NAADP mediates ATP-induced Ca^{2+} signals in astrocytes

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Abstract

Intracellular Ca^{2+} signals provide astrocytes with a specific form of excitability that enables them to regulate synaptic transmission. In this study, we demonstrate that NAADP-AM, a membrane-permeant analogue of the new second messenger Nicotinic Acid-Adenine Dinucleotide Phosphate (NAADP), mobilizes Ca^{2+} in astrocytes and that the response is blocked by Ned-19, an antagonist of NAADP signalling. We also show that NAADP receptors are expressed in lysosome-related acidic vesicles. Pharmacological disruption of either NAADP or lysosomal signalling reduced Ca^{2+} responses induced by ATP and endothelin-1, but not by bradykinin. Furthermore, ATP increased endogenous NAADP levels. Overall, our data provide evidence for NAADP being an intracellular messenger for agonist-mediated calcium signalling in astrocytes.

Key words: lysosomes, Ca^{2+} mobilization, glia, endotehlin-1, bradykinin, , Ned-19.

Abbreviations: cADPR: cyclic ADP-ribose; GPN: glycyl-L-phenylalanine 2-naphthylamide; IP_3: inositol 1,4,5-trisphosphate; NAADP: Nicotinic Acid-Adenine Dinucleotide Phosphate.

Short title: NAADP signalling in astrocytes

Introduction

After several years of intense research, classical paradigms on brain function have been challenged by data that demonstrate an active and key role for glial cells [1,2]. Based on Ca^{2+} signals rather than action potentials, astrocytes are excitable cells that assimilate information from different inputs and send instructive chemical signals to neighbouring cells. In this concept of an astrocyte-neuron network (or tripartite synapse), astrocytes sense neuronal activity as they express a wide range of functional neurotransmitter receptors capable of inducing intracellular Ca^{2+} increases. In turn, astrocytic Ca^{2+} increases trigger the release of many chemical transmitters [3], such as ATP, that signal back to neurons, modulating their excitability, or to astrocytes, reinforcing the Ca^{2+} wave and allowing transfer of information between them. Interestingly, although receptor-mediated Ca^{2+} signals in astrocytes mainly depend on stimulation of phospholipase C, inositol 1,4,5-triphosphate (IP_3) production, and Ca^{2+} release from the endoplasmic reticulum, they differ in magnitude and spatial- and temporal characteristics, which indicates that they are not equally capable of stimulating gliotransmitter release.

NAADP is the most recently discovered and most potent Ca^{2+} mobilizing second messenger [4,5] and has been shown to control cellular events such as insulin release from pancreatic β cells [6] or fertilization [7]. The main features of NAADP are its ability to mobilize Ca^{2+} from lysosome-related acidic organelles and its bell-shaped concentration-effect curve [4,6,8]. NAADP is also considered as a Ca^{2+} trigger signal in many cell systems. The molecular identity of NAADP receptors in mammals is still unresolved, although two-pore channels (TPC) have been demonstrated to be endogenous NAADP receptors in sea urchins and to act as NAADP-activating Ca^{2+} channels when they are overexpressed in particular mammalian cell lines [9,10]. It has also been suggested that transient receptor potential-mucolipin 1 channel (TRP-ML1), TRPM-2 and ryanodine receptors act as a NAADP-sensitive channels [10].

Widespread distribution of NAADP binding sites in the brain [11] and glutamate-induced NAADP increases in whole-brain tissue [12] support a role for this second messenger in the brain. But to date, there is no evidence for NAADP as an intracellular second messenger in astrocytes.

2. Materials and Methods

2.1. Materials

[^{32}P]Nicotinamide adenine dinucleotide ([^{32}P]NAD) (800 Ci/mmol) was purchased from Perkin-Elmer; lysotracker red, penicillin and streptomycin from Invitrogen, and endothelin-1 (human, porcine), bafilomycin A1 and 2-aminoethoxydiphenylborane (2-APB) from Tocris. Ned-19 and NAADP-AM were synthesized in house. All other compounds were from Sigma.

2.2. Tissue Culture

Primary cultures of cortical astrocytes were prepared from 1-day-old Sprague-Dawley rats following the protocol described previously [13]. Cells were plated into 35-mm dishes containing a coverslip at a density of 0.2 x 10^6 viable cells/ml in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin. The cultures were incubated at 37 ºC in a humidified atmosphere of 5% CO_2/95% air and used at 7- 20 days *in vitro*. Additionally, secondary cultures of cortical astrocytes were made by seeding cells in flasks. When these cells reached confluence, microglia and oligodendrocytes were removed by mechanically shaking and astrocytes were seeded onto coverslips.

2.3. Ca^{2+} Imaging

Astrocytes were incubated with fura-2/AM at 4 µM in Krebs buffer with the following composition (in mM): 119 NaCl, 4.75 KCl, 5 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 20 Hepes, 1.3 $CaCl_2$ and 5 glucose at pH 7.4. After 1 h, cells were washed and coverslips mounted in a static chamber on an inverted Nikon TE2000U microscope, and Ca^{2+} imaging performed as previously described [14]. Cells were incubated with antagonists for 20 min before stimulation. Cytosolic Ca^{2+} concentration is given as the ratio of fluorescence obtained exciting cells at 340 and 380 nm (F340:F380 ratio). 10-20 cells from at least three independent cultures were analysed. Data are expressed as mean ± SEM.

2.5. Immunohistochemistry and Confocal Microscope

Cells cultured on glass coverslips were loaded with 100 µM Ned-19 for 1 h at 37 ^{o}C and during the last 10 min 500 nM lysotracker red was added. Coverslips were mounted in a static chamber on a LSM 510 Meta confocal microscope (Zeiss) using a 40 x oil immersion lens. Lysotracker red fluorescence was visualized at 568 nm excitation (red, krypton laser) and emission collected with a 590 nm long-pass filter. Ned-19 fluorescence was monitored with excitation at 355 nm (UV, argon laser) and emission collected with a band-pass filter of 415 ± 30 nm. Image analysis was done with ImageJ 1.43r (Wayne Rasband, NIH), using Image CorrelationJ plugin (www.gcsca.net /IJ/ImageCorrelationJ.html).

2.7. [^{32}P]NAADP synthesis and NAADP radiobinding assay

[^{32}P]NAADP was synthesized in a two-step reaction as described previously [15]. To determine endogenous NAADP levels, we used a radioreceptor binding assay extensively described by us [15]. In brief, cells cultured in 60-mm dishes were stimulated with ATP or vehicle and NAADP content of samples was quantified using a competitive binding curve made with known concentrations of NAADP. Data were normalized to calculate the fold increases over basal NAADP levels.

3. Results

3.1. NAADP is an intracellular second messenger in astrocytes

To study whether NAADP is an endogenous molecule in astrocytes, we first determined intracellular NAADP levels. Our data showed that at rest conditions NAADP was present at very low concentrations, 5.09 ± 0.93 pmol/mg protein (n = 20). These results are in agreement with published results of resting NAADP levels in different tissues that range from 0.1 to 48 pmol/mg protein and particularly with resting NAADP levels detected in whole-brain cell suspensions (8.3 pmol/mg protein) [12].

We also investigated the presence of NAADP receptors in astrocytes. As their nature is not completely and unequivocally resolved in mammalian cells and the presence of multiple binding sites for NAADP has been suggested in the brain [11], we considered that Ned-19, a fluorescent highly

selective NAADP receptor antagonist [16], was the best tool for such studies. Figure 1 shows that Ned-19 binding sites, presumably the NAADP receptors, are present in intact astrocytes with a punctate distribution and with a very high degree of colocalization with labelling of acidic organelles with lysotracker (correlation coefficient of 0.62). It is worth noting that not all acidic vesicles stained with lysotracker express NAADP receptors, stressing the heterogeneity of these organelles. Conversely, a very small percentage of the punctate staining of NAADP receptors did not colocalize with lysotracker, in agreement with results suggesting that the putative NAADP TPC1 partly localizes in endosomes [10]. On the other hand, lack of complete overlap demonstrate that the colocalization is not an artefact of light bleed from one channel to the other.

We then investigated whether increases in the NAADP concentration induced Ca^{2+} responses by adding NAADP-AM, a cell permeable acetoxymethyl ester of NAADP, to astrocytes loaded with fura-2. NAADP-AM is a less invasive tool than micro-injection of NAADP or caged-NAADP and it has been shown to be effective in numerous mammalian cell types [17]. The addition of 50 nM or 100 nM NAADP-AM produced complex Ca^{2+} responses with a prominent oscillatory component (Fig. 2A). Consistent with reports in other cell types [4,6], higher NAADP-AM concentrations induced smaller responses, generating a "bell-shaped" concentration-response curve (Fig. 2B). NAADP-AM-elicited Ca^{2+} responses were detected from 2 to 20 min after addition of NAADP-AM and were abolished by pre-incubation of cells with Ned-19 (Fig. 2C), which can be considered diagnostic for NAADP responses. Conversely, the lysosomal disruptor compound glycyl-L-phenylalanine 2-naphthylamide (GPN) inhibited most NAADP-AM responses (no cells responded to NAADP-AM in five experiments and 38 % of cells responded in one experiment) (Fig.2C) demonstrating that intracellular NAADP acts on specific receptors mainly localized in lyosome-related Ca^{2+} stores. On the other hand, extracellular addition of NAADP also induced intracellular Ca^{2+} increases at high concentrations as previously reported [18,19]. Responses were heterogeneous. For example, some astrocytic cultures did not respond to 1 mM NAADP, others had 28 % of cells showing quick and small Ca^{2+} responses, and in other cultures, 66 % of cells exhibited slow and high Ca^{2+} increases (Figure 1SA and B). Moreover, Ca^{2+} responses induced by extracellular NAADP were not inhibited by Ned-19 in any tested culture (Fig. S1C), demonstrating that extra- and intracellular NAADP act on different molecular targets in astrocytes.

Taken together, these results suggest that intracellular NAADP is an endogenous molecule in astrocytes able to induce Ca^{2+} release by the activation of receptors present mainly in acidic lysosome-related vesicles.

3.2. NAADP and acidic organelles mediate agonist-induced Ca^{2+} responses

We next investigated whether the NAADP signalling pathway could be activated by extracellular stimuli in astrocytes. In particular, we analysed the Ca^{2+} responses elicited by ATP, endothelin-1 and bradykinin, agonists traditionally linked to phospholipase C activation and IP_3-mediated Ca^{2+} release from the endoplasmic reticulum. Endothelin-1, however, has also been reported to increase NAADP levels in smooth muscle [20].

The addition of 100 μM ATP, 10 nM endothelin-1 or 1 μM bradykinin to astrocytes elicited Ca^{2+} responses characterized by an initial peak followed by a plateau phase (Fig. 3). The latter was more sustained if induced by endothelin-1, and in all cases it was sensitive to extracellular Ca^{2+} (Fig. 4) [21,22]. To determine the participation of NAADP signalling, we first pre-treated the cells with 100 μM Ned-19 to block the NAADP receptor. As shown in Fig. 3A and Fig. 3B, Ned-19 reduced the initial peak of the Ca^{2+} responses induced by ATP and endothelin-1, but no effect was observed on the increases elicited by bradykinin (Fig. 3C). Similarly, osmotic lysis of the lysosome-related organelles with 50 μM GPN and Ca^{2+} depletion of acidic vesicles with 2 μM bafilomycin A1 inhibited the peak phase of ATP and endothelin-1, but not bradykinin-induced, Ca^{2+} responses (Fig. 3A, 3B and 3C).

Reduction of the magnitude of ATP and endothelin-1-induced Ca^{2+} responses by Ned-19 was similar to that achieved by blocking the IP_3-mediated Ca^{2+} mobilization with 100 μM 2-APB, although in this case the second phase of the responses were also inhibited (Fig. 5). This and the results with EGTA plotted in Fig. 4 suggest the involvement of three signalling pathways in the early phases of ATP and endothelin Ca^{2+} responses, which in turn, would be amplified by Ca^{2+} -induced Ca^{2+} release and capacitative Ca^{2+} entry [21,22]. On the contrary, 100 μM 8-Br-cADPR, an antagonist of the second messenger cADPR, had no statistically significant effect on the responses of ATP and endothelin-1 (Fig. 5A and B) but partly inhibited bradykinin-induced Ca^{2+} increases (Fig. 5C).

3.3. ATP increases NAADP levels

Our results constitute the first report linking activation of purinergic receptors and Ca^{2+} release from acidic organelles in mammalian cells. In order to further support our data we determined intracellular NAADP levels after stimulation with ATP. We used a sensitive radioreceptor assay with a very high degree of specificity and that excludes any interference of the added ATP [6,15]. Fig. 6 shows that 100 μM ATP stimulated the production of NAADP over time in astrocytes, whereas no increases were detected if Krebs buffer was added (control). The ATP-mediated NAADP increase was fast and transient. These data demonstrate that NAADP is involved in the early phase of ATP-induced Ca^{2+} responses.

4. Discussion

In this study we provide direct evidence that NAADP is a true intracellular second messenger in astrocytes. The low NAADP-AM concentrations needed to elicit a response, the bell-shaped dose-effect curve, together with the low intracellular NAADP levels and their fast and transient increase in response to purinergic stimulation, agree with the NAADP signalling characteristics described in other cell types [4,5,10]. Interestingly, we also showed localization of the NAADP receptors in lysosome-related organelles and found that GPN, a disruptor of lysosome-related stores, inhibited most NAADP-AM-induced Ca^{2+} responses, suggesting that NAADP releases Ca^{2+} from lysosome-related acidic vesicles.

On the other hand, in our astrocytic cultures extracellular addition of NAADP did not always elicit Ca^{2+} responses, even at 1 mM. Responsive astrocytes did not show a bell-shaped dose-effect curve, and Ca^{2+} increases were not inhibited by the antagonist of NAADP receptors Ned-19. Ca^{2+} increases after extracellular addition of NAADP to astrocytes have been reported previously [18,19], although in these studies Ca^{2+} responses were shown at 10 μM NAADP. In both reports, NAADP-elicited responses were heterogeneous and mediated by multiple and different signalling mechanisms. Moreover, Heidemann and co-workers also showed that extracellular NADP and NAAD elicited Ca^{2+} responses [18], and Singaravelu and colleagues questioned NAADP internalization in astrocytes [19]. Taken together all the data demonstrate that extra- and intracellular NAADP act on different molecular targets in glial cells.

Importantly, we are able to link intracellular NAADP signalling to neurotransmitter-evoked Ca^{2+} signals in astrocytes and to show an agonist specific profile in Ca^{2+} mobilization. The coupling of endothelin-1 receptors to NAADP signalling has been previously described in smooth muscle cells [20]. Now we report, for the first time, that purinergic receptors traditionally linked to phospholipase C also mediate NAADP-induced Ca^{2+} mobilization. Our conclusions are based on pharmacological experiments, as well as on data obtained from a radioreceptor assay. ATP-induced NAADP synthesis is large, rapid, and transient, in agreement with our data showing a reduction of the initial peak phase of ATP-induced-Ca^{2+} responses caused by Ned-19, GPN and bafilomycin A1. This suggests that NAADP-mediated Ca^{2+} release is an initial event that triggers further Ca^{2+}-induced Ca^{2+} responses in astrocytes, again a characteristic of this signalling pathway in other cell types [5]. These findings do not exclude other Ca^{2+} signalling pathways at the early phase of ATP responses, and in fact our data also point to the participation of IP_3-mediated Ca^{2+} release and extracellular Ca^{2+} entry. In agreement, channel chatter between receptors situated in different intracellular Ca^{2+} stores has been previously suggested [10].

It is worth noting that a previous report failed to detect increases in NAADP levels in response to ATP in whole brain cell suspensions [12], probably due to the presence of cell types other than cortical astrocytes, in which ATP might not be coupled to NAADP signalling. The aforementioned work also reported that in cultures of hippocampal glial cells, bafilomycin A1 did not reduce the magnitude of ATP-induced Ca^{2+} responses, [12] in contrast to our data showing a clear effect of bafilomycin A1 in ATP-mediated Ca^{2+} responses in cortical astrocytes. All together, these results may underline regional diversity within the glial lineage in respect to the nature of Ca^{2+} lysosomal-like stores and agonist recruitment of second messengers. Indeed, there is emerging data suggesting that astrocytes are not a homogenous cell population, and that unique characteristics of astrocytes of different regions may distinctly tune the neuronal activities [23]. However, one cannot exclude that discrepancies between our data and those reported in hippocampal astrocytes could be best explained by differences in the methodology used. Whereas the experiments here were performed in physiological-like conditions, Pandley and coworkers kept cells in Ca^{2+}-free medium. In such conditions lysosome-related Ca^{2+} homeostasis might also be altered.

Our results uncovering new intracellular signalling pathways linked to activation of purinergic receptors in astrocytes are of notable significance as ATP not only acts a main astrocyte-to-astrocyte messenger, but also as a unique neuron-to-glia mediator [2]. Moreover, NAADP signalling pathway involvement in selective neurotransmitter-induced responses demonstrates the complexity of astrocytic Ca^{2+} signalling and hence excitability. Importantly, future investigations *in situ* and *in vivo* based on our description of astrocytic NAADP signalling coupled to ATP signalling could shed light on the present debate on the role of astrocytes in synaptic transmission *in vivo* [24].

In summary, we report that NAADP is an authentic intracellular second messenger in cultured astrocytes and plays an important role in ATP-mediated Ca^{2+} signals.

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Figure Legends

Fig. 1. The NAADP receptor antagonist Ned-19 colocalizes with acidic vesicles. Panels (A) and (B) are micrographs showing vital fluorescence staining of cortical astrocytes with 500 nM LysoTracker Red and 100 μM Ned-19, respectively; panel (C) shows the composite image. Colocalization of green and red pixels was quantified with ImageJ, and is shown in panel D, with the hottest colours indicating highest degrees of colocalization. Panel (E) shows the correlation plot of images shown in (A) and (B).

Fig. 2. NAADP-AM, a cell-permeant analogue of NAADP, induces Ca^{2+} responses. Cells were incubated with different concentrations of NAADP-AM, and Ca^{2+} responses were recorded. Cells were also pre-treated with 100 μM Ned-19 or 50 μM GPN and then challenged with 100 nM NAADP-AM. Representative traces are shown in (A and C) and means of the increases of the Ca^{2+} concentration observed for each NAADP-AM concentration were calculated (B). Number of experiments analysed is indicated in parentheses, except for pre-treatment with Ned-19 and GPN, in which 3 and 6 experiments were performed, respectively.

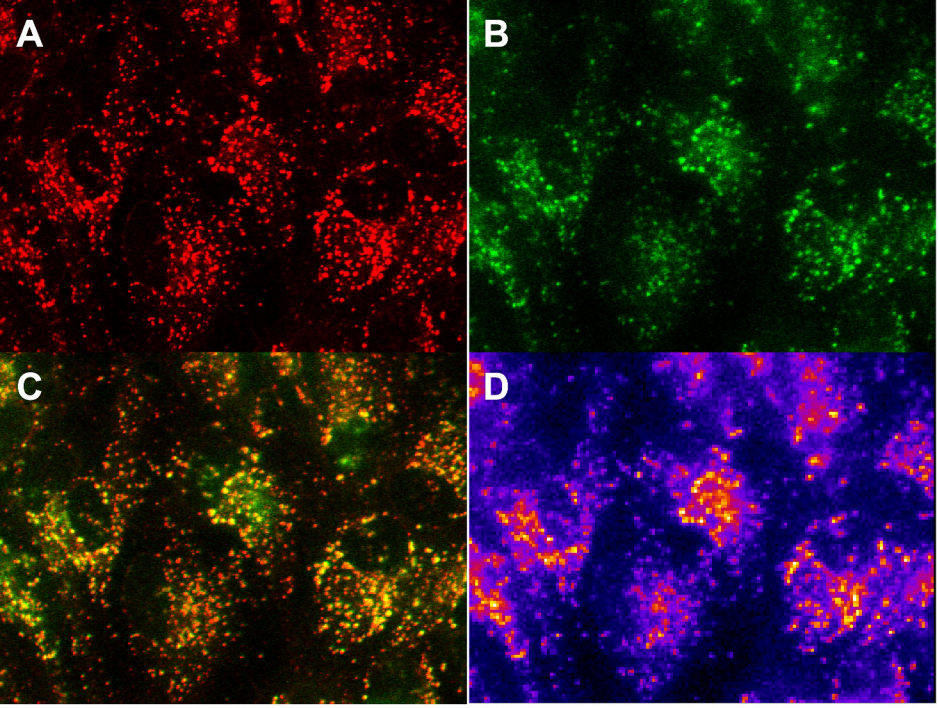
Fig. 3. NAADP and acidic organelles mediate ATP and endothelin-1 but not bradykinin-induced Ca^{2+} responses. Cells were challenged with agonists in the absence or in the presence of 100 μM Ned-19, 50 μM GPN or 2 μM bafilomycin A1 (Baf A1), and Ca^{2+} responses were induced by 100 μM ATP (A), 10 nM endothelin (ET-1) (B) or 1 μM bradykinin (BK) (C). Representative traces are shown on the left side of the figure. Middle panels show the means of the maximum ratio increase over basal Ca^{2+} concentrations as percentage of its respective control (ratio increase over basal Ca^{2+} concentrations induced by the agonist). Right panels show means of plateau responses (increase of Ca^{2+} concentrations at 3 minutes over basal Ca^{2+} concentrations) as percentage of their respective peak responses (the initial increase over basal Ca^{2+} concentrations). The number of experiments performed is indicated in parentheses. Statistics: *** $P<0.001$, two-tailed *t* test.

Fig. 4. Ca^{2+} responses induced by ATP, endothelin-1 and bradykinin in the presence of EGTA. Cells were stimulated with 100 μM ATP, 10 nM endothelin (ET-1) or 1 μM bradykinin (BK) in the presence of EGTA, a calcium chelator, to inhibit extracellular Ca^{2+} entry. Responses are reduced and above all, more transient.

Fig. 5. ATP, endothelin-1 and bradykinin release Ca^{2+} from the endoplasmic reticulum. Cells were challenged with agonists in the absence or in the presence of an antagonist of IP_3 receptors (100 μM 2-APB) or an antagonist of cADPR signalling (100 μM 8-Br-cADPR). Panels (A), (B) and (C) show Ca^{2+} responses induced by 100 μM ATP, 10 nM endothelin (ET-1) and 1 μM bradykinin (BK), respectivetly. Representative traces are shown on the left side of the figure. Middle panels show the means of the maximum ratio increase over basal Ca^{2+} concentrations as percentage of its respective control (ratio increase over basal Ca^{2+} concentrations induced by the agonist). Right panels show means of plateau responses (increase of Ca^{2+} concentrations at 3 minutes over basal Ca^{2+} concentrations) as percentage of their respective peak responses (the initial increase over basal Ca^{2+} concentrations). The number of experiments performed is indicated in parentheses. Statistics: *P<0.05 and ***P<0.001, two-tailed *t* test.

Fig. 6. ATP increases intracellular NAADP levels. ^{32}P-NAADP was used in a competitive assay to determine the endogenous NAADP levels at rest conditions (dotted line) and upon stimulation with 100 μM ATP (solid line). Values are means ± SEM (n=8) normalized to the absolute level of NAADP at time zero of each experiment.

Fig. S1. Extracellular NAADP-induced Ca^{2+} responses. Cells were incubated with different concentrations of NAADP added extracellularly and Ca^{2+} responses were recorded. In some experiments, 100 µM Ned-19 was applied 20 minutes before NAADP addition (C). Representative traces are shown in (A and C) and means of the increases of the Ca^{2+} concentration observed for each NAADP-AM concentration were calculated (B). Number of responding cells of at least 3 independent experiments is indicated in parentheses. Depending on the culture, two different responses of 1 mM NAADP were obtained, and both are shown. Data obtained in the presence of Ned-19 correspond to cells from cultures that showed high Ca^{2+} increases at 1mM NAADP.



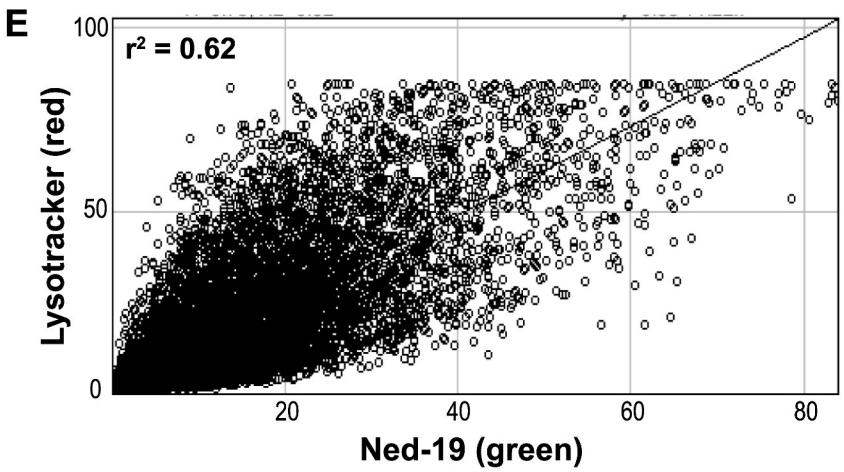


Figure 1

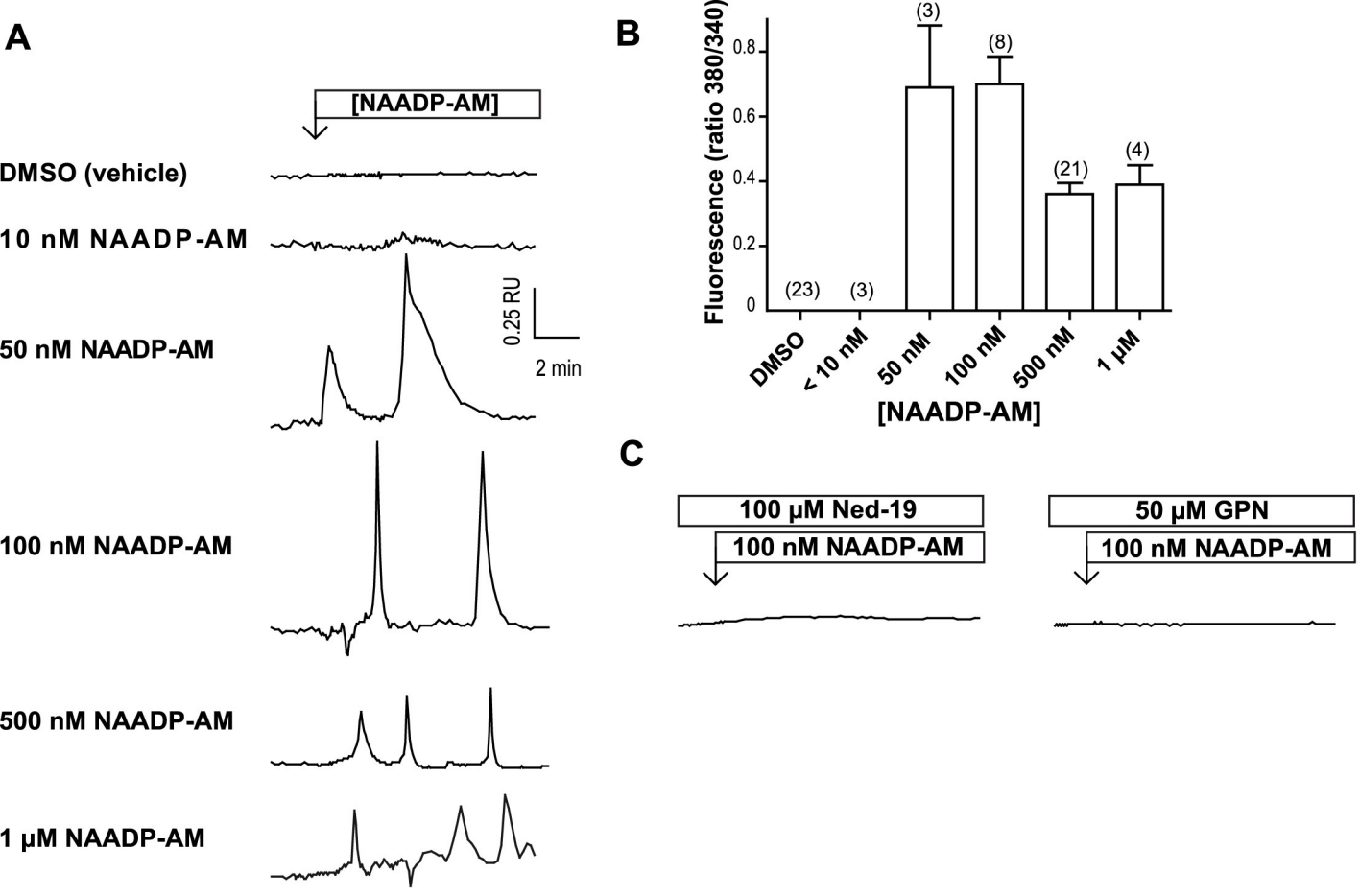

A
[NAADP-AM]
DMSO (vehicle)
10 nM NAADP-AM
50 nM NAADP-AM
100 nM NAADP-AM
500 nM NAADP-AM
1 µM NAADP-AM
0.25 RU
2 min
B
Fluorescence (ratio 380/340)
0
0.2
0.4
0.6
0.8
(23)
(3)
(3)
(8)
(21)
(4)
DMSO
< 10 nM
50 nM
100 nM
500 nM
1 µM
[NAADP-AM]
C
100 µM Ned-19
100 nM NAADP-AM
50 µM GPN
100 nM NAADP-AM

Figure 2

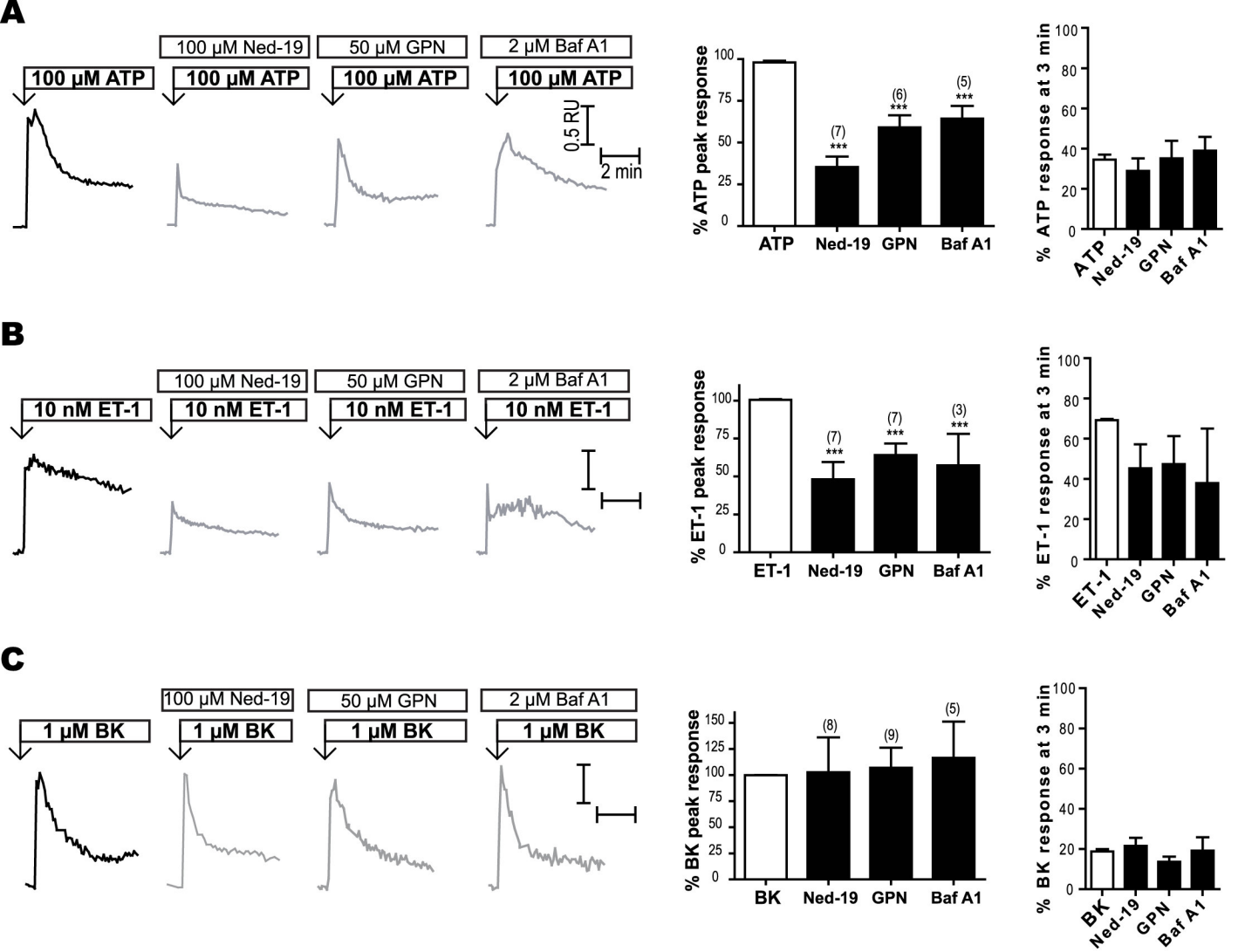

A
100 µM ATP
100 µM Ned-19
100 µM ATP
50 µM GPN
100 µM ATP
2 µM Baf A1
100 µM ATP
0.5 RU
2 min
% ATP peak response
ATP
Ned-19
GPN
Baf A1
% ATP response at 3 min
B
10 nM ET-1
100 µM Ned-19
10 nM ET-1
50 µM GPN
10 nM ET-1
2 µM Baf A1
10 nM ET-1
% ET-1 peak response
ET-1
Ned-19
GPN
Baf A1
% ET-1 response at 3 min
C
1 µM BK
100 µM Ned-19
1 µM BK
50 µM GPN
1 µM BK
2 µM Baf A1
1 µM BK
% BK peak response
BK
Ned-19
GPN
Baf A1
% BK response at 3 min

Figure 3

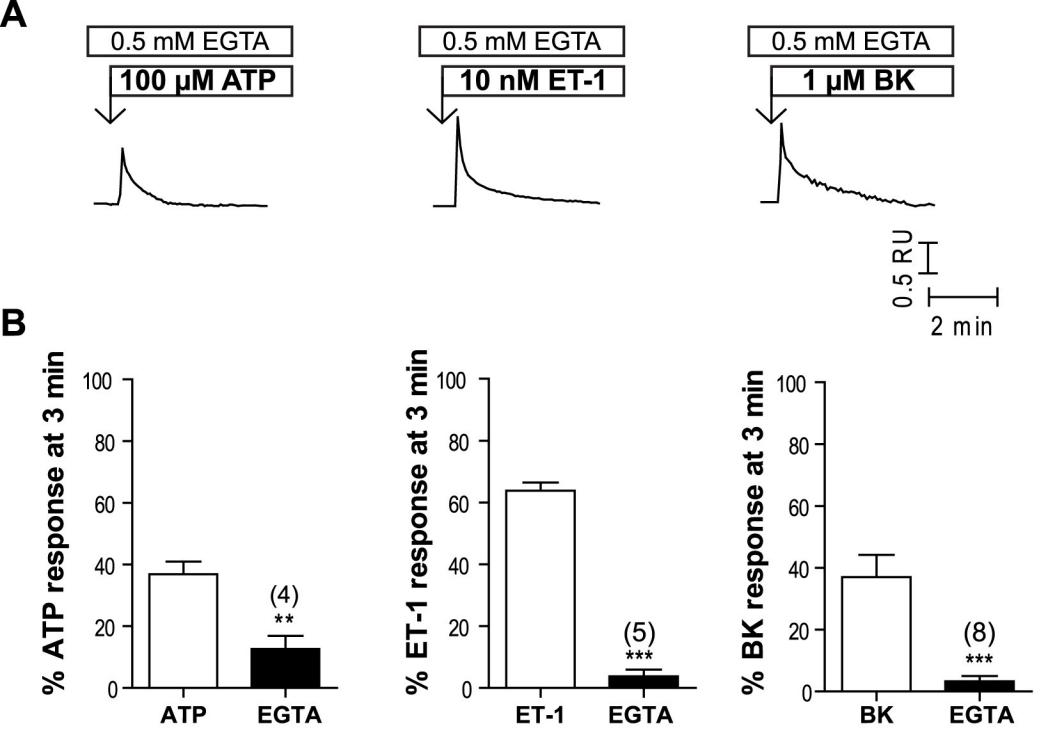

A
0.5 mM EGTA
100 µM ATP
0.5 mM EGTA
10 nM ET-1
0.5 mM EGTA
1 µM BK
0.5 RU
2 min
B
% ATP response at 3 min
100
80
60
40
20
0
(4)
**
ATP
EGTA
% ET-1 response at 3 min
100
80
60
40
20
0
(5)
***
ET-1
EGTA
% BK response at 3 min
100
80
60
40
20
0
(8)
***
BK
EGTA

Figure 4

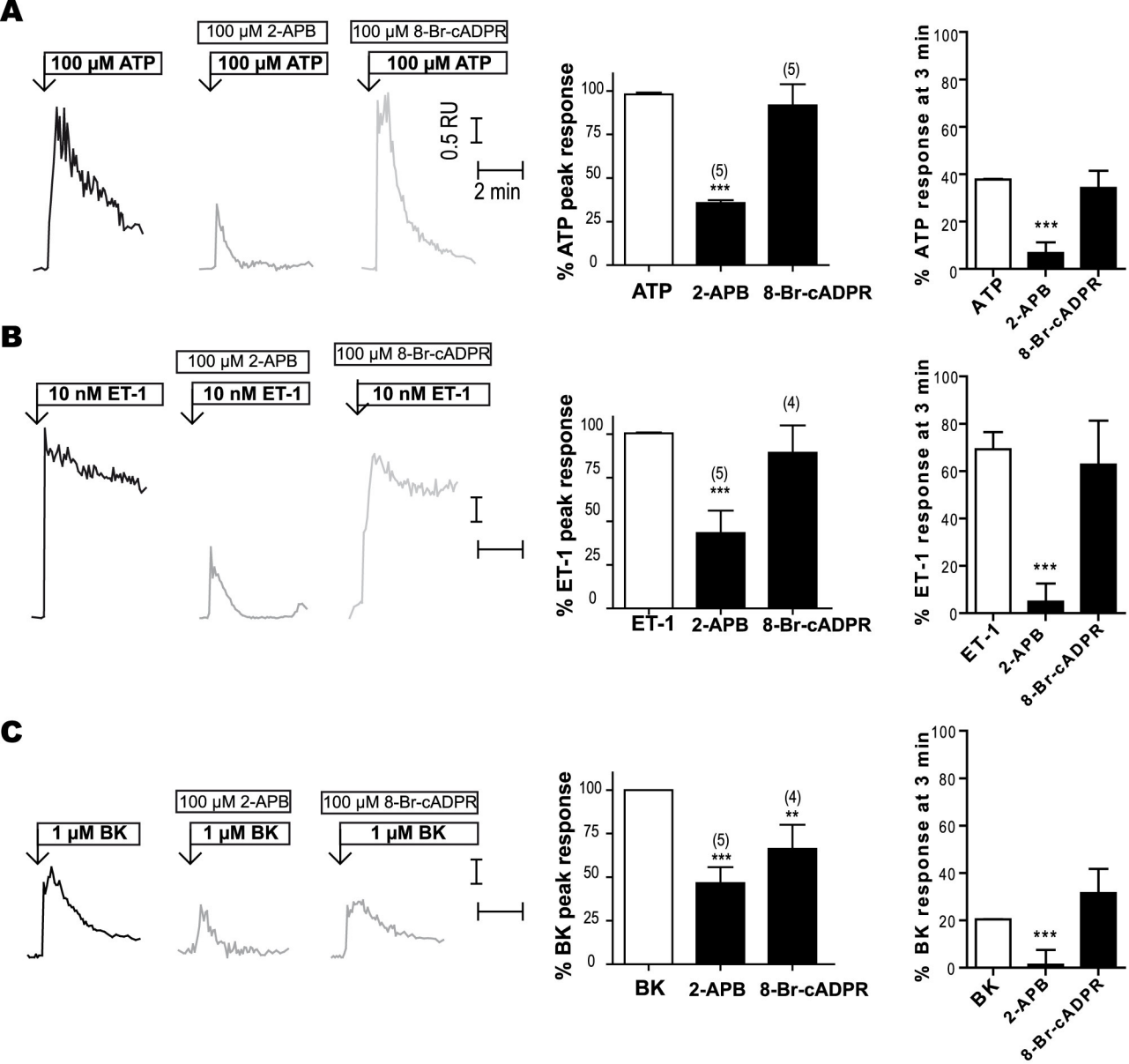

A
100 µM ATP
100 µM 2-APB
100 µM ATP
100 µM 8-Br-cADPR
100 µM ATP
0.5 RU
2 min
% ATP peak response
(5)
(5)
***
ATP
2-APB
8-Br-cADPR
% ATP response at 3 min
***
B
10 nM ET-1
100 µM 2-APB
10 nM ET-1
100 µM 8-Br-cADPR
10 nM ET-1
% ET-1 peak response
(4)
(5)
***
ET-1
2-APB
8-Br-cADPR
% ET-1 response at 3 min
***
C
1 µM BK
100 µM 2-APB
1 µM BK
100 µM 8-Br-cADPR
1 µM BK
% BK peak response
(4)
(5)
**
***
BK
2-APB
8-Br-cADPR
% BK response at 3 min
***

Figure 5

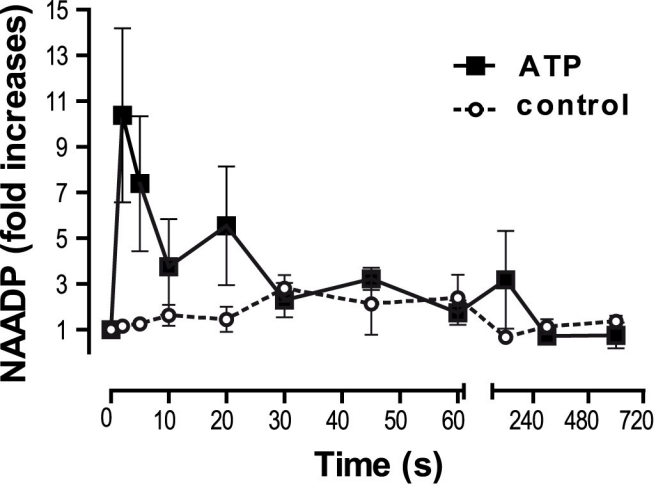

NAADP (fold increases)
15
13
11
9
7
5
3
1
ATP
control
0
10
20
30
40
50
60
240
480
720
Time (s)

Figure 6

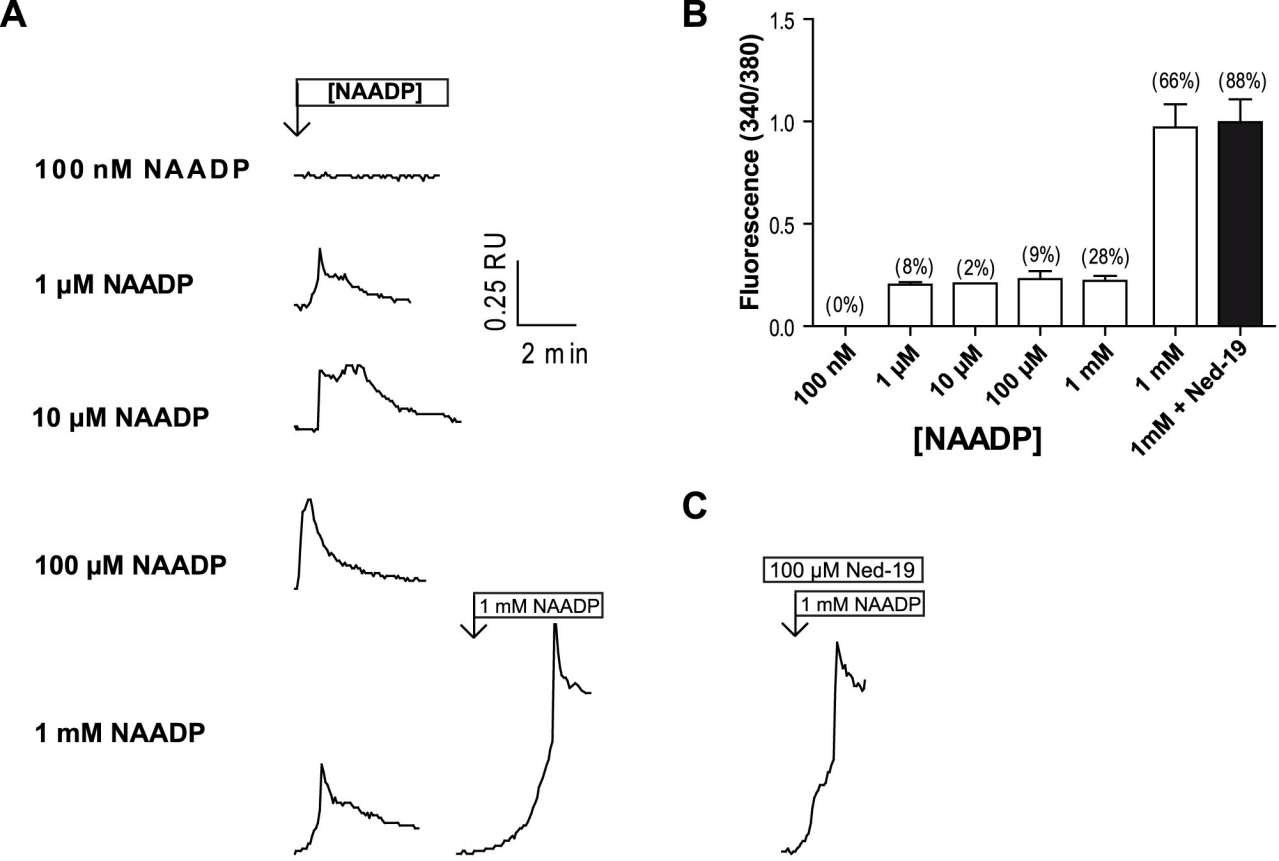

A
[NAADP]
100 nM NAADP
1 µM NAADP
10 µM NAADP
100 µM NAADP
1 mM NAADP
0.25 RU
2 min
1 mM NAADP
B
Fluorescence (340/380)
1.5
1.0
0.5
0.0
(0%)
(8%)
(2%)
(9%)
(28%)
(66%)
(88%)
100 nM
1 µM
10 µM
100 µM
1 mM
1 mM
1mM + Ned-19
[NAADP]
C
100 µM Ned-19
1 mM NAADP

Figure S1