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Calcium mobilization by nicotinic acid adenine dinucleotide phosphate (NAADP) in rat astrocytes

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Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) has been shown to release intracellular Ca^{2+} in several types of cells. We have used Ca^{2+}-sensitive fluorescent dyes (Fura-2, Fluo-4) to measure intracellular Ca^{2+} in astrocytes in culture and in situ. Bath-applied NAADP elicited a reversible and concentration-dependent Ca^{2+} rise in up to 90% of astrocytes in culture (EC_{50} = 7 μM). The NAADP-evoked Ca^{2+} rise was maintained in the absence of extracellular Ca^{2+}, but was suppressed after depleting the Ca^{2+} stores of the ER with ATP (20 μM), with cyclopiazonic acid (10 μM) or with ionomycin (5 μM). P_2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′4′-disulfonic acid (PPADS, 100 μM), IP_3 receptor blocker 2-aminoethoxydiphenyl borate (2-APB, 100 μM) and PLC inhibitor U73122 (10 μM) also reduced or suppressed the NAADP-evoked Ca^{2+} rise. NAADP still evoked a Ca^{2+} response after application of glycyl-L-phenylalanine-β-naphthylamide (GPN, 200 μM), which permeabilizes lysosomes, or preincubation with H^+-ATPase inhibitor bafilomycin A1 (4 μM) and of *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, 2 μM), that impairs mitochondrial Ca^{2+} handling. In acute brain slices, NAADP (10 μM) evoked Ca^{2+} transients in cerebellar Bergmann glial cells and in hippocampal astrocytes. Our results suggest that NAADP recruits Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores in mammalian astrocytes, at least partly by activating metabotropic P_2Y receptors.

Keywords: Fura-2; Cyclopiazonic acid; Ionomycin; Calcium stores; Endoplasmic reticulum

1. Introduction

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+} mobilizer from intracellular stores in a wide variety of cell types [1], ranging from invertebrate to mammalian cell systems [2–6]. NAADP was first reported to evoke Ca^{2+} release from sea urchin eggs [7,8], when it was injected into the cell. NAADP is synthesized enzymatically from nicotinamide adenine dinucleotide phosphate (β-NADP) by ADP-ribosyl cyclase, which is widespread in mammalian tissues [9–11], and results from the base-exchange reaction between $NADP^+$ and nicotinic acid [12].

In most non-excitable cells as well as many excitable cells, Ca^{2+} signaling is mediated by endogenous second messengers like inositol 1,4,5-triphosphate (IP_3) and cyclic ADP-ribose (cADPR). In sea urchin eggs, NAADP, unlike IP_3 and cADPR, mobilizes Ca^{2+} from acidic compartments, presumably lysosomes [13]. However, in different cell types NAADP has been reported to challenge Ca^{2+} stores which are sensitive to thapsigargin, such as the endoplasmic reticulum (ER) Ca^{2+} stores [14] or insensitive to thapsigargin [15].

NAADP, which seems to act intracellularly, has not yet been identified as a functional messenger in the brain and is still unidentified in nerve and glial cells. The present study aimed to identify NAADP as a possible messenger in astrocytes, the major non-excitable cell type in the brain. We have measured Ca^{2+} in astrocytes, both in culture and in acute brain slices, and found that bath-applied NAADP evoked a Ca^{2+} response in the majority of these cells, presumably due to Ca^{2+} release from IP_3-sensitive ER stores. Blocking P_2 and IP_3 receptors significantly reduced the NAADP-induced Ca^{2+} response and inhibiting phospholipase C fully abolished the response. NAADP still evoked a Ca^{2+} response

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after disruption of lysosomes and inhibition of vacuolar H^+-ATPases.

2. Materials and methods

2.1. Cell culture

For preparing primary cultures of astrocytes, cerebral cortices of new-born rats (P0–P3) were rapidly removed and maintained in a chilled solution that contained (mM): NaCl 137, KCl 5.4, KH_2PO_4 0.22, Na_2HPO_4 0.2, glucose 5.6 and saccharose 58.4, pH 7.3–7.4. Cells were dissociated by mechanical chopping and passed through the membranes of pore size, 210 and 132 μm, respectively. Then the isolated cells were cultured according to the method described by Fischer [16]. After the cells had reached confluence (in about 10 days), the cells were dissociated by 0.25% of trypsin and this step was stopped by fetal calf serum (10%). Then the cells were incubated with DNAse (grade I), centrifuged, resuspended in RPMI 1640 medium (supplemented with the aliquot of RPMI-1640, 2.0 g $NaHCO_3$, 2.0 g glucose, 1 ml penicillin or streptomycin and 5% fetal calf serum), plated on glass coverslips coated with poly-D-lysin (0.001%), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The experiments were performed on secondary astrocyte culture between 2 and 14 days after plating at room temperature (21–24 °C).

2.2. Slice preparation

Hippocampal brain slices were prepared by following the method of Edwards et al. [17]. In brief, a juvenile rat (P8–P12) was decapitated and the brain was removed rapidly and prepared in an ice-cold, bicarbonate-buffered (5% CO_2/95% O_2, pH 7.4), Ca^{2+}-reduced (0.5 mM; $MgCl_2$ increased to 2.5 mM) saline. Frontal slices (300 μm thick) of the forebrain hemispheres in the area of the hippocampus were cut with a vibratom (Leica VT1000S; Campden, Loughborough, UK), and stored for 1 h in gassed Ca^{2+}-reduced saline at 30 °C before dye loading and later at room temperature (21–24 °C). Cells of interest were astrocytes of the *stratum radiatum* and the *stratum lacunosum-moleculare* in the CA1 area of the hippocampus. Cerebellar slices were prepared from juvenile rats (P8–P13) by following the same procedure. Sagittal slices of the vermis (250 μm thick) were obtained using the above-described method. The glial cells were identified by their cell shape and/or by their Ca^{2+} response to 0 mM K^+ [18,19].

2.3. Dye loading

Cultures of rat cerebral astrocytes were incubated in Fura-2-AM containing saline (1 μM, dissolved in DMSO to make a 2 mM stock solution) (Molecular probes, Eugene, OR, USA) for 30 min in the dark at room temperature. Cultured astrocytes were also incubated with Fluo-4-AM (2 μM, dissolved in DMSO to make a 2 mM stock solution) (Molecular Probes) for 45 min. Hippocampal slices were loaded with a solution containing 2 μM Fluo-4-AM (dye dissolved in DMSO to make a 2 mM stock solution) in a petri dish inside a small oxygenated closed box for 60 min at 20–24 °C. Cerebellar slices were also incubated in Fluo-4-AM, but with a concentration of 3 μM dye (dissolved in DMSO and pluronic acid to make a 3 mM stock solution). After dye-loading the slices were kept on a mesh of nylon in ACF. All saline for acute brain slices were gassed with 5% CO_2/95% O_2 to maintain pH and oxygen level at a physiological level.

2.4. Calcium imaging

Experiments on cultured cells were performed with an Olympus upright microscope (BX50W1) using a Ca^{2+} imaging system (TILL Photonics, Munich-Martinsried, Germany). Monochromator setting and data acquisition were controlled by software for a personal computer system. Fura-2-loaded cells were excited by monochoromatic wavelengths of 340 and 380 nm. The fluorescence emissions of several regions of interest (ROI, each covering one single cell body) were simultaneously recorded with the CCD camera IMAGO (TILL Photonics, Munich-Martinsried, Germany), using a 440 nm longpass filter. The signals were sampled at 0.2–0.5 Hz, computed into relative ratio units, and converted into values of absolute Ca^{2+} concentrations as described previously by Jung et al. [20].

Experiments with cells of acute brain slices and cultures were done with a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Germany). The Ca^{2+}-sensitive dye Fluo-4 was excited by the 488 nm line of a krypton–argon laser. Ca^{2+} images were taken with a frequency of 0.3–1.0 Hz. Excitation and emission signals were separated by a dichroic mirror. The emission signal was truncated by a 505 nm optical band pass filter. The sequence of fluorescence images was sampled in one focal plane for Ca^{2+} measurements. Regions of interest (ROIs) were defined in the first image, and the normalized fluorescence changes F/F_0 (%), where F is the fluorescence intensity at any time and F_0 is the basic fluorescence intensity at the beginning of the experiment, were measured throughout the series. All settings of the laser, optical filter and microscope as well as data acquisition were controlled by PC software (Zeiss). Further details have been described previously [19].

2.5. Solutions

The standard saline for cultured astrocytes contained (mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 10, Hepes 10, pH 7.4 set by addition of NaOH. Ca^{2+}-free saline was prepared by replacing $CaCl_2$ by equimolar amounts of $MgCl_2$ and by adding 0.5 mM EGTA. The experimental chamber containing cell cultures was continuously perfused with the saline at room temperature (21–24 °C). Standard saline for acute brain slices (artificial cerebrospinal fluid, ACF) contained (mM): NaCl 125,

KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 25, $NaHCO_3$ 26, NaH_2PO_4 1.25, L-lactate 0.5, gassed with 5% CO_2/95% O_2 to adjust the pH to 7.4. Glycyl-L-phenylalanine-β-naphthylamide (GPN, Sigma), cyclopiazonic acid (CPA; Alexis) and ionomycin (Sigma) were dissolved in dimethyl sulfoxide (DMSO) as stock solutions of 200, 100 and 10 mM and added at a final concentration of 200,10 and 5 μM, respectively. 2-Aminoethoxydiphenyl borate (2-APB, Tocris), 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122, Tocris), bafilomycin A1 (Tocris) and *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, Tocris) were dissolved in DMSO as stock solutions of 100, 5, 4 and 2 mM and added at a final concentration of 100, 10, 4 and 2 μM, respectively. Nictotinic acid adenine dinucleotide phosphate (β-NAADP, Sigma), nictotinamide adenine dinucleotide phosphate (β-NADP, Sigma), adenosine diphosphate (ADP, Sigma), adenosine triphosphate (ATP, Sigma) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, Sigma) were dissolved in distilled water to make a stock solution of 100 mM each and used at concentrations as indicated. All drugs were added to the experimental saline immediately before use.

2.6. Statistics

All data are expressed as the means ± S.E.M.; *n* indicating cells (and culture plates or brain slices) analyzed with the statistical program either by Sigma-Plot or Origin 7.0 (OriginLab Corp., Northampton, USA). Statistical differences were made using Student's *t*-test in Origin 7.0.

3. Results

3.1. NAADP-induced Ca^{2+} increase in cultured rat astrocytes

Bath application of NAADP for 1 min elicited a Ca^{2+} response in cultured rat cerebral astrocytes in a concentration-dependent manner (Fig. 1). There appeared two types of response patterns in the cells; the cells could show repetitive Ca^{2+} transients (Ca^{2+} oscillations; Fig. 1A), a monophasic, transient Ca^{2+} increase and/or biphasic Ca^{2+} responses consisting of an initial large transient followed by a smaller sustained component ('shoulder'; Fig. 1B). Due to these different response patterns, it was difficult to compare amplitudes of the responses. Therefore, in order to quantify the effect of NAADP, we counted the number of cells responding to the agonist. Forty-eight cells of four cell cultures were analyzed in terms of concentration-dependent action of NAADP (Fig. 1C and D). Only 2 (4%) and 7 (15%) cells responded to 1 and 3 μM NAADP, respectively, while 10 μM NAADP evoked a Ca^{2+} response in 35 cells (73%). Higher NAADP concentrations (30, 100 μM) evoked responses in 85 and

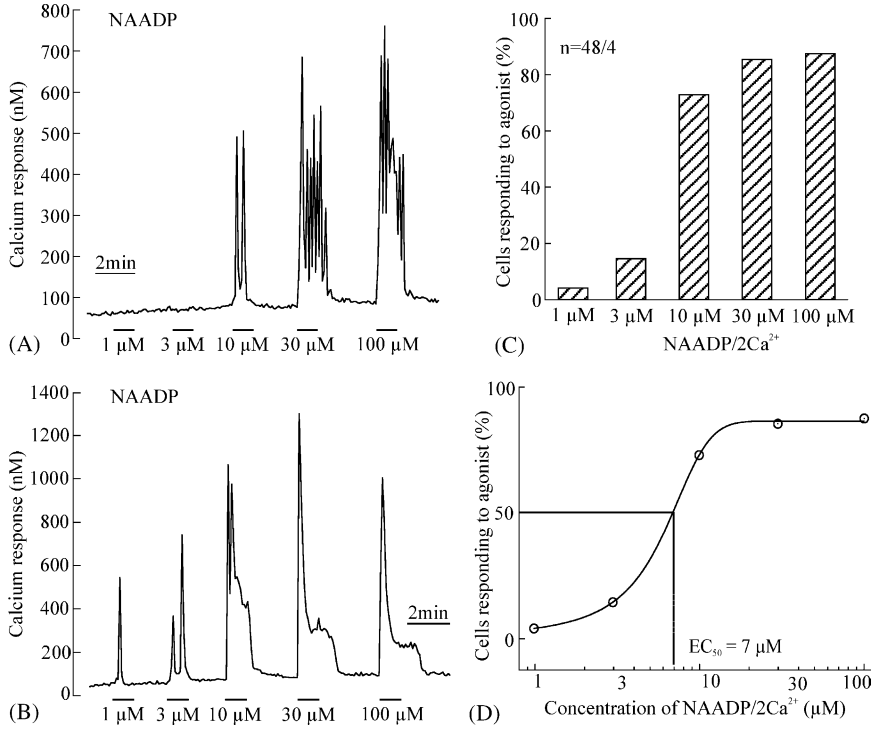

Fig. 1. Ca^{2+} response patterns of cortical astrocytes in culture stimulated by sequential application of 1, 3, 10, 30 and 100 μM concentrations of NAADP (1 min) in the presence of extracellular Ca^{2+}. Astrocytes showed two types of response patterns, repetitive Ca^{2+} transients (A), a monophasic, transient Ca^{2+} increase and/or biphasic Ca^{2+} responses consisting of an initial large transient component followed by a smaller sustained component. (B and C) The number of responding cells to different concentrations of NAADP. (D) A fit to the dose–response curve yielded an EC_{50} value of 7 μM. *n* indicates the number of cells/culture used for statistical analysis.

88% of the cells, respectively. From the dose–response curve, the half-maximal effect of NAADP, where 50% of the cells responded, was determined to be 7 μM (EC_{50} value). In most of our subsequent experiments, we used 10 μM NAADP to compare the number of responding cells under various conditions.

3.2. NAADP elicits Ca^{2+} release from intracellular stores

NAADP elicited a reversible Ca^{2+} rise in cultured rat astrocytes, even in the absence of extracellular Ca^{2+} (Fig. 2). Repeated application of 10 μM NAADP in the presence and absence of extracellular Ca^{2+} evoked a response in 54 and 48% of the cells, respectively ($n = 58$ cells of 4 cultures; Fig. 2A and B). Subsequent application of NAADP after re-addition of extracellular Ca^{2+} evoked a response in 62% of the cells. A second NAADP application in the absence of extracellular Ca^{2+} still evoked a response in 60% of the cells, which had responded to the first NAADP application in Ca^{2+}-free saline ($n = 67$). These experiments show that the response to NAADP is due to Ca^{2+} release from intracellular stores rather than from Ca^{2+} influx. The frequency of the response to NAADP could, however, be affected not only by the availability of stored Ca^{2+}, but also by some desensitization of NAADP response pathway. NADP, which acts as a precursor of NAADP, also elicited a reversible Ca^{2+} rise in cultured astrocytes. Sixty-three and 57% (10 μM, 1 min; $n = 97$; data not shown here) of the cells responded in the presence and absence of extracellular Ca^{2+}, respectively.

In order to substantiate this finding, we tried to identify the intracellular Ca^{2+} stores, from which Ca^{2+} is released in response to NAADP. First, we used a well-known agonist of IP_3-mediated Ca^{2+} release from ER stores, ATP, which activates the metabotropic cascade via P_2Y receptors in astrocytes [21]. ATP (20 μM for 5 s) was applied in alternating sequence with NAADP in the presence and absence of extracellular Ca^{2+} (Fig. 2C and D). Both ATP and NAADP induced a Ca^{2+} rise in the presence as well as in the absence of external Ca^{2+}; ATP in 100% ($n = 88$) and NAADP in 66% ($n = 58$) of the cells. Once ATP was applied in Ca^{2+}-free saline, there was no Ca^{2+} rise to subsequent applications of NAADP any more, although ATP still evoked a Ca^{2+} rise at least one more time. If ATP was applied first in the absence of Ca^{2+} and then NAADP, a Ca^{2+} rise to ATP (100%), but not to NAADP (0%) was observed ($n = 44/2$; not shown). These experiments suggest that NAADP presumably recruits Ca^{2+} from IP_3-sensitive, ATP-targeted stores in rat astrocytes. NAADP, however, cannot deplete these stores, suggesting that this messenger may only access part of the ATP-sensitive ER stores.

3.3. NAADP acts via P_2 receptors

Since extracellular NAADP evoked Ca^{2+} release from ATP-sensitive stores, we were interested to know whether NAADP activates a receptor in the cell membrane. So we tried

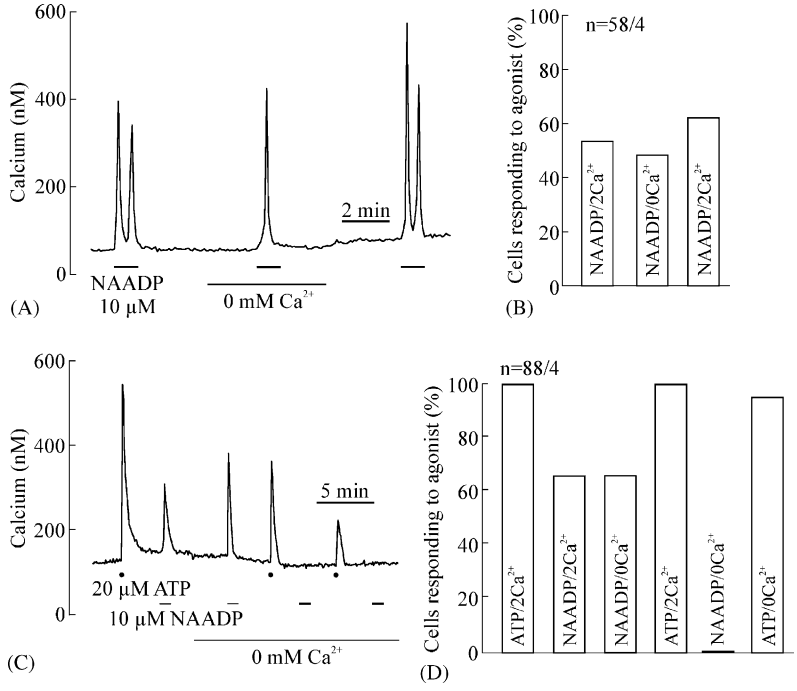

Fig. 2. Ca^{2+} release elicited by NAADP in astrocyte culture: (A) NAADP (10 μM, 1 min) elicited a reversible Ca^{2+} rise in the presence and absence of external Ca^{2+}. (B) The percentage of cells responding in 2 mM Ca^{2+} was similar as in Ca^{2+}-free saline (0 mM Ca^{2+}). (C and D) Purinergic receptor agonist ATP (20 μM, 5 s) depleted the NAADP-sensitive stores. Once ATP was applied in Ca^{2+}-free saline, there was no subsequent rise in Ca^{2+} by NAADP, indicating that ATP exhausted NAADP-sensitive stores.

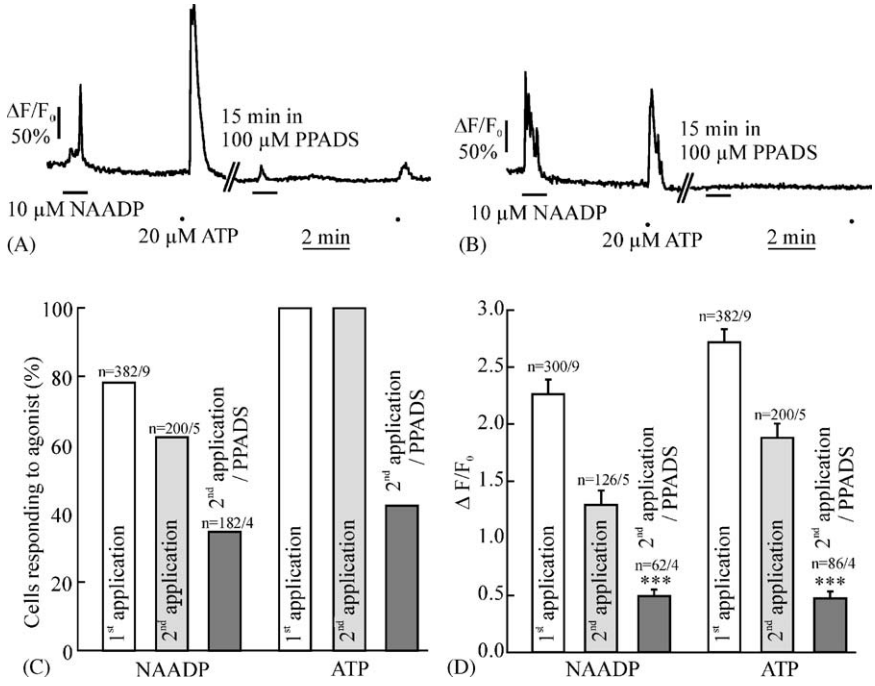

Fig. 3. Purinergic receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) on the NAADP- and the ATP-evoked Ca^{2+} responses in astrocytes. Astrocytes were exposed to NAADP and ATP first alone, and then in the presence of PPADS (100 μM, 15 min). PPADS reduced (A) or suppressed (B) the NAADP- and the ATP-evoked Ca^{2+} response. (C) The number of cells responding to NAADP and ATP in PPADS were significantly reduced in comparison to control. (D) Amplitude of NAADP- and ATP-evoked Ca^{2+} response under control conditions and in the presence of PPADS.

to block purinergic receptors using the P_2 receptor antagonist, PPADS (Fig. 3). NAADP and ATP were applied twice in the presence of extracellular Ca^{2+} as control. To test the effects of PPADS, astrocytes were exposed to NAADP and ATP again, the second time in the presence of PPADS (100 μM; Fig. 3A and B), preincubated for at least 15 min. PPADS led to partial (Fig. 3A) or complete (Fig. 3B) suppression of NAADP- and ATP-evoked Ca^{2+} response. In the control, NAADP evoked a response in 79% ($n = 382$) and 63% ($n = 200$) of the cells in subsequent applications. When P_2 receptors were blocked with PPADS, the number of cells responding to the second application of NAADP decreased to 34% ($n = 182$). When ATP was applied twice to the astrocytes, all cells (100%, $n = 382$) responded in both applications. After the treatment of PPADS, only 43% ($n = 182$) of cells responded to ATP (Fig. 3C). The amplitude of the Ca^{2+} responses to NAADP and ATP still present in PPADS decreased to less than 50% of the control amplitude in the absence of PPADS ($P < 0.001$; Fig. 3D). These results suggest that extracellular NAADP, like ATP, could act, at least partially, via P_2 receptors in plasma membrane to evoke Ca^{2+} response from intracellular stores. There was, however, a PPADS-resistant component of the Ca^{2+} response for both agonists.

3.4. The Ca^{2+} stores challenged by NAADP are CPA- and ionomycin-sensitive

IP_3-sensitive Ca^{2+} stores can be depleted by thapsigargin or cyclopiazonic acid (CPA), which inhibit the SERCA-Ca^{2+} pump. NAADP was applied before and after depleting the stores with CPA (10 μM) to see whether these two drugs challenge the same intracellular Ca^{2+} stores (Fig. 4). Before CPA addition, NAADP evoked Ca^{2+} responses in the presence and absence of extracellular Ca^{2+} in 84 and 81% of the cells ($n = 114$; Fig. 4A and B). CPA elicited a slow transient Ca^{2+} rise in all cells, which is due to the leakage of Ca^{2+} out of the ER stores and lack of Ca^{2+} re-uptake into the stores. After depletion of the CPA-sensitive Ca^{2+} stores, less than 5% of the cells responded to NAADP, indicating that NAADP presumably challenges the same stores as CPA.

In another approach to test whether NAADP-sensitive stores are part of CPA-sensitive stores, NAADP was applied in the presence of CPA, when the Ca^{2+} stores had not been fully depleted yet (Fig. 4C). Interestingly, all 25 cells in 2 cultures responded to NAADP under these conditions, as they did to ATP before (Fig. 4D), and the CPA-induced Ca^{2+} transient was shortened by the NAADP-evoked Ca^{2+} transient. This confirms that NAADP challenges CPA-sensitive Ca^{2+} stores in rat astrocytes.

Similar results were obtained with ionomycin (5 μM, 2 min). The Ca^{2+} ionophore ionomycin empties endoplasmic reticulum Ca^{2+} stores. NAADP was applied before and after depleting the ionomycin-sensitive stores (Fig. 4E). Again, Ca^{2+} responses were elicited by NAADP before adding ionomycin, both in the presence (74%) and absence (64%) of extracellular Ca^{2+}, but no cell responded to NAADP after depleting the stores with ionomycin in Ca^{2+}-free saline

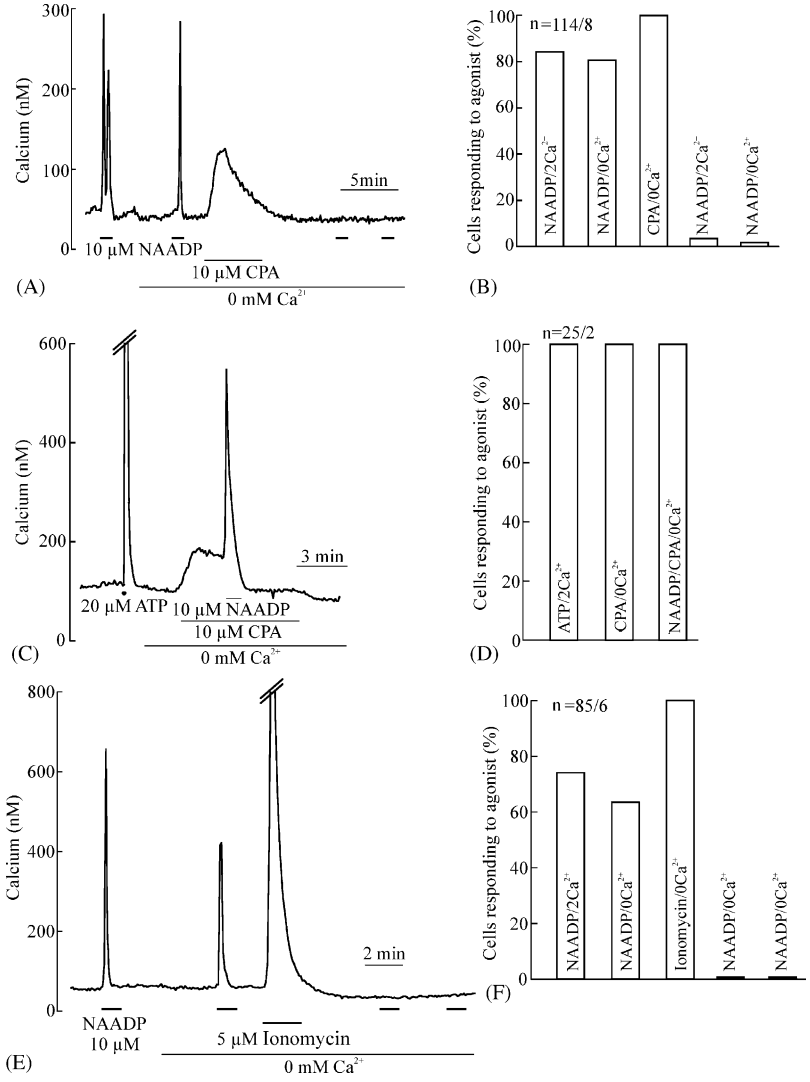

Fig. 4. (A) NAADP-sensitive stores are cyclopiazonic acid (CPA)-sensitive in astrocytes. In Ca^{2+}-free saline, the stores sensitive to CPA (10 μM, 5 min) were depleted and subsequent applications of NAADP evoked a Ca^{2+} response in less than 5% of the cells (B), indicating that NAADP targets the same stores as CPA. (C and D) If NAADP was applied in the presence of CPA before the stores were fully emptied, the cells still responded to NAADP. (E and F) After depletion of Ca^{2+} stores by the Ca^{2+} ionophore ionomycin (5 μM, 2 min), NAADP (10 μM, 1 min) did not evoke Ca^{2+} response any more.

($n = 85$; Fig. 4F). This indicates that ionomycin completely depletes NAADP-sensitive stores.

3.5. NAADP-evoked Ca^{2+} response depends on PLC/IP_3 pathway

To test whether PLC/IP_3 pathway is involved in the NAADP-induced Ca^{2+} rise, IP_3 receptor inhibitor 2-APB [22] and the PLC inhibitor U73122 [23] were used. First, we applied NAADP and ADP (20 μM, 5 s) twice in the presence of extracellular Ca^{2+} as control. When 2-APB (100 μM) was added to block IP_3-receptors in the ER, the NAADP- and the ADP-evoked Ca^{2+} rise were greatly reduced (Fig. 5A) or completely suppressed (Fig. 5B). NAADP evoked a Ca^{2+} rise in nearly 80% of the cells ($n = 225$) during the first application, and 64% ($n = 116$) during the next. After treatment with 2-APB (10 min), the number of responding cells to NAADP was reduced to 12% ($n = 109$). All cells (100%; $n = 225$) responded to both applications of ADP, which challenges P_2Y receptors, whereas after blocking IP_3 receptors with 2-APB, only 14% of the cells still responded to ADP with a Ca^{2+} response ($n = 109$; Fig. 5C). The mean amplitude of NAADP and ADP-evoked Ca^{2+} responses, which were still present in 2-APB, was significantly reduced in comparison to the control without 2-APB by 10% ($P < 0.001$; Fig. 5D).

The NAADP-induced Ca^{2+} response was also suppressed by preincubation with the phospholipase C inhibitor U73122 (10 μM, 30 min), which also suppressed the Ca^{2+} responses to ATP ($n = 113$; Fig. 5E and F). Seventy-five percent of the cells responded to NAADP and 100% of the cells to ATP under control conditions ($n = 109$; Fig. 5F). These results suggest that activation of receptors by NAADP, like ATP, would generate IP_3 and subsequently IP_3-induced Ca^{2+} release from intracellular stores.

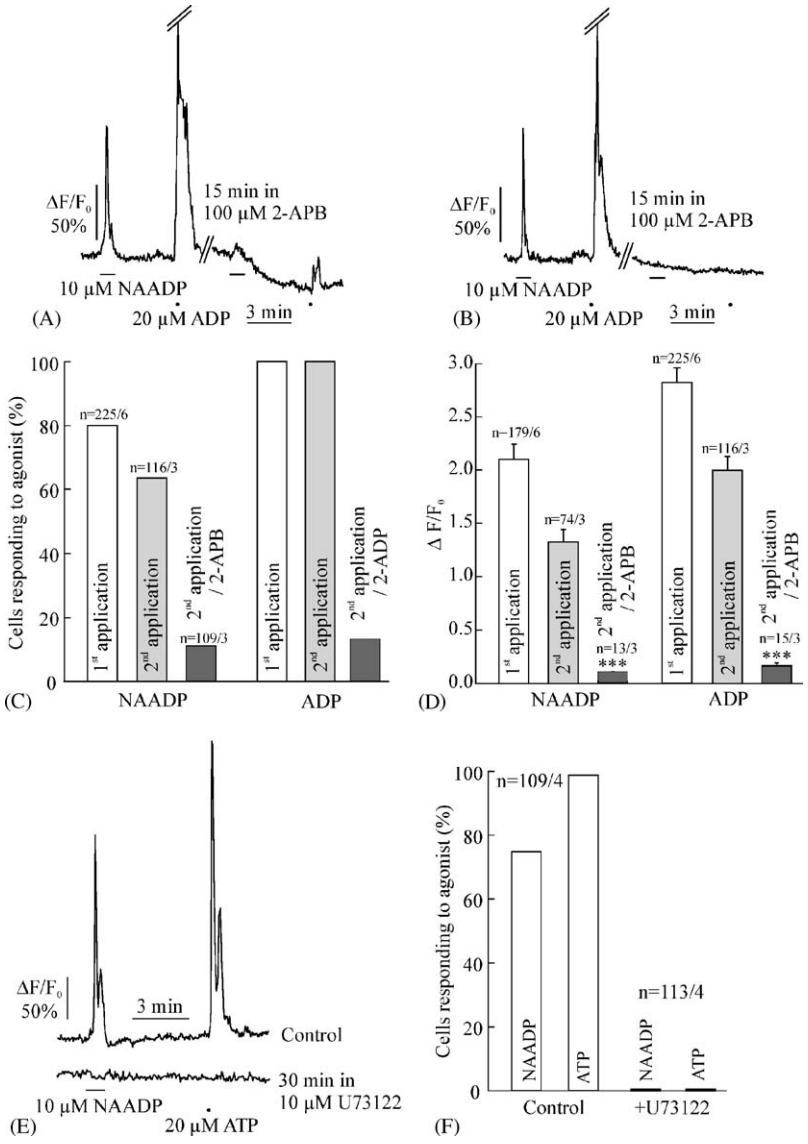

Fig. 5. IP_3 receptor blocker 2-aminoethoxydiphenyl borate (2-APB) on the NAADP- and the ADP-evoked Ca^{2+} response in astrocytes. Cells were exposed to NAADP and ADP (20 μM, 5 s) first alone, and then in the presence of 2-APB (100 μM, 10 min). Blockade of IP_3 receptors reduced (A) or suppressed (B) the NAADP- and the ADP-evoked Ca^{2+} response. (C) The number of cells responding to NAADP and ATP in PPADS were significantly reduced in comparison to the control. (D) Amplitude of NAADP- and ADP-evoked Ca^{2+} response under control conditions and in the presence of 2-APB. (E and F) Preincubation with phospholipase C inhibitor U73122 (10 μM, 30 min) suppressed the NAADP- and ATP-evoked Ca^{2+} response. A significant number of astrocytes responded to NAADP and ATP under control conditions.

3.6. Effects of GPN, bafilomycin A1 and FCCP on NAADP-sensitive stores

Glycyl-L-phenylalanine-β-naphthylamide (GPN) is a substrate of lysosomal cathepsin C [24,25], whose cleavage results in osmotic lysis and subsequent release of Ca^{2+} from lysosomes [26]. GPN (200 μM, 5 min), applied in a Ca^{2+}-free saline, itself evoked a slow transient Ca^{2+} rise in 95% of the cells ($n = 134$; Fig. 5A and B). In this experimental series, NAADP evoked a Ca^{2+} response in 84% of the cells in the presence, and in 69% of the cells in the absence of extracellular Ca^{2+} before the addition of GPN. After depleting the GPN-sensitive Ca^{2+} stores, still 53% of the cells responded to NAADP, indicating that the NAADP-sensitive stores were not depleted by GPN. Bafilomycin A1, which is the inhibitor of vacuolar proton pump (V-type H^+-ATPase) dissipates the proton gradient into acidic organelles. It has been demonstrated that Ca^{2+} uptake into acidic organelles like lysosomes is driven by proton gradients maintained by V-type H^+-ATPase [27,28]. Preincubation of astrocytes with bafilomycin A1 (4 μM, 60 min) did not block NAADP-evoked Ca^{2+} rise ($n = 99$; Fig. 6C). Eighty-two percent of the cells to NAADP and 100% to ATP showed a Ca^{2+} response, which was similar as under control conditions ($n = 112$; Fig. 6D).

The proton ionophore *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) is a powerful mitochon-

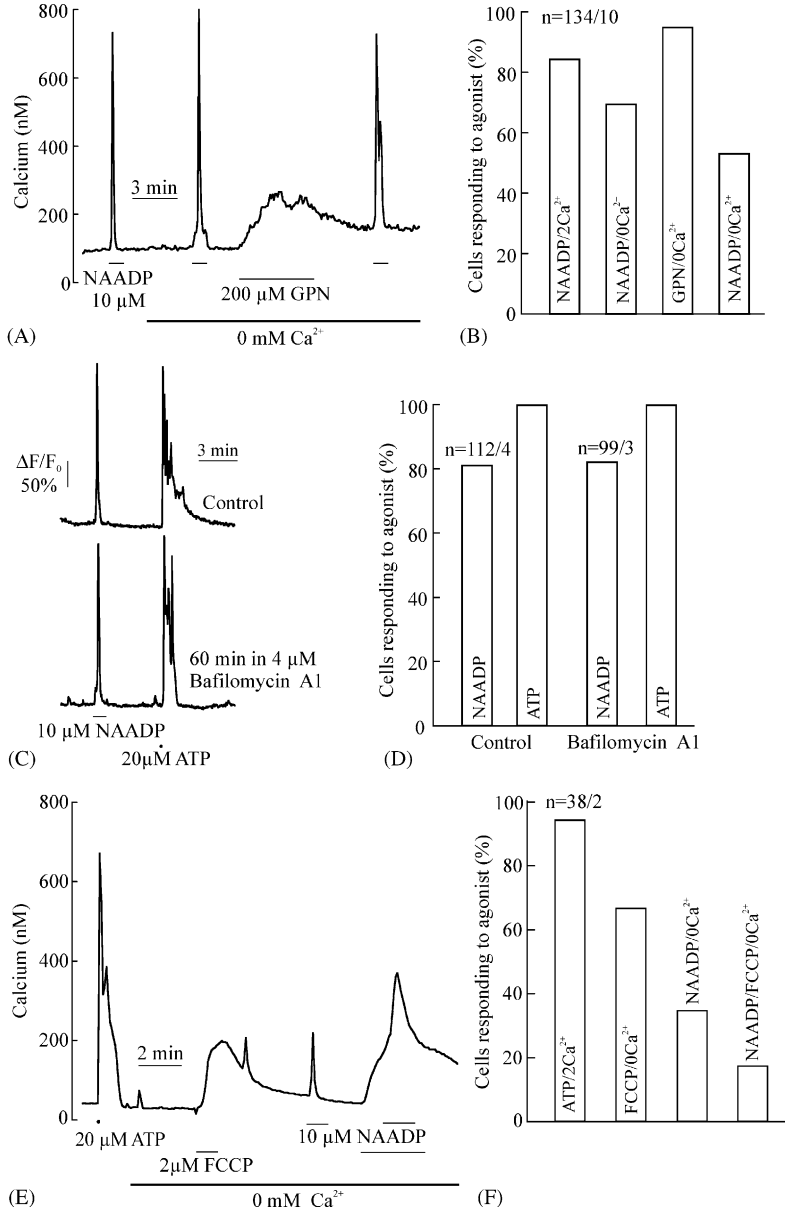

Fig. 6. (A and B) Glycyl-L-phenylalanine-β-naphthylamide (GPN, 200 μM, 5 min) induced Ca^{2+} release itself, but did not suppress the NAADP-evoked Ca^{2+} response, indicating that NAADP-sensitive stores are not depleted by GPN. (C and D) Pretreatment with proton pump inhibitor bafilomycin A1 did not block the NAADP- and the ATP-evoked Ca^{2+} response. (E and F) After the mitochondrial uncoupler *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, 2 μM) induced Ca^{2+} rise in the absence of external Ca^{2+}, a significant number of cells still responded to NAADP with a Ca^{2+} transient, even during the Ca^{2+} rise induced by FCCP.

drial uncoupling agent, which enters mitochondria in the protonated form, discharging the pH gradient, and thereby dissipating the mitochondrial membrane potential. In this way, FCCP eliminates the ability of mitochondria to sequester Ca^{2+}. Perfusion of FCCP (2 μM) and NAADP elicited a Ca^{2+} rise in the absence of extracellular Ca^{2+}, indicating the release of Ca^{2+} from intracellular stores (Fig. 6E). NAADP could still evoke a Ca^{2+} transient after the treatment of FCCP in 34% of the cells, and 18% of the cells also during a second exposure to FCCP ($n = 38$; Fig. 6F). In this series 94% of the cells responded to ATP (20 μM, 5 s) at the beginning of the experiment. These results suggest that FCCP impairs the cells' responsiveness to NAADP, but make it unlikely that mitochondria were the source of the Ca^{2+} released in response to NAADP.

3.7. NAADP also evokes a Ca^{2+} rise in astrocytes in situ

In order to confirm that the response to bath-applied NAADP was not restricted to cultured astrocytes, but also to astrocytes in situ, we applied the agonist to acute brain slices of the hippocampus and the cerebellum. Cytosolic Ca^{2+} was measured in hippocampal astrocytes and cerebellar Bergmann glial cells. The glial cells were identified by their

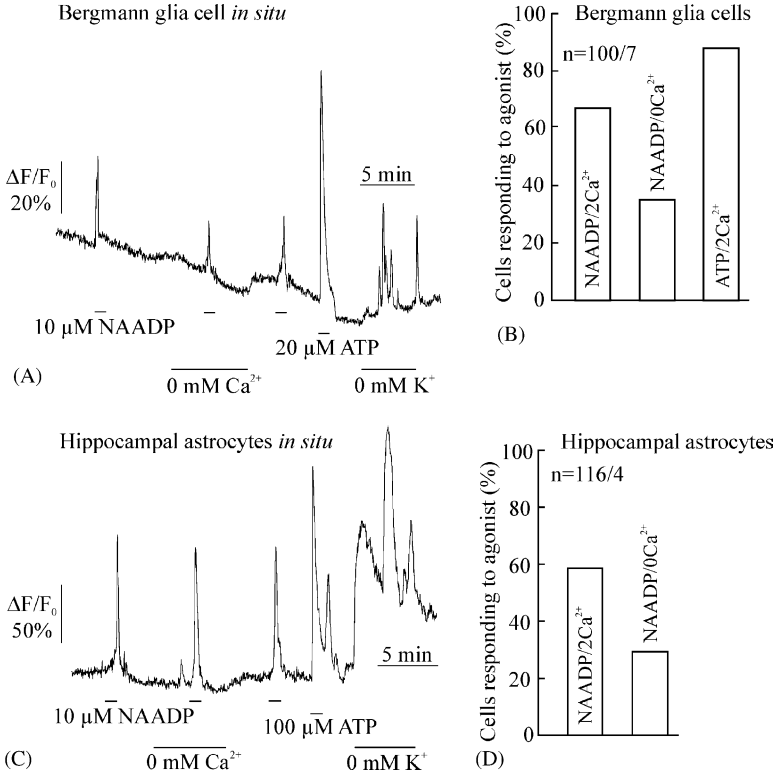

Fig. 7. NAADP-induced Ca^{2+} rise in astrocytes in situ. NAADP (10 μM, 1 min) elicited a reversible Ca^{2+} rise in both Bergman glial cells of acute cerebellar brain slices (A and B) and in astrocytes of acute hippocampal brain slices (C and D) in the presence and absence of external Ca^{2+}. ATP (20 μM or 100 μM, 1 min) evoked a Ca^{2+} response in the majority of cells and was used as a control, while 0 mM K^+ (5 min) was applied to identify astrocytes.

typical location, their shape, and by their response to 0 mM K^+, which results in a Ca^{2+} rise [18,19]. NAADP evoked reversible Ca^{2+} rises in 67% of the Bergmann glial cells in the presence of Ca^{2+} ($n = 100$; Fig. 7A and B). In comparison, 90% of the astrocytes responded with robust Ca^{2+} transient to ATP in these preparations. In the absence of extracellular Ca^{2+}, the number of cells responding to NAADP decreased to 35%.

Similarly, NAADP evoked a Ca^{2+} rise in 59% of the hippocampal astrocytes in the presence of extracellular Ca^{2+}, and in 29% of the cells in the absence of Ca^{2+} ($n = 116$; Fig. 7C and D). Increasing the NAADP concentration to 30 μM did not significantly increase the number of cells responding to the agonist ($n = 30$; not shown). These results indicate that 10 μM NAADP evoked a Ca^{2+} response in the majority of astrocytes in situ, suggesting that this agonist functions as a Ca^{2+} mobilizer also in acute brain slices.

4. Discussion

In the present study, we have employed the novel messenger NAADP to challenge Ca^{2+} signals in rat astrocytes in culture and in situ. Although NAADP has so far been described as an intracellular, *second* messenger, NAADP was able to evoke Ca^{2+} release from intracellular stores in astrocytes, when applied extracellularly via the bath perfusion. We could show that NAADP evoked a Ca^{2+} response, when applied to cultured astrocytes; in acute brain slices of the cerebellum and hippocampus, NAADP elicited Ca^{2+} transients in a significant number of glial cells.

The response to NAADP documented here differed in several aspects from those reported in most cells, where NAADP was usually microinjected into cells or applied to cellular fragments [29–31]. In most cells investigated so far, NAADP targets an intracellular Ca^{2+} store, presumably related to acidic compartments such as lysosomes, but distinct from IP_3- and thapsigargin-sensitive ER stores. In astrocytes, NAADP evoked a Ca^{2+} transient or Ca^{2+} oscillations due to Ca^{2+} release from IP_3- and CPA-sensitive ER stores by the following criteria: (1) NAADP evoked a Ca^{2+} response in the presence and absence of external Ca^{2+}; (2) the Ca^{2+} response was suppressed by depleting the stores with CPA or ionomycin, but was still present, when the stores had not been emptied completely; (3) NAADP challenged the same stores as ATP, which elicits Ca^{2+} release from ER stores via IP_3 formation; (4) the Ca^{2+} response to NAADP was suppressed by blocking P_2; (5) IP_3 receptors, and (6) was fully abolished after inhibition of phospholipase C. Neither GPN nor FCCP, which target acidic organelles or mitochondria, respectively, could suppress the NAADP-induced Ca^{2+} response, while impairing the vacuolar H^+-ATPase activity by

bafilomycin A1 had also no effect on NAAPD-evoked Ca^{2+} response.

4.1. Ca^{2+} release elicited by NAADP

The discovery of a highly potent intracellular second messenger NAADP has provided new insights into the complex Ca^{2+} signaling patterns [32–34]. Surprisingly, NAADP evokes Ca^{2+} responses in astrocytes, when applied extracellularly. This infers that NAADP is either actively transported into the astrocytes to act as an intracellular messenger, or activates a receptor in the cell membrane, which then initiates a signaling cascade leading to intracellular Ca^{2+} release. NADP, a precursor of NAADP, also evokes Ca^{2+} responses in astrocytes. In guinea-pig taenia coli, it was reported that NADP acts in a similar way as a P_2-purinoceptor agonist [35].

The maximal effects of NAADP in cultured astrocytes were observed with increasing concentrations of NAADP ($\geq$10 μM), leading to a non-desensitizing response. In most other cell types, intracellular NAADP acts in nanomolar concentrations with prominent self-inactivation at higher concentrations [10]. In rat mesangial cell microsomes, NAADP evoked Ca^{2+} responses with half-maximal concentration at 3 μM [36], which is similar to the 7 μM found in the present study for cultured astrocytes. However, in mesangial cell microsomes, in contrast to astrocytes, the NAADP-induced Ca^{2+} response was not suppressed by inhibitors of IP_3- or ryanodine-sensitive receptors.

Yet another pathway activated by NAADP has been described in isolated pancreatic acinar nuclei, where NAADP releases Ca^{2+} from thapsigargin-sensitive stores in the nuclear envelope by activating ryanodine receptors [14]. The nuclear envelope membrane is continuous with the ER membrane, and also contains both ryanodine and IP_3 receptors. We have recently shown that astrocytes do not contain functional caffeine-sensitive stores [19], which infers that ryanodine-sensitive stores as target for NAADP are unlikely in these cells.

In the nervous system, NAADP has been shown to release Ca^{2+} from stores in presynaptic terminals of identified neurons in *Aplysia* [37] and at the frog neuromuscular junction [38], resulting in increased transmitter release. A recent report using rat cortical neurons showed that NAADP, applied in liposomes, evokes a cytosolic Ca^{2+} increase and potentiates neurite outgrowth in response to serum and nerve growth factor, which was both abolished after blockade of IP_3 and ryanodine receptors [39].

The present study provides evidence that NAADP also evokes Ca^{2+} responses in astrocytes due to Ca^{2+} release from CPA- and ionomycin-sensitive stores. NAADP appears to challenge the same stores as ATP, although NAADP apparently cannot fully recruit the ATP-sensitive Ca^{2+} stores. We suggest that NAADP activates the metabotropic P_2Y receptors in the plasma membrane of astrocytes, which then generates PLC-coupled IP_3 formation and subsequently results in IP_3-induced Ca^{2+} release.

4.2. NAADP-sensitive stores are different from GPN- and FCCP-sensitive stores

Recent studies have proposed that the NAADP-sensitive Ca^{2+} store might be the acidic lysosomes [13,40]. In some cell types, NAADP evokes Ca^{2+} signals from bafilomycin A1-sensitive lysosomal stores, co-localized with a fraction of the sarcoplasmic reticulum expressing ryanodine receptors. These lysosome–sarcoplasmic reticulum junctions comprise a highly specialized trigger zone for NAADP [41]. However, the NAADP targeted stores are heterogenous in different cell types [42]. Our study suggests that NAADP challenges neither acidic organelles nor mitochondria in astrocytes. The NAADP-evoked Ca^{2+} response in astrocytes is bafilomycin insensitive in contrast to mammalian neurons [39]. Disrupting lysosomes and dissipating the mitochondrial proton gradient are also ineffective in suppressing the NAADP-induced Ca^{2+} response. In line with this are the experiments with ionomycin, which does not target acidic compartments, because protons would compete with Ca^{2+} for binding of the ionophore at the inner surface of the membrane [43]; yet, ionomycin depleted the NAADP-sensitive stores. This suggests that the acidic stores are different from NAADP-sensitive stores in cultured astrocytes.

Taken together, our results show that NAADP activates P_2Y receptors in the cell membrane of astrocytes, which then initiate a metabotropic pathway leading to Ca^{2+} release from ATP-sensitive stores via the IP_3-dependent cascade. It appears that NAADP is a much more wide-spread messenger leading to Ca^{2+} responses in neurons and glial cells, and that NAADP may operate via different mechanisms, both in the brain and other tissues, to release Ca^{2+} from intracellular organelles.

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