Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Regulates Autophagy in Cultured Astrocytes

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca\(^{2+}\)-mobilizing messenger that in many cells releases Ca\(^{2+}\) from the endolysosomal system. Recent studies have shown that NAADP-induced Ca\(^{2+}\) mobilization is mediated by the two-pore channels (TPCs). Whether NAADP acts as a messenger in astrocytes is unclear, and downstream functional consequences have yet to be defined. Here, we show that intracellular delivery of NAADP evokes Ca\(^{2+}\) signals from acidic organelles in rat astrocytes and that these signals are potentiated upon overexpression of TPCs. We also show that NAADP increases acidic vesicular organelle formation and levels of the autophagic markers, LC3II and beclin-1. NAADP-mediated increases in LC3II levels were reduced in cells expressing a dominant-negative TPC2 construct. Our data provide evidence that NAADP-evoked Ca\(^{2+}\) signals mediated by TPCs regulate autophagy.

Increases in cytosolic Ca\(^{2+}\) regulate a myriad of cellular functions including information processing in the central nervous system (1). In many cells, these changes can be driven by mobilization of intracellular Ca\(^{2+}\) stores (2). Much attention has focused on the endoplasmic reticulum as a Ca\(^{2+}\) store (3), but accumulating evidence also implicates acidic organelles such as lysosomes in the control of Ca\(^{2+}\) dynamics (4). In particular, NAADP\(^{2}\) has emerged as a novel intracellular Ca\(^{2+}\)-mobilizing messenger that links cell surface stimulation to the release of Ca\(^{2+}\) from acidic Ca\(^{2+}\) stores (5).

Changes in the concentration of cytosolic Ca\(^{2+}\) in glial cells are key for bidirectional control of neuronal activity (1). Previous studies have shown that extracellular application of NAADP can evoke Ca\(^{2+}\) signals in astrocytes, consistent with a messenger role for NAADP in this cell type, following its internalization (6). Interpretation of these results, however, is clouded by the demonstrated lack of specificity with respect to related nucleotides (6) and by the potential activation by NAADP of cell surface purinergic receptors (7). Whether NAADP acts as an intracellular messenger in astrocytes is therefore unclear.

Although the role of inositol trisphosphate and ryanodine receptors is established in mediating Ca\(^{2+}\) release from the ER in response to inositol trisphosphate and cyclic ADP-ribose, respectively (2), the molecular basis for Ca\(^{2+}\) release by NAADP from acidic organelles is less certain (8). In a series of recent studies, however, a novel family of Ca\(^{2+}\) channels, known as the two-pore channels (TPCs), have emerged as likely targets (9). Thus, TPCs localize to endosomes and/or lysosomes through an identified targeting motif and enhance NAADP-mediated cytosolic Ca\(^{2+}\) signals when overexpressed (10–12). Inhibition of TPC expression/function using siRNA (10), TPC knock-out mice (11), or a dominant-negative TPC construct (10) reduces NAADP-evoked Ca\(^{2+}\) signals, and biophysical analyses indicate that TPCs are NAADP-gated Ca\(^{2+}\)-permeable channels (13). Moreover, a functional role for TPCs has been identified in events such as smooth muscle contraction (14) and differentiation (15) in which NAADP had been previously implicated (16, 17). Nevertheless, the molecular mechanism of action of NAADP remains controversial and may involve direct activation of ryanodine receptors or certain members of the transient receptor potential channel family (18).

Macroautophagy (referred to from here on as autophagy) is a catabolic process essential for the turnover of cytosolic proteins and organelles, such as mitochondria and peroxisomes. It involves the formation of a double membrane structure known as the autophagosome (or autophagic vacuole) around a targeted region of the cytosol (19). Autophagosomes derive from phosphatidylinositol 3-phosphate-rich membranes and require phosphatidylinositol 3-kinase and the ATG family of proteins including microtubule-associated protein 1 light chain 3 (LC3/ATG8) and beclin-1 (ATG6) (20, 21). Once formed, autophagosomes mature and eventually fuse with lysosomes, thereby delivering their contents to lysosomal hydrolases for degradation. A key regulator of autophagy is the mTOR complex, which

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\[2\] The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide phosphate; NAADP-AM, NAADP-acetoxyethyl; TPC, two-pore channel; ER, endoplasmic reticulum; LC3, light chain 3; mTOR, mammalian target of rapamycin; mRFP, monomeric red fluorescence protein; ANOVA, analysis of variance; 3-MA, 3-methyladenine; AVO, acidic vesicular organelle.
under normal nutrient-rich conditions exerts an inhibitory effect on autophagy. Deregulated autophagy has been implicated in several diseases including neurodegeneration (22). Consequently a better understanding of its regulation is vital.

Agents such as bafilomycin, NH₄Cl, chloroquine, and nigericin are commonly used to inhibit autophagy (23, 24). These agents collapse the pH gradient across the lysosome, and it is this change in the pH that is thought to compromise autophagosome-lysosome fusion. Notably, Ca²⁺ uptake into acidic organelles will also be affected by alkalinization (25). Indeed, bafilomycin has been shown to inhibit NAADP-evoked Ca²⁺ signals in a variety of cells (26) including neurons (27). This raises the possibility that NAADP-mediated Ca²⁺ signals may be involved in the autophagic process. Here, we provide evidence that NAADP mobilizes Ca²⁺ from acidic Ca²⁺ stores through TPCs in primary cultured rat astrocytes and present evidence that NAADP-evoked Ca²⁺ signals regulate autophagy.

MATERIALS AND METHODS

Cell Isolation and Culture—Astrocytes were prepared from cortices of 4-day-old rats, as described previously (28). After 7–10 days, cells were plated in Petri dishes for fluorescence microscopy.

Plasmid Constructs and Transfection—Constructs encoding human TPC1 or TPC2 tagged at their C termini with GFP or monomeric red fluorescence protein (mRFP) and C-terminally GFP-tagged TPC2 L265P were previously described (10, 12). To monitor autophagosomes and autolysosomes, GFP-LC3 or mCherry-LC3 was used (29). Cells were transiently transfected with 1 μg of cDNA using FuGENE® (Roche Applied Sciences) according to the manufacturer’s instructions. Experiments were performed 1–2 days after transfection.

Microinjection and Ca²⁺ Measurements—Astrocytes were loaded with 3 μM Fura-2AM (Molecular Probes, Eugene, OR) for 30 min in microscopy buffer and then washed with intracellular buffer used previously (30). Fura-2 fluorescence microscopy and microinjection were conducted as described (30). Micropipettes were supplemented with intracellular buffer with or without the indicated concentration of NAADP. The injection parameters were: 30° angle; 40-hectopascal compensation pressure; 60-hectopascal injection pressure; 0.5-s injection time corresponding to an injection volume of 40–70 femtoliters.

Confocal Microscopy—Fluorescence images of astrocytes were acquired using a confocal microscope (LSM 510 META; Carl Zeiss, Germany) and a 40× NA 1.3 oil immersion objective. The excitation and emission wavelengths were 488 and 505–550 nm for GFP constructs and 543 and 560–615 nm for mRFP constructs. In some experiments, cells were labeled with LysoTracker® Green (500 nm, 30 min) and imaged using the GFP settings. The percentages of LC3-positive cells with LC3 puncta were quantified as described (29).

Flow Cytometry—Populations of astrocytes were washed in PBS and incubated with acridine orange (1 μg/μl, 15 min). Cells were analyzed on a flow cytometer (10⁶ events) using the CellQuest software (BD Biosciences).

Western Blot Analysis—Experiments were performed as described previously (29). The primary antibodies used were anti-LC3B (1:1000, Cell Signaling) or anti-BECN1 (1:100; Santa Cruz Biotechnology). Densitometric analyses were performed using the QuickScan 2000 WIN software (Helena Laboratories).

RT-PCR—Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was prepared using a SuperScript™ III reverse transcriptase (Invitrogen) with oligo(dT) primers. Oligonucleotide primers were designed to the nucleotide sequences of rat TPCs (10). Reactions were denatured for 3 min at 94 °C followed by 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 50 °C for TPC1 and 52 °C for TPC2), and extension (7 min at 72 °C) using DNA polymerase (Platinum Taq high fidelity; Invitrogen).

For quantitative PCR, samples were analyzed using SYBR Green and the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Amplification conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were obtained at the end of the amplification to confirm specificity. Each sample was run in triplicate. The average cycle threshold (Ct) was determined with Applied Biosystems software. Data were analyzed by the comparative ΔΔCt method (ABI PRISM User Bulletin 2; Applied Biosystems). Data are expressed as mean ± S.E. of the 2⁻ΔΔCt values from 3–4 cDNAs from different animals. Expression levels were normalized to the expression of β-actin after parallel amplification. The oligonucleotide primer sequences to TPCs were described in Ref. 10.

Statistical Analysis—All values are given as mean ± S.E. Significance was tested by ANOVA followed by Dunnett’s or Bonferroni’s test. Values of p < 0.05 were considered significant.

Chemicals—NAADP-AM was synthesized as described previously (31). Other reagents were from Sigma-Aldrich.

RESULTS AND DISCUSSION

To clarify the role of NAADP in astrocytes, we measured cytosolic Ca²⁺ levels in response to direct intracellular delivery of NAADP. As shown in Fig. 1A, microinjection of NAADP (pipette concentration of 100 nm) evoked a prompt increase in cytosolic Ca²⁺ levels. The NAADP-evoked Ca²⁺ rise was sub-
stastically reduced after preincubation with NED-19 (Fig. 1A), a recently identified NAADP antagonist (32). The effects of NAADP were also inhibited by bafilomycin A1 (Fig. 1A). These data are consistent with the involvement of acidic organelles in mediating the effects of NAADP. The residual Ca\(^{2+}\) signal in the presence of NED-19 or bafilomycin A1 was comparable with that evoked by buffer microinjection (Fig. 1B). Similar experiments using a range of NAADP concentrations revealed the typical bell-shaped concentration-effect relationship (Fig. 1B) whereby micromolar concentrations of NAADP are ineffective in evoking Ca\(^{2+}\) signals (5). Taken together, the above results suggest that NAADP-sensitive Ca\(^{2+}\) channels are functionally expressed in astrocytes and display the hallmark features with respect to their pharmacology, functional location, and agonist sensitivity.

Emerging studies implicate TPCs as credible targets for NAADP within the endolysosomal system (9). To probe the role of TPCs in NAADP action in astrocytes, we overexpressed TPC1 and TPC2 and determined their subcellular distribution and sensitivity to NAADP. As shown in Fig. 1C, both isoforms displayed a punctate intracellular distribution. There was partial colocalization of TPC1 with the weak fluorescent base LysoTracker Green, whereas TPC2 showed near complete colocalization (Fig. 1C). These data are consistent with a lysosomal and non-lysosomal (likely endosomal) location for TPC1 and a predominantly lysosomal location for TPC2 as reported in breast cancer cells (10). Importantly, NAADP-evoked Ca\(^{2+}\) signals were substantially larger in cells overexpressing either isoform (Fig. 1D), consistent with the effects of NAADP being mediated by TPCs in this cell type. Indeed, NAADP-evoked Ca\(^{2+}\) signals in TPC2-expressing cells were reduced after preincubation with bafilomycin A1 (data not shown). Endogenous transcripts were substantially reduced after preincubation with that evoked by buffer microinjection (Fig. 1B). Similar results were observed in cells incubated with rapamycin (Fig. 2, A and D). Incubation of cells with 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid prevented puncta formation by NAADP-AM (supplementary Fig. 1), suggesting that the effects of NAADP were Ca\(^{2+}\)-dependent.

To determine the specificity of action of NAADP-AM, we used NED-19 (32), which as shown above inhibited NAADP-mediated Ca\(^{2+}\) release (Fig. 1, A and B). NED-19 completely blocked the increase in AVO number induced by NAADP-AM (Fig. 2, B and C) and substantially reduced NAADP-AM-induced GFP-LC3 puncta formation (Fig. 2, B and D). We also examined the effect of 3-methyladenine (3-MA), which inhibits autophagy by targeting class III PI3K, which is essential for the initiation of autophagy via recruitment of other ATG proteins (34). As with NED-19, 3-MA blocked the stimulatory effects of NAADP-AM on both acridine orange and GFP-LC3 labeling (Fig. 2, B–D). Additionally, we determined the effects of NAADP-AM on endogenous levels of LC3. As shown in the Western blot in Fig. 2E, NAADP-AM increased the LC3-II/LC3-I ratio. NAADP-AM treatment also caused a modest but significant increase in levels of beclin-1, another autophagy marker (Fig. 2F). Taken together, the above results obtained using three independent methods uncover a selective and previously unrecognized role for NAADP in regulating autophagy.

Because TPCs were shown to be located in acidic organelles and capable of driving Ca\(^{2+}\) release by NAADP, we probed the relationship between TPCs and autophagy. We focused on TPC2 because it showed a greater colocalization with LysoTracker Green than with TPC1 (Fig. 1C). In cells co-transfected with TPC2 and LC3, we noted marked colocalization of the two expressed proteins after NAADP-AM treatment (Fig. 3A). We quantified the number of GFP-LC3-positive structures in control cells and cells expressing TPC2 (Fig. 3B). In the absence of treatment, there was little difference in GFP-LC3 staining between the two cell types. Intriguingly, although the number of puncta increased ~6-fold upon NAADP-AM treatment, there was no significant difference between control and TPC2-expressing cells (Fig. 3B) despite demonstrably larger (global) Ca\(^{2+}\) signals in the latter (Fig. 1). As in untransfected cells (Fig. 2), the effects of NAADP-AM on LC3 puncta formation were
point mutation was recently shown to block NAADP-evoked mutated within the putative pore region (TPC2 L265P). This in sufficient numbers to maximally increase LC3-II levels. vide further evidence that the effects of NAADP on autophagy an analogous residue (Leu-273) was similarly mutated (10). In sup-
dominant-negative manner as reported for TPC1 in which the ing cells. These data are consistent with TPC2 L265P acting in a lowed by Bonferroni’s post hoc test).

In summary, we report the presence of functional NAADP-sensitive Ca^{2+}-permeable channels in astrocytes on acidic stores and show that their activation is associated with an increase in autophagic markers. Our intracellular NAADP delivery methods extend previous studies where NAADP was applied extracellularly and shown to have pleiotropic actions (6, 7). A signaling role for mobilization of acidic Ca^{2+} stores by NAADP reported here is consistent with a recent report demonstronstrating that astrocytic Ca^{2+} signals in response to glutamate are reduced following pretreatment with baflomycin A1 and glycyrl-1-phenylalanine-β-naphthylamide (GPN) (35). The mechanism whereby NAADP regulates autophagy remains to be further investigated. Of note is a previous study demonstrating that elevations in cytosolic Ca^{2+} can induce autophagy via inhibition of mTOR. However, this effect was ascribed to the mobilization of ER Ca^{2+} stores (36). Although NAADP targets acidic Ca^{2+} stores, their mobilization is often associated with subsequent mobilization of ER Ca^{2+} stores through the process of Ca^{2+}-induced Ca^{2+} release (5). Indeed, NAADP-evoked Ca^{2+} signals in astrocytes were partially sensitive to depletion of ER Ca^{2+} stores with thapsigargin (data not shown). Thus, indirect mobilization of ER Ca^{2+} stores by NAADP may regulate autophagy through subsequent activation of Ca^{2+}/calmodulin-dependent kinase-β and AMP-activated protein kinase (36). Alternatively, NAADP may regulate autophagy by local Ca^{2+} release events that promote fusion of autophagosomes and lysosomes. Indeed, several homotypic and heterotypic fusion events between organelles within the endolysosomal system required for trafficking are sensitive to 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (a fast Ca^{2+} chela-tor) but not EGTA (a slow Ca^{2+} chelator), indicative of spatially restricted Ca^{2+} elevations close to the fusion machinery (37). Regardless of its exact mechanism of action, our data implicate NAADP and its target channels as novel regulators of autophagy.

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