<sub>i</sub> was 86 ± 2.6 nM (n = 126). To test for the presence of NAADP-sensitive Ca<sup>2+</sup> channels, cells were perfused with NAADP-AM, a cell-permeant analogue of NAADP that has recently been shown to induce Ca<sup>2+</sup> signals in cortical neurons, cardiomyocytes and lymphoblasts [20, 38, 43]. In medulla oblongata neurons, NAADP-AM (500 nM) increased [Ca<sup>2+</sup>]<sub>i</sub> in 84 out of the 126 cells examined (Fig. 1). Two profiles of [Ca<sup>2+</sup>]<sub>i</sub> increase were identified: i) [Ca<sup>2+</sup>]<sub>i</sub> oscillations with a mean amplitude increase of 417 ± 7.3 nM (n = 63, Fig. 1A) and ii) slow and sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> with a mean amplitude of 483 ± 9.4 nM (n = 21, Fig. 1B). These data provide direct evidence for the presence of functional NAADP receptors in the medulla neurons consistent with our

previous autoradiography analysis [36]. In  $\text{Ca}^{2+}$ -free saline, application of NAADP-AM (500 nM) produced either  $[\text{Ca}^{2+}]_i$  oscillations (Fig. 1C, solid line;  $n = 26/35$ ) or rapid transient responses (Fig. 1C, dotted line;  $n = 9/35$ ). Thus, NAADP mobilizes intracellular  $\text{Ca}^{2+}$  stores as shown previously [9, 16]. The mean amplitude of both responses in  $\text{Ca}^{2+}$ -free medium ( $302 \pm 4.6$  nM,  $n = 26$  and  $327 \pm 3.4$  nM,  $n = 9$ ) was, however, reduced relative to those in  $\text{Ca}^{2+}$ -containing medium (see above). These data indicate that NAADP stimulates both  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  influx.

In many intact cells, NAADP-mediated  $\text{Ca}^{2+}$  signals are sensitive to inhibitors of inositol trisphosphate/ryanodine receptors or depletion of endoplasmic reticulum calcium stores release [11, 16-20]. These data are consistent with functional coupling of NAADP receptors to endoplasmic reticulum  $\text{Ca}^{2+}$  channels but evidence for a more direct effect of NAADP on ryanodine receptors has also been proposed [44]. That NAADP-mediated  $\text{Ca}^{2+}$  signals in several cell types are also inhibited by interfering with acidic compartments [7, 9-15], however, is inconsistent with the latter mechanism. To determine the mechanism of action of NAADP in medulla neurons, we examined the effects of the V-type  $\text{H}^+$ -ATPase inhibitor bafilomycin A1 on NAADP responses. As shown in Fig. 1D, pretreatment with bafilomycin A1 (1  $\mu\text{M}$ , 60 min) completely abrogated the response to NAADP-AM ( $n = 114$ ). These data suggest that NAADP targets acidic  $\text{Ca}^{2+}$  stores. To define the contribution of endoplasmic reticulum  $\text{Ca}^{2+}$  channels to NAADP responses, we examined the effect of pre-treating cells with the ryanodine receptor antagonist, ryanodine (1  $\mu\text{M}$ , 10 min) or the inositol trisphosphate receptor blocker, xestospongine C (10  $\mu\text{M}$ , 15 min) on the responses to NAADP. As shown in Fig. 1E, only small amplitude ( $168 \pm 3.4$  nM;  $n = 76/107$ ) transient responses were observed in response to NAADP-AM in the presence of ryanodine (Fig. 1E). In marked contrast, NAADP responses in  $\text{Ca}^{2+}$ -free saline were unaffected by xestospongine C; both  $\text{Ca}^{2+}$  oscillations ( $n = 25/33$ ; Fig. 1F, solid line) and transient responses ( $n = 8/33$ ; Fig. 1F, dotted line) were observed that were similar in magnitude ( $308 \pm 3.7$  nM,  $n = 25$  and  $319 \pm 4.2$  nM,  $n = 8$ ) to those in the absence of the drug (Fig. 1C). Xestospongine C inhibited  $\text{Ca}^{2+}$  responses to microinjected inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) by 72 % ( $n = 6$ ; Supplementary Fig. 1). Taken together, the above data suggest that NAADP receptors in medulla neurons are localized on acidic  $\text{Ca}^{2+}$  stores, the activation of which results in selective recruitment of ryanodine receptors on the endoplasmic reticulum in a manner similar to that reported in smooth muscle cells [9].

To determine the sub-cellular localization of acidic compartments in medulla neurons, we performed confocal microscopy of cells loaded with fluorescent weak base LysoTracker red. As shown in Figure 2, small discrete vesicles arranged in either a perinuclear fashion (Fig. 2A) or distributed more sparsely throughout the cytoplasm (Fig. 2B, E) were observed. Pretreatment with bafilomycin A1 (1  $\mu\text{M}$ , 60 min) substantially reduced staining, as expected (Fig. 2C). In order to compare the distribution of acidic compartments with the endoplasmic reticulum, we imaged cells co-loaded with LysoTracker and ER-Tracker. As shown in Fig. 2D-F, the distribution of the two organelles was distinct with individual acidic vesicles surrounded by the dense endoplasmic reticulum.

The reduction of NAADP-mediated  $\text{Ca}^{2+}$  signals upon removal of extracellular  $\text{Ca}^{2+}$  (Fig. 1C) prompted us to probe for possible functional coupling of NAADP receptors to plasma membrane channels. We therefore measured electrical responses in nucleus ambiguus neurons in brainstem slices by the whole-cell patch-clamp technique. The mean resting membrane potential and input resistance of nucleus ambiguus neurons were  $-55.9 \pm 1.3$  mV and  $715.4 \pm 45.9$  M $\Omega$ , respectively ( $n = 58$ ). Nearly all neurons were silent; and injection of depolarizing currents (100 – 500 pA, 300 ms) elicited repetitive firings followed by a hyperpolarization (data not shown) as previously reported [45]. In the presence of tetrodotoxin (1  $\mu\text{M}$ ), NAADP-AM (500 nM) application depolarized 11 out of 19 neurons

studied. The response of an exemplar neuron is shown in Fig. 3A, where NAADP-AM caused a depolarization of 10 mV. NAADP-mediated depolarization was reduced in amplitude in  $\text{Ca}^{2+}$ -free saline (Fig. 3B) consistent with possible  $\text{Ca}^{2+}$  entry.

Because all of the above experiments were performed in the presence of intracellular EGTA, the effects of NAADP-AM on membrane potential are unlikely to be mediated by global changes in  $[\text{Ca}^{2+}]_i$  since these would be effectively buffered by the chelator [46]. We therefore considered the possibility that the effects of NAADP may be due to local  $[\text{Ca}^{2+}]_i$  changes. To test this, we performed patch-clamp experiments using an intracellular solution containing the fast calcium chelator, BAPTA in place of EGTA [46]. Under these conditions, cells failed to depolarize in response to NAADP-AM (Fig. 3C, F). These data suggest that the effects of NAADP on membrane potential are not direct but instead secondary to local changes in cytosolic  $\text{Ca}^{2+}$ . To probe the role of acidic  $\text{Ca}^{2+}$  stores in this process, cells were pretreated with bafilomycin A1 (1  $\mu\text{M}$ , 60 min) prior to NAADP-AM challenge. As shown in Fig. 3D, depolarization in response to NAADP-AM was abolished. The effects of NAADP-AM on membrane potential were also inhibited by pretreatment with ryanodine (1  $\mu\text{M}$ , 10 min; Fig. 3E). Thus, the pharmacology of NAADP-mediated depolarization (summarized in Fig. 3F) and NAADP-mediated changes in global cytosolic  $\text{Ca}^{2+}$  (Fig. 1) are similar. Combined with the sensitivity of NAADP-mediated depolarization to slow and fast calcium chelators, we conclude that the effects of NAADP on membrane potential are due to coupled activation of subplasmalemmal NAADP-sensitive  $\text{Ca}^{2+}$  channels and ryanodine receptors. Thus,  $\text{Ca}^{2+}$  entry through this pathway might contribute to global cytosolic  $\text{Ca}^{2+}$  changes to NAADP, perhaps in conjunction with store-operated  $\text{Ca}^{2+}$  entry (Fig. 1). Although, our localization studies suggested that acidic compartments in these cells are predominantly cytoplasmic (Fig. 2) it is possible that a small subpopulation of peripheral acidic vesicles might not be resolvable using LysoTracker. Indeed, even in the large eggs of the sea urchin, acidic vesicles are relatively uniformly distributed, yet imaging of luminal pH clearly demonstrates heterogeneity in the spatial organization of NAADP-sensitive  $\text{Ca}^{2+}$  stores [47].

To further investigate the mechanism of NAADP-mediated depolarization, we examined current-voltage relationships. Subtraction of the two I-V curves obtained before and during perfusion with NAADP-AM reveals the presence of an inward current activated by NAADP (Fig. 4A-F). The mean reversal potential was  $\sim 0$  mV ( $n = 6$ ). As with depolarization, the NAADP-induced currents were markedly reduced in the presence of intracellular BAPTA in  $\text{Ca}^{2+}$ -free saline (Fig. 4G) and following pretreatment with ryanodine (Fig. 4H). Since in our experimental conditions, the equilibrium potential for potassium ions is  $-98$  mV and for sodium ions is  $+56$  mV, the reversal potential of the NAADP-AM-induced current suggests an involvement of a non-selective cationic conductance. Indeed, the inward component of the NAADP-AM-induced current was abolished and the reversal potential shifted to more negative values ( $-40$  mV) when extracellular  $\text{Na}^+$  was replaced with N-methyl-L-glucamine (NMDG) (Fig. 4I). Additionally, inward NAADP-mediated currents were readily detectable (but slightly reduced) when  $\text{K}^+$  in the intracellular solution was replaced with  $\text{Cs}^+$  to block voltage-gated  $\text{K}^+$  channels, and the slices pretreated with apamin and charybdotoxin to block  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Supplementary Fig. 2). Although depolarization was clearly reduced upon removing extracellular  $\text{Ca}^{2+}$  (Fig. 3B), we were unable to record an inward  $\text{Ca}^{2+}$  current, in the presence of NMDG (Fig. 4I). These data might suggest block of the channel by NMDG as reported for some non-selective cation channels [48] and/or the presence of an extracellular facilitatory  $\text{Ca}^{2+}$  binding site. Taken together, these data demonstrate functional coupling of NAADP-sensitive  $\text{Ca}^{2+}$  channels to  $\text{Ca}^{2+}$  activated non-selective cation channels.

NAADP-induced depolarization has previously been observed in starfish oocytes [49] however in those cells, the response was abolished upon removal of extracellular  $\text{Ca}^{2+}$  and

the reversal potential of recorded currents was +50 mV suggesting activation of a  $\text{Ca}^{2+}$  current [50]. It is possible that the currents we observe are due to activation of TRPM2 channels which are  $\text{Ca}^{2+}$  permeable non-selective cation channels that show little permeability in the presence of NMDG and which are regulated by both cytosolic and extracellular  $\text{Ca}^{2+}$  [51] consistent with the properties we describe (Fig. 4). Indeed, TRPM2 channels (previously named TRPC7) are expressed in the medulla [52]. Although previous studies have proposed direct modulation of TRPM2 by NAADP [51], we note that whole-cell recordings were performed in intracellular solutions in which  $[\text{Ca}^{2+}]_i$  was unbuffered raising the possibility that the observed effects might be secondary store release as reported here. Intriguingly, such a mechanism offers an alternative explanation for the sensitivity of NAADP-mediated currents to a cyclic ADP-ribose antagonist [51] if mobilization of NAADP-sensitive stores is coupled to activation of ryanodine receptors.

In summary, we provide evidence for the presence of acidic NAADP-sensitive  $\text{Ca}^{2+}$  stores in medulla neurons. We show that activation of NAADP receptors in these cells using a novel cell permeable analogue is intimately associated with selective recruitment of an endoplasmic reticulum  $\text{Ca}^{2+}$  channel to mediate global cytosolic  $\text{Ca}^{2+}$  changes. Additionally, we provide new evidence for recruitment of non-selective cation channels on the cell surface by NAADP resulting in depolarization. Based on differential sensitivity of the latter to slow and fast  $\text{Ca}^{2+}$  chelators, we infer that this “chatter” is due to local sub membrane  $\text{Ca}^{2+}$  fluxes again involving coordinated mobilization of acidic and endoplasmic reticulum  $\text{Ca}^{2+}$  stores. Indeed, it is notable that such a cortical NAADP-sensitive store has been recently visualized in sea urchin eggs [47]. The dependence of the cortical flash in sea urchin eggs on both NAADP and L-type calcium channels is consistent with NAADP acting indirectly possibly through prior mobilization of cortical calcium stores and subsequent depolarization. NAADP-mediated currents in starfish oocytes might also involve a cortical (endoplasmic reticulum)  $\text{Ca}^{2+}$  store based on their sensitivity to thapsigargin [50]. Thus, strategic placing of NAADP-sensitive  $\text{Ca}^{2+}$  stores may allow coordination of signaling events not only in the cell interior, but also at cell surface (Fig. 5). NAADP-mediated depolarization described here could provide a novel mechanism for excitation of cardiac vagal neurons possibly through NAADP synthesis via excitatory glutamatergic and/or cholinergic inputs [53].

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## FIGURE LEGENDS

### Figure 1 Effect of NAADP-AM on cytosolic $\text{Ca}^{2+}$ concentration in cultured rat medullary neurons.

Typical changes in  $[\text{Ca}^{2+}]_i$  of individual isolated medulla oblongata neurons stimulated with NAADP-AM either in the presence (A-B) or absence (C-F) of extracellular  $\text{Ca}^{2+}$ . Cells were pretreated with bafilomycin A1 (1  $\mu\text{M}$ , 60 min; D), ryanodine (1  $\mu\text{M}$ , 10 min; E) or xestospongine C (10  $\mu\text{M}$ , 15 min; F) as indicated.

### Figure 2. Localization of acidic compartments and endoplasmic reticulum in cultured rat medullary neurons.

(A-B) Confocal images of LysoTracker Red fluorescence showing two different patterns of staining. Bafilomycin A1 (1  $\mu\text{M}$ , 60 min) was added prior to addition of LysoTracker Red in C. (D-F) Co-localization of ER-tracker (D) and LysoTracker (E) fluorescence in the same cell. A merge of the two images is shown in F.

### Figure 3. Effect of NAADP-AM on membrane potential in nucleus ambiguus neurons.

(A-E) Effect of NAADP-AM on resting membrane potential of nucleus ambiguus neurons from rat medullary slices. Experiments were performed either in the presence of extracellular  $\text{Ca}^{2+}$  (A, E) or  $\text{Ca}^{2+}$ -free medium (B-D); tetrodotoxin (TTX, 1  $\mu\text{M}$ ) was included in the perfusion solution. Recordings in C were made using an intracellular solution which contained BAPTA in place of EGTA. Slices in D and E were pre-treated with bafilomycin A1 (BAF, 1  $\mu\text{M}$ , 60 min) or ryanodine (1  $\mu\text{M}$ , 10 min), respectively. Downward deflections are hyperpolarizing electrotonic potentials induced by constant current pulses (30 pA amplitude, 300 ms duration) used to monitor the membrane resistance. NAADP-AM was administered for the period indicated by the solid bar. F, Pooled data from the indicated number of neurons quantifying the extent of depolarization under the various conditions. \* statistically significant difference as compared to the depolarizations in regular Krebs. \*\* statistically significant difference as compared to depolarizations in  $\text{Ca}^{2+}$ -free Krebs solution ( $P < 0.05$ ).

### Figure 4. Effect of NAADP-AM on whole-cell currents recorded in nucleus ambiguus neurons.

(A-B) Typical whole-cell currents before (-NAADP-AM) and during (+NAADP-AM) administration of NAADP-AM, in response to voltage command steps from a holding potential of -60 mV to potentials varying from -120 mV to +60 mV (C). Steady-state I-V relationships before (○) and during (●) NAADP-AM superfusion are shown in panel D and the difference in currents in panel E. (F-I) Pooled difference currents (mean  $\pm$  s.e.m from 4-6 neurons perfused with NAADP-AM in control Krebs solution (F),  $\text{Ca}^{2+}$ -free Krebs solution and BAPTA-containing intracellular solution (G), control Krebs solution supplemented with ryanodine (1  $\mu\text{M}$ ) 10 min prior to recording (H) and modified Krebs solution in which NaCl was replaced with N-methyl-L-glucamine (NMDG) (I).

### Figure 5. Channel “chatter” in the medulla oblongata.

Proposed model of channel cross-talk whereby NAADP receptors localized on acidic  $\text{Ca}^{2+}$  stores are functionally coupled to ryanodine receptors to mediate global  $\text{Ca}^{2+}$  signals in the cell interior and local  $\text{Ca}^{2+}$  signals in the plasma membrane to mediate depolarization.

Figure 1

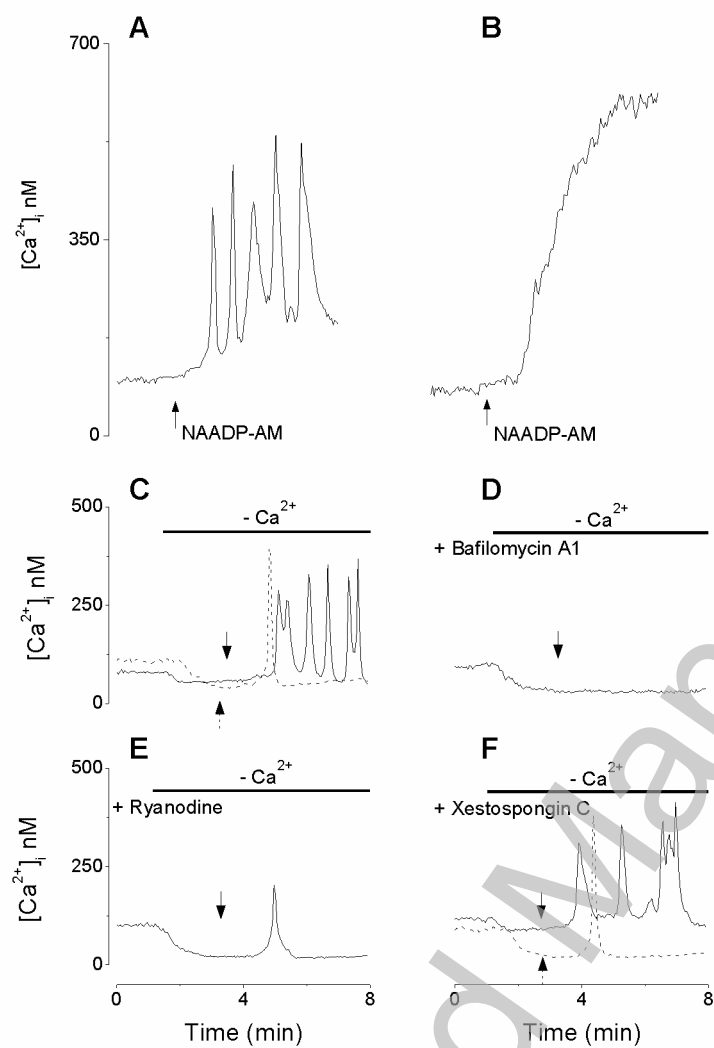
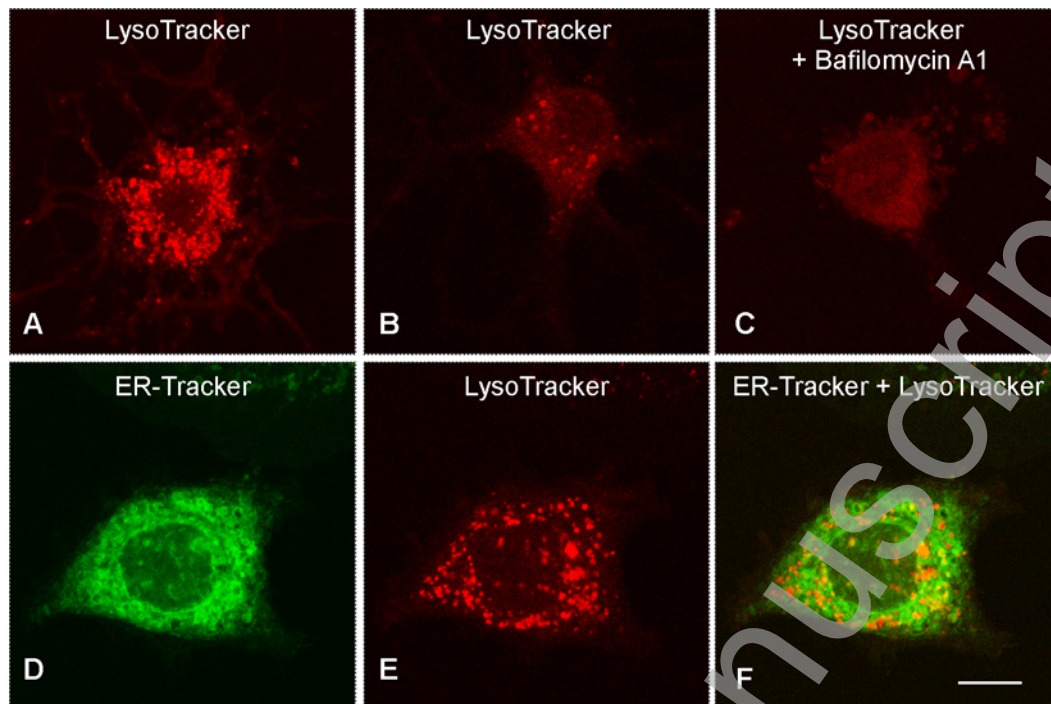
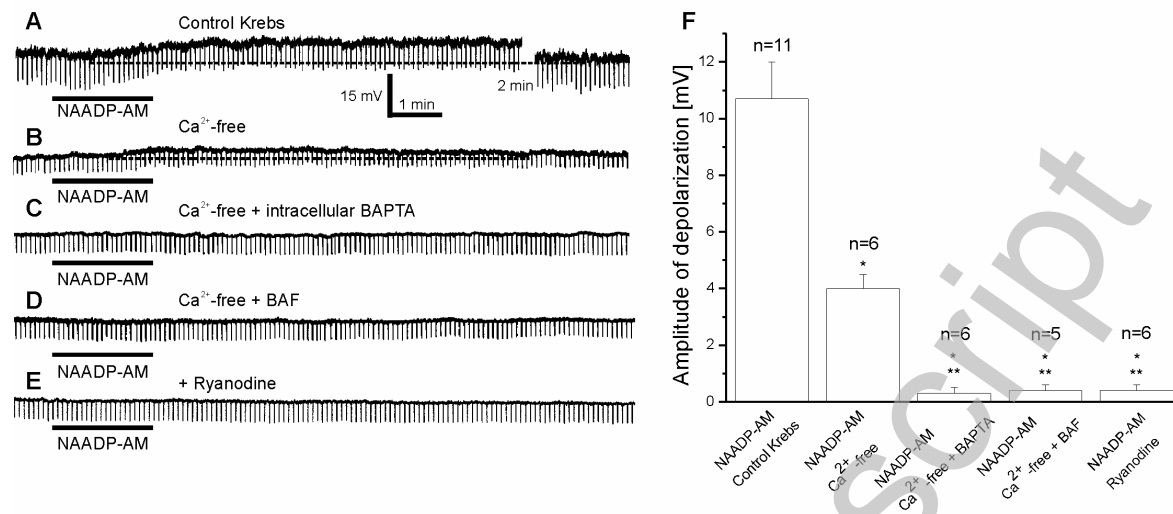


Figure 2



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Figure 3



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Figure 4

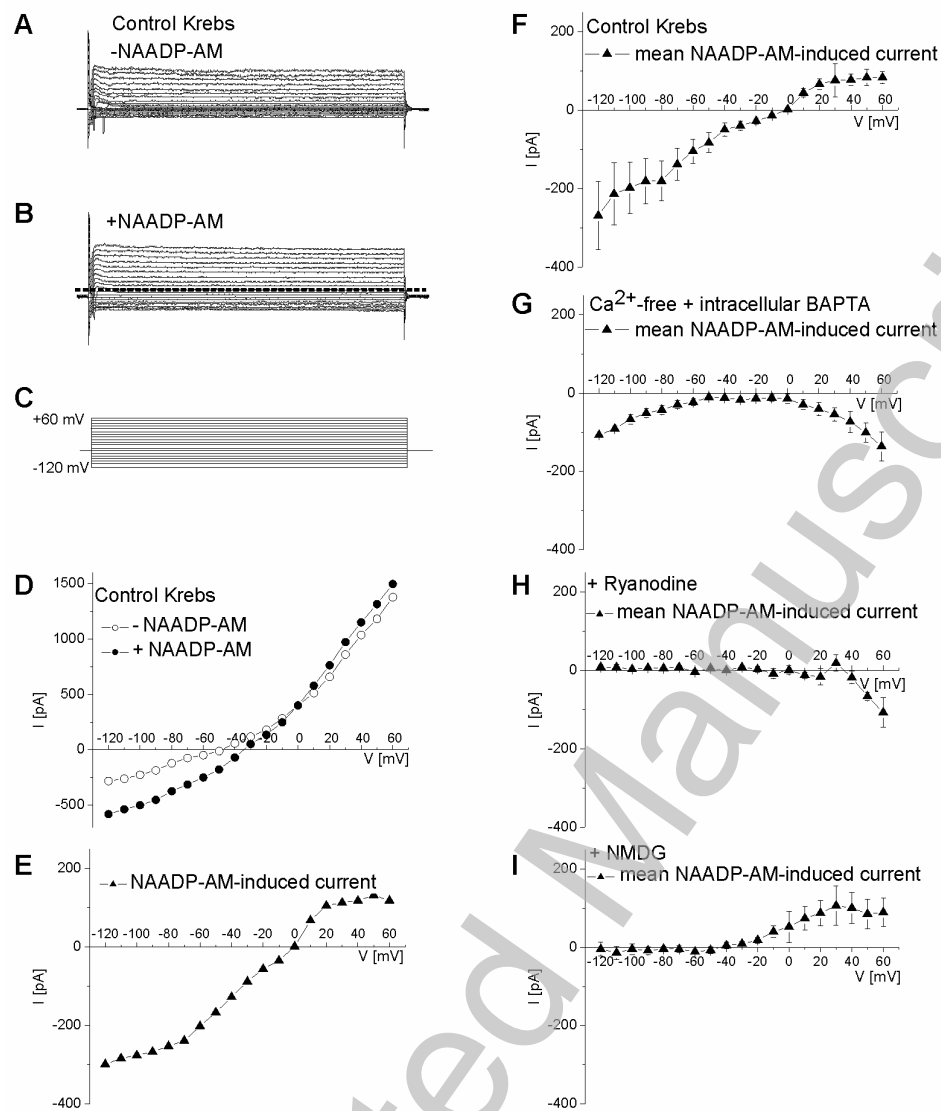




Figure 5

