NAADP receptors are present and functional in the heart

Judit Bak*†, Richard A. Billington*, Gyoergy Timar†, Alice C. Dutton* and Armando A. Genazzani*

Alongside the well-studied inositol 1,4,5 trisphosphate and ryanodine receptors, evidence is gathering that a new intracellular release mechanism, gated by the pyridine nucleotide nicotinic acid adenine dinucleotide phosphate (NAADP), is present in numerous organisms, ranging from plant to mammalian cells (reviewed in [1]). Most cells have been shown to express at least two Ca^{2+}-release mechanisms controlled by different messengers, and this can lead to redundancy, convergence, or divergence of responses. One exception appears to be muscle and heart contractile tissues. Here, it is thought that the dominant intracellular channel is the ryanodine receptor, while IP_3 receptors are poorly expressed and their role appears to be negligible. We now report that NAADP receptors are functional and abundant in cardiac microsomes. NAADP binds specifically and with high affinity (130 pM and 4 nM) to two sites on cardiac microsomes and releases Ca^{2+} with an apparent EC_{50} of 323 ± 14 nM. Furthermore, binding experiments show that this receptor displays both positive and negative cooperativity, a peculiarity unique among intracellular Ca^{2+} channels. Therefore, we show that the heart possesses multiple mechanisms to increase the complexity of Ca^{2+} signaling and that NAADP may be integral in the functioning of this organ.

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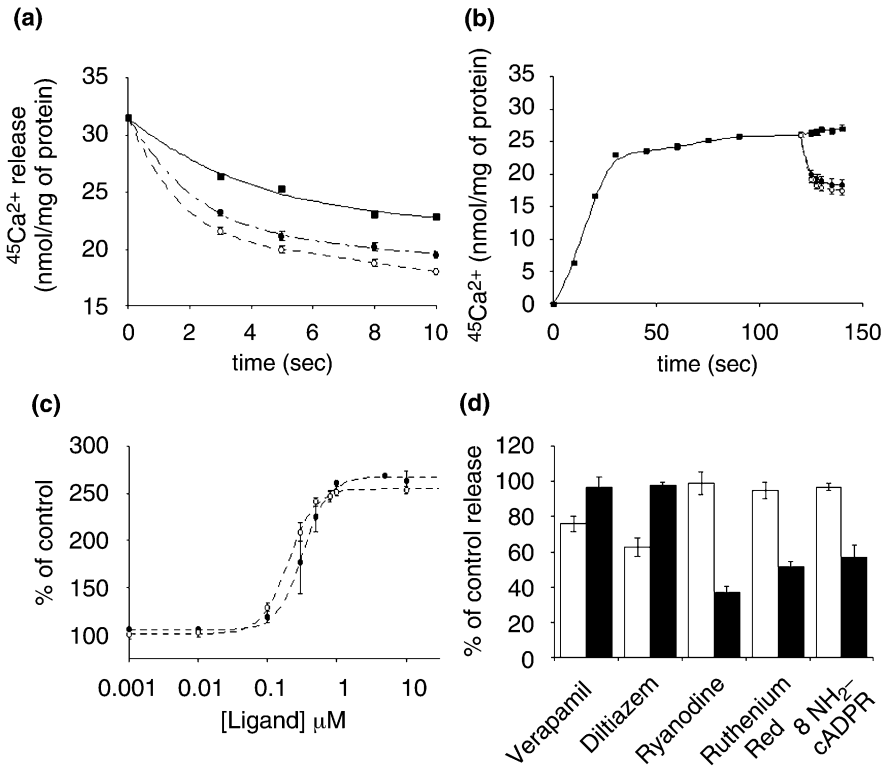
Results and discussion

First, we investigated whether NAADP could induce Ca^{2+} release from adult rabbit heart microsomes loaded passively with $^{45}Ca^{2+}$ (see Materials and methods in the Supplementary material available with this article online) and compared it to cADPR-induced Ca^{2+} release. In this assay, both NAADP (1 μM) and cADPR (1 μM) induced a fast Ca^{2+} efflux from the microsomes, which differed significantly from control microsomes (Figure 1a). In the first 3 seconds after its addition, NAADP released 8.3 ± 0.31 nmol Ca^{2+}/mg protein, while cADPR released 9.9 ± 0.23 nmol Ca^{2+}/mg protein, compared to the 5.2 ± 0.32 nmol Ca^{2+}/mg protein released by the control release solution (Figure 1a). It is thought that the latter release occurs in part due to Ca^{2+}-induced Ca^{2+} release (CICR), since an optimal concentration of this ion is present in the release buffer (1 μM). To evaluate the efficacy of release by pyridine nucleotides, we compared Ca^{2+} release to that induced by caffeine, an established agonist of the ryanodine receptor. Caffeine (5 mM) released 10.9 ± 0.88 nmol Ca^{2+}/mg protein in 3 s (44.3% of ionomycin release, n = 3). In the same set of experiments, cADPR released 9.9 ± 0.32 nmol Ca^{2+}/mg protein (91% of caffeine release, n = 3), and NAADP released 8.6 ± 0.22 nmol Ca^{2+}/mg protein (80% of caffeine release, n = 3). Similar to previous data in brain microsomes [2], NAADP appeared insensitive to the extravesicular Ca^{2+} concentrations, while controls, as expected, appeared to be the most sensitive (10 nM Ca^{2+} in the buffer induced 63% ± 7.2% of 1 μM Ca^{2+}). When Ca^{2+} was buffered to 10 nM, cADPR released 9.2 ± 0.65 nmol Ca^{2+}/mg protein (143% of the control), while NAADP released 10.12 ± 0.93 nmol/mg (158% of the control; data not shown) after 3 s. This would suggest that NAADP-mediated Ca^{2+} release is robust and could play a modulatory role toward other release mechanisms. This possibility is further substantiated by the data from other model systems, where NAADP-induced Ca^{2+} release is capable of recruiting other release mechanisms [3–5]. To further discriminate between the effect of NAADP and any artifacts of the technique, we loaded microsomes in an active manner with 1 mM ATP in the medium. Ca^{2+} ATPases rapidly induced Ca^{2+} uptake in microsomes (half-maximal uptake occurred between 15 and 20 s, and maximal uptake occurred after 30–40 s). After 2 min of incubation with ATP, stores were fully loaded, and release was initiated. In this scenario, NAADP (2 μM) and cADPR (2 μM) released similar amounts of Ca^{2+} (5.2 ± 0.56 and 6.4 ± 0.56 nmol/mg protein after 5 s, respectively; Figure 1b). Total NAADP and cADPR release was 32%–38% and 40%–43% of ionomycin-releasable Ca^{2+}, respectively (calculated after 20 s of the addition of the agonist in two different cardiac preparations). It has been previously suggested that ATP and cADPR might compete for the same site on the ryanodine receptor complex [6], but in our experimental conditions, we could detect cADPR release in the presence of 1 mM ATP at room temperature. When a concentration-response curve

Figure 1

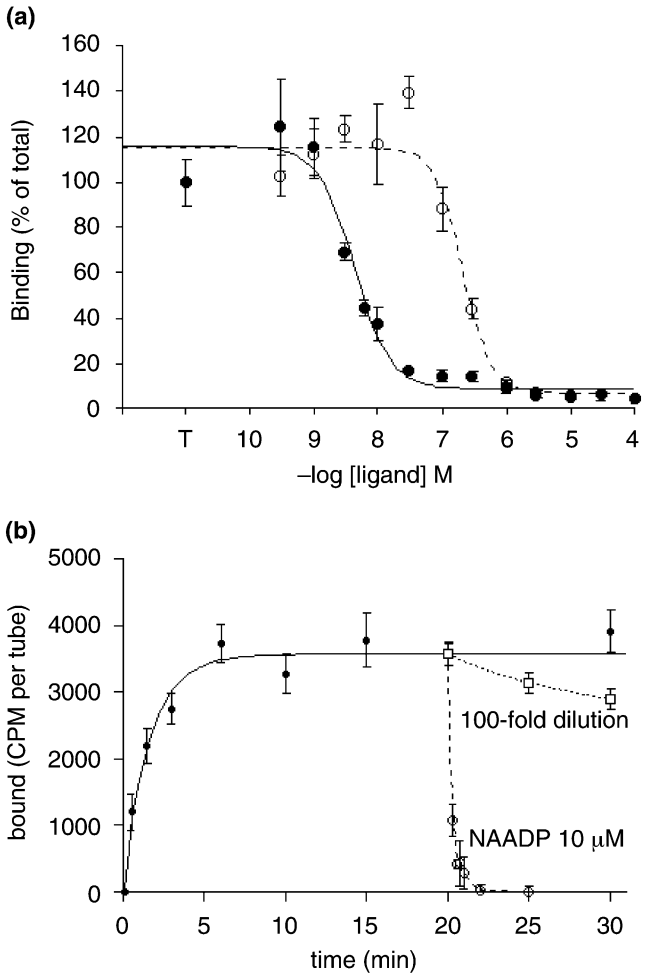


(a) $^{45}Ca^{2+}$ release from passively loaded cardiac microsomes induced by CICR (solid line, filled squares), NAADP (1 μM; broken line, filled circles), and cADPR (1 μM; broken line, open circles). **(b)** Active $^{45}Ca^{2+}$ uptake into cardiac microsomes. Release by NAADP (2 μM; filled circles) and cADPR (2 μM; open circles). **(c)** The concentration dependence of NAADP- (filled circles) and cADPR- (open circles) induced Ca^{2+} release. **(d)** A comparison of the effects of various pharmacological agents on NAADP- (1 μM; open bars) and cADPR- (1 μM; filled bars) induced release. Pharmacological agents include verapamil, 100 μM; diltiazem, 100 μM; ryanodine, 100 μM; ruthenium red, 5 μM; and 8-NH_2-cADPR, 10 μM. Values are mean ± SEM of 3–9 determinations.

was performed after passive loading and the release at 3 s was used as a measure of the total response induced by NAADP, it was found that NAADP releases Ca^{2+} with an EC_{50} of 323 ± 14 nM and a Hill slope of 2.32 ± 0.21 (Figure 1c). cADPR appeared to be slightly more potent, with an EC_{50} of 199 ± 6.1 nM and a Hill slope of 2.2 ± 0.11. NAADP has been shown to release Ca^{2+} via a distinct channel in all systems examined thus far, although interplay between Ca^{2+}-release systems has been reported. Similarly, ruthenium red and 8-NH_2-cADPR, antagonists of the ryanodine receptor-mediated mechanism, had no effect on NAADP-induced Ca^{2+} release, while these compounds were capable of partially inhibiting cADPR-mediated responses (Figure 1d). High concentrations of verapamil and diltiazem (100 μM) selectively, but only partially, inhibited NAADP responses versus cADPR responses, consistent with data from earlier reports in sea urchin eggs [7] (Figure 1d). Although these antagonists did not completely abolish NAADP-induced release, it did not seem appropriate to increase their concentrations, since 100 μM is approximately two orders of magnitude higher than the concentration required to selectively block L-type Ca^{2+} channels and already influences non-specifically numerous channels. Furthermore, the coaddition of heparin and 8-NH_2-cADPR did not affect NAADP-induced Ca^{2+} release (data not shown).

If NAADP, as suggested by our data, releases Ca^{2+} by a separate mechanism from that of ryanodine and IP_3 receptors, then we would expect to find a specific binding site in cardiac microsomes. Indeed, [^{32}P]NAADP bound avidly to its receptor (see Supplementary material for a [^{32}P]NAADP production and binding protocol) with an apparent K_d of 4.2 ± 0.71 nM (Figure 2a). Furthermore, the Hill slope was close to 2 (2.0 ± 0.89), mimicking closely the cooperativity observed for Ca^{2+} release. The discrepancy between the EC_{50} of Ca^{2+} release and the K_d is consistent with sea urchin egg data [1], has been shown for other ligands (e.g., IP_3 [8]), and might be attributable to the different kinetics of release versus binding [8]. Our data also suggests that there is an additional specific displacement when more than 1 μM cold ligand was used (Figure 2a), which could represent an $NADP^+$ binding protein. When adding low concentrations of cold ligand to the 150 pM [^{32}P]NAADP, we observe an increase in bound molecules (up to 150% of the control value, Figure 2a). This suggests a positive cooperativity similar to the one observed for some plasma membrane receptors (e.g., insulin). When the data obtained was linearized by Scatchard analysis (data not shown), we detected the presence of at least three binding sites. Alternatively, this could be interpreted as the presence of a single class of binding sites whose affinity changes with receptor occupancy, i.e., negative cooperativity (see below). Alongside a low-affinity site (identified to be the same as the one mentioned above), another two sites were identifiable. They showed

Figure 2



(a) The displacement of [^{32}P]NAADP binding by NAADP (solid line, filled circles) and NADP (broken line, open circles; purified once as described in Supplementary material). **(b)** The association and dissociation kinetics of [^{32}P]NAADP binding. Dissociation was performed by either 100-fold dilution (open squares) or the addition of excess cold ligand (open circles). Values are mean ± SEM of 6–9 determinations.

affinity constants of approximately 130 pM and 4 nM, with B_{max} values of 25 and 507 fmol/mg, respectively. Recently, a similar assay has been used to investigate NAADP binding in rat brain [9]. Although, in this report, binding appeared specific, the K_d was found to be considerably higher (~200 nM), and the Hill slope differed considerably (~1). It, therefore, appears that, similar to IP_3 and ryanodine receptors, different NAADP receptor subtypes may be expressed in different tissues.

Not surprisingly, cADPR had no influence on [^{32}P]NAADP binding. The possibility that this binding site was not specific to NAADP was further tested by challenging [^{32}P]NAADP binding with high concentrations of structurally similar compounds (Table 1). NAD^+ (10 μM) lacked affinity for the binding site, as did NAAD, suggesting that, as in sea urchin eggs, the phosphate group is critical for receptor-ligand recognition [10]. On the other hand, $NADP^+$ and ADP ribose phosphate (ADPRP) showed some affinity for the receptor. $NADP^+$ had an affinity of 206 ± 24.5 nM (Hill slope of 1.97 ± 0.12; Figure 2a), while ADPRP had no effect at a concentration of 100 nM but displaced 60% ± 3.9% of the bound [^{32}P]NAADP at 1 μM. The $NADP^+$ effect is similar to that observed in sea urchin eggs [11, 12], where it displays a 500-fold lower affinity than NAADP, and part of this effect has been attributed to the contamination present in the commercial sources of $NADP^+$, which is extremely resistant to purification [13]. In support of this, $NADP^+$ (20 μM) bought from commercial sources (Sigma) released 34% of the Ca^{2+} released by NAADP (1 μM), suggesting that NAADP is present in the sample. However, ADPRP seems to be recognized differently by the mammalian receptor compared to that in sea urchin, where the K_d appears to be greater than 10 μM (R.A.B. and A.A.G., unpublished data). Nonetheless, this data supports the notion that NAADP binding is specific, that the phosphate group is crucial for recognition, and that the pyridine moiety dictates affinity. We, therefore, suggest that it is likely that the binding site in the heart is not identical to that present in sea urchin eggs.

Table 1

Displacement of [^{32}P]NAADP binding by structural analogs and Ca^{2+} mobilizing agents.

Ligand	% Bound
Total	100 ± 1.23
10 μM NAADP	9.22 ± 0.71
10 μM NAD	128 ± 17.74
10 μM NAAD	125 ± 15.2
10 μM cADPR	113 ± 7.70
10 μM ryanodine	107 ± 17.3
100 nM ADPRP	96.7 ± 5.27
10 μM ADPRP	16.3 ± 2.16

Values are ± SEM of 3–6 determinations.

We then investigated the association and dissociation kinetics of [^{32}P]NAADP binding. NAADP associated rapidly to its receptor on ice, with a $t_{1/2}$ of 0.6 min (Figure 2b). Once again, this is in stark contrast to brain, where association appeared to be ~10-fold slower [9]. To measure dissociation, we allowed full association and then added an excess amount of cold NAADP. Unlike in sea urchin, [^{32}P]NAADP was displaced rapidly, with ~56.0 ± 6.5% of specific counts displaced after 15 s of addition of the displacing ligand. To further analyze this, we allowed full association for 20 min and diluted the microsomes 100-fold. This procedure, by reducing the concentration of free ligand in the medium, should induce a similar dissociation to that shown with the addition of cold ligand. Al-

most no dissociation was measured during the time course of the experiment. To our knowledge, the only other proteins that display similar characteristics are plasma membrane peptide receptors (e.g., insulin, GDNF), and this is the first report of an intracellular mechanism displaying such a property. In the case of insulin receptors, the presence of negative cooperativity, whereby the ligand binding site appears to be able to switch between a high-affinity, slow-dissociating state to a low-affinity, fast-dissociating state as their occupancy increases, has been postulated [14–16]. It is highly likely that a similar situation occurs with NAADP. Our data parallels functional observations in pancreatic and submandibular acinar cells [4, 5, 17], where 100 μM NAADP does not elicit responses, but 50 nM does. Nonetheless, whether and how these two observations are mechanistically linked is unclear at the present time.

From our data it appears that NAADP signaling could play a role in cardiac Ca^{2+} homeostasis. This possibility is strengthened by the recent finding that the enzyme responsible for the production of NAADP is present on the intracellular membranes of the myocardium [18]. Furthermore, a preliminary report suggests that NAADP enhances spark occurrence in permeabilized cardiomyocytes [19]. This contribution, together with a recent report that IP_3 receptors might play a role in the atria in excitation-contraction coupling [20], suggests that the heart possesses multiple release mechanisms. Whether NAADP-sensitive signaling represents a "backup" system for ryanodine receptor-mediated signaling, whether it represents a signal that is decoded differently (allowing the myocyte to discriminate between a signal to contract and a signal to perform a different task), or whether it coordinates ryanodine-receptor signaling (as proposed recently in pancreatic acinar cells, starfish, and sea urchins) remains to be established.

Supplementary material

The Materials and methods section, including information on microsome preparation, radioligand binding, and Ca^{2+}-release assays, can be found with the Supplementary material, which is available at http://images.cellpress.com/supmat/supmatin.htm.

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