Widespread Distribution of Binding Sites for the Novel Ca\(^{2+}\)-mobilizing Messenger, Nicotinic Acid Adenine Dinucleotide Phosphate, in the Brain*

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca\(^{2+}\)-mobilizing agent in invertebrate eggs that has recently been shown to be active in certain mammalian and plant systems. Little, however, is known concerning the properties of putative NAADP receptors. Here, for the first time, we report binding sites for NAADP in brain. In contrast to sea urchin egg homogenates, \([^{32}P]\)NAADP bound reversibly to multiple sites in brain membranes. The rank order of potency of NAADP, 2',3'-cyclic NAADP and 3'-NAADP in displacing \([^{32}P]\)NAADP was, however, the same in the two systems and in agreement with their ability to mobilize Ca\(^{2+}\) from homogenates. These data indicate that \([^{32}P]\)NAADP likely binds to receptors mediating Ca\(^{2+}\) mobilization. Autoradiography revealed striking heterogeneity in the distribution of \([^{32}P]\)NAADP binding sites throughout the brain. Our data strongly support a role for NAADP-induced Ca\(^{2+}\) signaling in the brain.

Increases in cytosolic Ca\(^{2+}\) can show both temporal and spatial inhomogeneity giving rise to the phenomena of Ca\(^{2+}\) waves and oscillations (1, 2). In non-excitable cells, a variety of extracellular stimuli mediate changes in cytosolic Ca\(^{2+}\) through the mobilization of intracellular Ca\(^{2+}\) stores (3). This is achieved through the concerted activation of a family of related intracellular Ca\(^{2+}\)-channels/receptor complexes for inositol 1,4,5-trisphosphate (IP\(_3\)) (4, 5) and ryanodine (6). The latter are thought to be activated and/or modulated by cyclic ADP-ribose (cADPR) (7, 8). Depletion of intracellular Ca\(^{2+}\) stores activates Ca\(^{2+}\) entry from the extracellular space that serves to refill them, thereby sustaining the Ca\(^{2+}\) signal (9). Conversely, in excitable cells such as neurons, Ca\(^{2+}\) increases are mediated primarily through voltage- and ligand-gated Ca\(^{2+}\) channels located on the plasma membrane. However, accumulating evidence also implicates a role for intracellular Ca\(^{2+}\) stores in neuronal Ca\(^{2+}\) homeostasis (10, 11). Thus, in all cells, interplay between mobilization of stored Ca\(^{2+}\) and Ca\(^{2+}\) influx across the plasma membrane is likely important in shaping cytosolic Ca\(^{2+}\) signals.

Recently, a novel Ca\(^{2+}\)-mobilizing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) has been described in sea urchin eggs (7), which, based on cross-desensitization experiments (12) and distinct pharmacology (13), is thought to release Ca\(^{2+}\) independently of IP\(_3\) or ryanodine receptor activation. NAADP-induced Ca\(^{2+}\) mobilization in the sea urchin egg is unique in several respects. First, sub-threshold concentrations of NAADP completely desensitize homogenates to subsequent challenge with maximal concentrations of NAADP that normally evoke full Ca\(^{2+}\) release (13, 14). Secondly, NAADP releases Ca\(^{2+}\) from a pool distinct from that mobilized by IP\(_3\) and cADPR (12, 15, 16), and finally, NAADP-induced Ca\(^{2+}\) mobilization is not regulated by Ca\(^{2+}\) (16, 17), a property that underlies regenerative Ca\(^{2+}\) release via IP\(_3\) and ryanodine receptors (1, 2). A binding site for NAADP has been previously demonstrated in sea urchin egg microsomes (14); however, little is known concerning the molecular identity or distribution of putative NAADP receptors.

The actions of NAADP have now been extended to mammalian (18, 19) and plant (20) preparations. In acinar cells of the pancreas, NAADP is thought to underlie complex Ca\(^{2+}\) signals in response to the brain-gut peptide, cholecystokinin, by providing a "trigger" Ca\(^{2+}\) release that is subsequently propagated by ryanodine and IP\(_3\) receptors (18, 21). NAADP can also mobilize Ca\(^{2+}\) from crude brain microsomes (19) raising the possibility that NAADP signaling, like the phosphoinositide pathway (10, 11), may be active in the brain. Indeed, NAADP metabolism has been characterized in brain preparations (19, 22).

Here we have characterized binding sites for \([^{32}P]\)NAADP in the brain. This is the first report of NAADP binding sites in a mammalian tissue. Our data support a general role for NAADP-mediated Ca\(^{2+}\) signaling in the brain.

**EXPERIMENTAL PROCEDURES**

Synthesis of Radioiodode-[\([^{32}P]\)NAADP synthesis described here is a variation of the procedure reported previously (14). \([^{32}P]\)NAADP was synthesized from \([^{32}P]\)NAD (2 µCi/mmol; Amersham Pharmacia Biotech) by incubation with NAD kinase (50 units/ml; Sigma) and 10 mM MgATP for 2 h at 37 °C in a buffer containing 5 mM HEPES (pH 7.5). The remaining ATP and ADP was converted to AMP by incubation with apyrase (2 units/ml; Sigma) for 1 h at 37 °C. The reaction was then diluted 5-fold into a medium containing 10 mM MES (pH 5.0), 19 mM nicotinamide, and ADP-ribose cyclase (2 µg/ml; Sigma) and incubated for another 2 h to convert \([^{32}P]\)NADP to \([^{32}P]\)NAADP by base exchange. The final mixture was separated by anion exchange high performance liquid chromatography on a 3 × 150-mm column packed with AGMP1 (Bio-Rad). Elution was performed at a flow rate of 1 ml/min using a gradient of trifluoroacetic acid (TFA) that increased linearly from 0–2% over the first 6 min, to 4% at 11 min, to 8% at 16 min, to 16% at 21 min, to 32% at 26 min, and to 100% (150 mM TFA) at 26.1 min. Fractions were collected every minute and neutralized by the addition of Tris base (final concentration 75 mM), and their radioactivity was determined by Cerenkov counting. A major peak was observed at 25.6 ± 0.4 min (n = 3) that corresponded to authentic

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The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; cADPR, cyclic ADP-ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; TFA, trifluoroacetic acid; MES, 4-morpholineethanesulfonic acid; NAAD, nicotinic acid adenine dinucleotide.
NAADP (determined by monitoring absorbance at 254 nm) run on a parallel AGMP1 column (elution time 25.1 ± 0.2 min).

Tissue Preparation—Whole brains from male CD1 mice or Harlan Sprague-Dawley rats were homogenized at 4 °C using an UltraTurax homogenizer in binding medium composed of 20 mM HEPES (pH 7.2) and 1 mM EDTA. Equilibrated samples were then centrifuged (10 min; 20,000 g) and stored at −20 °C until use.

Collection of sea urchin (Lytechinus pictus) eggs and preparation of homogenates was typically 125 fmol/mg of protein (~95% of total binding). Most error bars in A and C were smaller than the symbols.

Fig. 1. Binding sites for [32P]NAADP in brain. A, association kinetics of [32P]NAADP binding. Membranes were incubated with [32P]NAADP for the indicated times prior to determination of bound radiolabel (n = 3). B, specificity of [32P]NAADP binding. Effect of NAADP (100 μM), NADP (100 μM), NAAD (100 μM), cADPR (100 μM), and IP3 (20 μM) on [32P]NAADP binding (n = 3). Data are expressed relative to binding of [32P]NAADP in the absence of competitor. C, dissociation kinetics of [32P]NAADP binding. Membranes were equilibrated with [32P]NAADP for 60 min, and 100 μM NAADP was added at time 0 to initiate dissociation of the radioligand (n = 3). Specific binding (2000–4000 cpm/incubation) to brain membranes was typically 7 fmol/mg of protein (~70% of total binding). Most error bars in A and C were smaller than the symbols.

 Autoradiography—Male Harlan Sprague Dawley rats (340–360 g) were killed by cervical dislocation, and brains were rapidly removed, frozen in isopentane, and stored at −70 °C prior to sectioning. Cryostat sections (12 μm) were thaw-mounted on to gelatin-coated slides and returned to −70 °C for storage. Binding of [32P]NAADP (1–2 nM) to the sections was performed for 2 h at 20 °C in a medium composed of 20 mM HEPES (pH 7.2) and 1 mM EDTA. Equilibrated samples were then washed with HEPES buffer at 4 °C (10 min; pH 7.2), air dried, and exposed to Hyperfilm (Amersham Pharmacia Biotech) for 12–24 h at −80 °C. Autoradiograms were analyzed using Scion Image (Scion Corporation, Frederick, MD). Nonspecific binding of [32P]NAADP (determined in the presence of 100 μM unlabeled NAADP) to sections cut at the level of the frontal cortex (see Fig. 3A) and thalamus (data not shown) was virtually undetectable.

Other Methods—2′,3′-Cyclic NAADP and 3′-NAADP were synthesized and purified as described (23). Protein was determined using bicinchoninic acid (Sigma). Data are presented as means ± standard error of the mean.
RESULTS AND DISCUSSION

Enzymatically prepared [32P]NAADP bound to crude membrane preparations from brain (Fig. 1). Association of [32P]NAADP with membranes followed monophasic kinetics with a first order association rate constant (k_{1a}) of 9.4 ± 1 × 10^{7} M^{-1} min^{-1} (n = 3; see Fig. 1A). Binding of [32P]NAADP was specific for NAADP, because a 100,000-fold molar excess of the related nucleotides, NAD, nicotinic acid adenine dinucleotide (NAAD), and cADPR did not displace [32P]NAADP (Fig. 1B). Binding of [32P]NAADP was also insensitive to IP_{3} (20 μM) (Fig. 1B). These data indicate that [32P]NAADP binds to a unique site distinct from that of known Ca^{2+}-mobilizing messengers. Apparent (partial) displacement of the radioligand was observed with NAADP (100 μM), but this effect, however, is likely to be due to NAADP contamination in commercial NADP preparations (14).

From competitive equilibrium displacement analysis (Fig. 2), the concentration of NAADP causing half-maximal displacement of the radioligand (IC_{50}) from brain membranes was 200 ± 17 nM (n = 11). In contrast, the IC_{50} for sea urchin egg homogenates was 0.47 ± 0.06 nM (n = 12). Differences were also observed in the Hill coefficient (n_{H}) for binding in the two tissues; n_{H} was 0.76 ± 0.04 (n = 11) and 1.05 ± 0.02 (n = 12) in brain and sea urchin eggs, respectively. Thus, [32P]NAADP likely binds to a single site in the egg preparations (probably the inactivation site; see below), whereas in the brain, multiple binding sites for [32P]NAADP may exist.

[32P]NAADP binding to sea urchin egg microsomes has been reported to be essentially irreversible (14). This was confirmed in the present studies using crude homogenates (data not shown). This unusual property is likely to underlie the remarkable inactivation properties of NAADP-induced Ca^{2+} release in these cells (13, 14). In stark contrast, however, [32P]NAADP binding to brain membranes was completely reversible (Fig. 1C). Thus, an excess of unlabeled NAADP (100 μM) initiated dissociation of bound [32P]NAADP with a first order dissociation rate constant (k_{-1}) of 0.07 ± 0.008 min^{-1} (n = 3). The clear differences in the reversibility of NAADP binding in the brain and sea urchin egg likely explain the ~1000-fold difference in apparent affinity for NAADP (Fig. 2).

We next examined the effects of the NAADP analogues, 2′,3′-cyclic NAADP and 3′-NAADP (23), on [32P]NAADP binding. Both the analogues fully displaced [32P]NAADP but were less potent than NAADP (Fig. 2). The observed rank order of potency (NAADP > 2′,3′-cyclic NAADP > 3′-NAADP) was the same in brain membranes and sea urchin egg homogenates (Fig. 2). IC_{50} values for NAADP, 2′,3′-cyclic NAADP, and 3′-NAADP were 240 ± 40 nM, 531 ± 26 nM, and 2518 ± 104 nM (n = 3), respectively, for brain membranes. The corresponding values for sea urchin egg homogenates were 0.18 ± 0.008 nM, 0.64 ± 0.03 nM, and 1.3 ± 0.35 nM (n = 3). Additionally, the reported rank order of potency of NAADP and the analogues in stimulating Ca^{2+} release from sea urchin egg homogenates is also identical (23). Taken together, these data strongly suggest that [32P]NAADP binds to physiologically relevant receptors linked to NAADP-induced Ca^{2+} mobilization (19).

Autoradiographic analysis revealed that NAADP binding sites were distributed throughout the brain (Fig. 3). Strikingly, the localization of [32P]NAADP binding sites was far from homogeneous. Binding of [32P]NAADP was particularly high in the medulla (Fig. 3H), midbrain (Fig. 3G), and thalamus (Fig. 3E). Individually, these areas are known to be involved in a diverse range of functions and collectively, contain pathways involved in the processing of somatosensory information. Precisely localized levels of binding were also detected in anterior corti-

cerebral regions and globus pallidus (Fig. 3D). In contrast to inositol trisphosphate and ryanodine receptor distribution (24), NAADP binding was low in the striatum (Fig. 3C) and hippocampus (Fig. 3F). Furthermore, [32P]NAADP binding levels in the cerebellum were moderate (Fig. 3H), whereas this region is particularly enriched in the other intracellular Ca^{2+} release channels (24). Interestingly, [32P]NAADP binding was apparent in both gray and white matter (e.g. corpus callosum; see Fig. 3C), possibly indicating the presence of the binding sites in both neuronal and non-neuronal cells. Whether the observed regional differences in [32P]NAADP binding sites results from differences in expression levels and/or affinity of NAADP receptors remains to be established. Nevertheless, our data point to possible functional heterogeneity in NAADP signaling within the brain.

This study is the first to demonstrate specific binding sites for NAADP in a mammalian tissue. Remarkably, whereas the NAADP analogue specificity was similar in brain and sea urchin eggs, the reversibility of binding between the two systems was in stark contrast. These data indicate differential regulation and/or the existence of multiple NAADP receptor subtypes. Indeed, the apparent affinity for NAADP (and the analogues) were markedly different in brain and egg preparations, and displacement curves in the brain were somewhat shallow. Furthermore, we have demonstrated intriguing heterogeneity in the distribution of NAADP binding sites within the brain and found key differences between the localization of these sites and that of known intracellular Ca^{2+} release channels. The present data, together with our recent demonstration of NAADP-induced Ca^{2+} release from brain microsomes (19), and the ability of brain homogenates to both synthesize and degrade NAADP (19, 22), strongly support a signaling role for NAADP in the brain.

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