Regulation of calcium signalling by adenine-based second messengers

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Abstract
cADPR (cyclic ADPR (ADP-ribose)), NAADP (nicotinic acid–adenine dinucleotide phosphate) and ADPR belong to the family of adenine-containing second messengers. They are metabolically related and are all involved in the regulation of cellular Ca\textsuperscript{2+} homeostasis. Activation of specific plasma membrane receptors is connected to cADPR formation in many cell types and tissues. In contrast receptor-mediated formation of NAADP and ADPR has been shown only in a few selected cellular systems. The intracellular Ca\textsuperscript{2+} channel triggered by cADPR is the RyR (ryanodine receptor); in the case of NAADP, both activation of RyR and a novel Ca\textsuperscript{2+} channel have been proposed. In contrast, ADPR opens the non-specific cation channel TRPM2 [TRP (transient receptor potential) melastatin 2] that belongs to the TRP family of ion channels.

Adenine-based second messengers
The first second messenger discovered in 1957 was cAMP [1]. This cyclic adenine-containing nucleotide has since been shown to mediate important intracellular effects, including activation of glycogenolysis in hepatocytes and mobilization of fatty acids in adipose tissue. However, except in cardiac myocytes, cAMP is not connected to increases in [Ca\textsuperscript{2+}]; (cytosolic free Ca\textsuperscript{2+} concentration). On the other hand changes in [Ca\textsuperscript{2+}], have been early recognized as one of the most important, powerful and versatile mechanisms of intracellular signal transduction since such changes occur in response to many extracellular signals, e.g. hormones, mediators, cell–cell contacts or physical stimuli in many different cells and organisms. Accordingly, research has focused on putative second messengers regulating rapid movements of Ca\textsuperscript{2+} ions across the membranes of intracellular Ca\textsuperscript{2+} stores, e.g. the ER (endoplasmic reticulum)/SR (sarcoplasmic reticulum), or across the plasma membrane. These rapid directed movements of Ca\textsuperscript{2+} ions are possible since SERCAs (SR/ER Ca\textsuperscript{2+}-ATPases) or PMCs (plasmamembrane Ca\textsuperscript{2+}-ATPases) continuously build up significant gradients by pumping Ca\textsuperscript{2+} ions into the stores or into the extracellular space.

In 1983, Schulz and Berridge discovered that InsP\textsubscript{3} releases Ca\textsuperscript{2+} from the ER [2]. InsP\textsubscript{3} has since then been shown to be involved in the generation of both local and global Ca\textsuperscript{2+} signals in many different cellular signal transduction processes (reviewed in [3]).

After the discovery of the InsP\textsubscript{3}/Ca\textsuperscript{2+} signalling system, it came as a surprise that additional small endogenous molecules are able to release Ca\textsuperscript{2+} from intracellular stores. In their pioneering work, Lee and co-workers [4–6] discovered two such compounds, cADPR [cyclic ADPR (ADP-ribose)] and NAADP (nicotinic acid–adenine dinucleotide phosphate).

Interestingly, there is a relatively close metabolic relationship between cADPR and NAADP. The main pathway to cADPR formation proceeds from NAD\textsuperscript{+} to cADPR catalysed by ADPRC (ADP-ribose cyclase) activity (Figure 1; reviewed in [7]). In addition, it is well known that NAD\textsuperscript{+} can be converted into NADP\textsuperscript{+} by NAD kinase (Figure 1). NAADP formation by a base-exchange reaction is then catalysed by ADPRC, the same class of enzymes that also synthesizes cADPR (Figure 1). In addition to the Ca\textsuperscript{2+}-releasing second messengers cADPR and NAADP, in 2001 the modulatory activity of a third adenine-containing nucleotide involved in Ca\textsuperscript{2+} homeostasis, ADPR, was discovered [8,9]. ADPR acts on a non-specific cation channel of the plasma membrane termed TRPM2 [TRP (transient receptor potential) melastatin 2].

The cADP-ribose/Ca\textsuperscript{2+} signalling pathway
In-depth review papers dealing with the cADPR/Ca\textsuperscript{2+} signalling system have been published in recent years [10–13]; readers interested in additional details or aspects not covered by the current review may refer to these.

The cADPR/Ca\textsuperscript{2+} signalling pathway has been detected in several cell types, including smooth, skeletal and cardiac muscles, neuronal and neuronal-related cells, haemopoietic cells, acinar cells and oocytes (for a more complete list refer to [10]). Activation of ADPRC is induced by ligation of receptors localized in the plasma membrane (Figure 2) and leads to increased concentrations of cADPR within the cell.

Key words: ADP-ribose (ADPR), cyclic ADP-ribose (cADPR), calcium signalling, nicotinic acid–adenine dinucleotide phosphate (NAADP), second messenger, transient receptor potential melastatin 2 (TRPM2).

Abbreviations used: ADPR, ADP-ribose; ADPRC, ADP-ribosyl cyclase; [Ca\textsuperscript{2+}], cytosolic free Ca\textsuperscript{2+} concentration; cADPR, cyclic ADPR; CRACM1, Ca\textsuperscript{2+} channel modulator 1; ER, endoplasmic reticulum; NAADP, nicotinic acid–adenine dinucleotide phosphate; N\textsuperscript{i}-cIDPR, N\textsubscript{i}-cyclic inosine diphosphoribose; PARP, poly(ADPR) polymerase; RyR, ryanodine receptor; SERCA, sarco/Endoplasmic reticulum Ca\textsuperscript{2+} ATPase; TRPM2, TRP melastatin 2.

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Figure 1 | Metabolic relationships between the Ca\textsuperscript{2+} messengers cADPR, ADPR and NAADP
Abbreviations used: NicAmide, nicotinamide; NicAcid, nicotinic acid; NAADP-R, proposed NAADP receptor.

Figure 2 | Receptor-mediated formation and sites of action of adenine-based, Ca\textsuperscript{2+}-modulating second messengers
Dotted lines indicate minor pathways or relationships not generally accepted or proven. NADase, NAD\textsuperscript{+} glycohydrolase; NudT9-H, NudT9 homology region.
The identity of the mammalian ADPRC forming cADPR inside cells is still somewhat controversial since the only molecularly known mammalian ADPRCs, CD38 and CD157 (reviewed in [7]), are ectoenzymes. For CD38, De Flora et al. [12] have solved this ‘topological paradox’ as depicted in Figure 2: NAD\textsubscript{2} is transported into the extracellular space via connexin 43. There, it is converted by ecto-CD38 into cADPR that is directly transported by CD38 or, more likely, by nucleoside transporters into the cytosol (reviewed in [12]). Uptake systems for cADPR also open the possibility for paracrine action of cADPR; indeed, paracrine- and cADPR-mediated relationships between several cell systems have been demonstrated (reviewed in [12]).

Recent work on CD38 revealed the structure of its extracellular domain at high resolution [14]. However, experiments in tissues from CD38 knockout mice indicate that additional cADPR-forming enzymatic activities are expressed [15]. Another interesting recent finding relates to novel adenine nucleotides of the Ap2A-type (adenosine–PP\textsubscript{i}–adenosine) formed by ADPRC in the presence of adenine; these compounds interfere with Ca\textsuperscript{2+} signalling in \textit{vitro} and display cytotoxic properties [16].

Once cADPR is formed, it increases [Ca\textsuperscript{2+}]. The most well-known targets for cADPR are RyRs (ryanodine receptors), one of the principal classes of intracellular, ligand-operated ion channels. Whether cADPR binds directly to RyR or first attaches to a soluble binding protein remains to be determined: evidence for a cADPR-binding protein has been presented by photo-affinity labelling studies [17] and by direct binding of cADPR to FKBP 12.6 (FK506 binding protein 12.6; [18]). Although the molecular identity of the binding site of cADPR is still unknown, a huge number of derivatives of cADPR, either synthesized chemically or by chemoenzymatic synthesis, have added significantly to the understanding of its structure–activity relationship. One important observation was the almost identical biological activity, in terms of Ca\textsuperscript{2+} release, of N1-cIDPR (N1-cyclic inosine diphosphoribose) as compared with cADPR [19]. Derivatives of N1-cIDPR are also agonists, but with varying potency [20]. Another interesting observation relates to the two ribose moieties connecting the base and the PP\textsubscript{i} bridge. Zhang and co-workers [21–23] have synthesized derivatives of N1-cIDPR in which either the ribose connected to N1 of hypoxanthine (also called ‘northern ribose’) or both ribose moieties have been replaced by simple diethyl ether or alkane strands. The diethyl ether and alkane strands partially mimic the ribose, at least the spacer function between the base and the PP\textsubscript{i} bridge. Surprisingly, these derivatives are biologically active and, owing to their more hydrophobic diethyl ether chains, are membrane-permeant [21–23].

In addition to Ca\textsuperscript{2+} release, cADPR appears to be involved in Ca\textsuperscript{2+} entry too. In human T-lymphocytes, sustained Ca\textsuperscript{2+} entry [24] was blocked by the membrane-permeant cADPR antagonist 7-deaza-8-Br-cADPR and by antisense RNA-mediated knock down of type 3 RyR [25]. Furthermore, application of membrane-permeant agonists to intact cells induced sustained Ca\textsuperscript{2+} entry [20–23]. In neutrophils from wild-type mice the chemotactic peptide fMLP (N-formyl-methionyl-leucylphenylalanine; ‘chemotactic peptide’) induced biphasic calcium signalling: calcium release followed by calcium entry [26]. The calcium entry phase was blocked by the cADPR antagonist, 8-Br-cADPR, and was absent from neutrophils from Cd38\textsuperscript{−/−} mice. The Cd38\textsuperscript{−/−} neutrophils lack the ability to produce both cADPR and ADPR [26], indicating that cADPR and/or ADPR are essentially involved in neutrophil Ca\textsuperscript{2+} entry. Two possible mechanisms exist: on the one hand store depletion by cADPR may activate capacitative Ca\textsuperscript{2+} entry (Figure 2). Experiments in DT40 lymphocytes lacking expression of all three types of InsP\textsubscript{3}R suggest activation of a current characteristic of capacitative Ca\textsuperscript{2+} entry by cADPR [27]. Proteins involved in capacitative Ca\textsuperscript{2+} entry have been identified recently as Stim1 and Orai1/CRACM1 (Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel modulator 1) [28,29]. Stim1 is a protein connecting the ER lumen with the putative Ca\textsuperscript{2+} channel Orai1/CRACM1 (Figure 2). In addition to Ca\textsuperscript{2+} entry via indirect activation of Orai1/CRACM1, cADPR may also act as co-activator of TRPM2 (see the section ‘The ADPR/TRPM2/Ca\textsuperscript{2+} signalling pathway’ below).

The NAADP/Ca\textsuperscript{2+} signalling pathway
In contrast with cADPR, NAADP has only recently been accorded full status of a second messenger by demonstrating rapid formation of NAADP in response to certain extracellular stimuli. Although NAADP had initially been described as a Ca\textsuperscript{2+} mobilizing endogenous nucleotide in 1995 [6], it took quite a while for suitable extraction protocols and quantification methods for NAADP to be developed. Accurate quantification of the minute amounts of NAADP in cells is either achieved by a competitive protein binding assay using a specific NAADP-binding protein from sea urchin egg [30] or by enzymatic cycling assays [31,32].

Endogenous basal concentrations of NAADP have been found to be in the low nanomolar range [30,32,33]. Upon specific stimulation in pancreatic acinar cells and T-lymphocytes a very rapid and transient increase in NAADP was observed at approx. 10–30 s post stimulation [30,32]. This indicates rapid regulation of an NAADP-synthesizing and an NAADP-metabolizing enzyme (Figure 2). The only known enzyme to synthesize NAADP is the ectoenzyme CD38; however, it is unclear how the enzymatic activity of CD38 is regulated. Thus the question as to which enzymes are involved in the fast metabolism of NAADP remains open. In T-cells, besides the rapid formation, a sustained increase in cellular NAADP was observed [32], indicating also a role in the sustained Ca\textsuperscript{2+} signalling phase.

The target organelle and the target receptor/Ca\textsuperscript{2+} channel for NAADP are also a matter of debate (Figure 2). Data originally obtained in sea urchin eggs indicate that the target organelle for NAADP is an acidic, lysosome-related compartment [34]. This has been confirmed in some mammalian
The ADPR/TRPM2/Ca\(^{2+}\) signalling pathway

TRPM2 is a plasma membrane channel conducting Ca\(^{2+}\) (and Na\(^{+}\)) inflow. Although the presence of ADPR in cells was already reported for erythrocytes in the early 1990s [42], it took another 10 years until activation of TRPM2 was proposed by Perraud et al. [8] and Sano et al. [9].

The rationale to investigate the influence of ADPR on TRPM2 function arose from the observation that the cytosolic C-terminal region of the channel protein contains a domain that is homologous with the mitochondrial ADPR-metabolizing enzyme NUDT9 (Figure 3; [8]). Structural and mutational analysis revealed direct binding of ADPR into a cleft formed by the NUDT9 homology region, thereby inducing the channel opening of TRPM2. Since the enzymatic ADPR hydrolase activity is largely reduced in the NUDT9 homology region of the channel [8], this motif primarily serves as an interaction site for ADPR.

Free ADPR is generated by different cellular pathways (Figure 3): (i) the hydrolysis of NAD\(^{+}\) directly yields ADPR and nicotinamide. This reaction is catalysed by NAD\(^{+}\) glycohydrolases (NADases), including the cell surface ectoenzymes CD38 and CD157 (reviewed in [7]) as well as a proposed mitochondrial NADase [43]; (ii) the subsequent action of both PARPs [poly(ADPR) polymerases; PARP enzymes] and poly(ADPR) glycohydrolases (PARG enzymes) indirectly generates ADPR via the formation and hydrolysis of

cell types, but other investigators found, at least in addition to a lysosomal store, also sensitivity of the ER towards NAADP [35]. Regarding the NAADP receptor/Ca\(^{2+}\) channel, there is clear pharmacological evidence from the sea urchin egg system that inhibitors of RyR do not block the effect of NAADP [6]. Ongoing attempts to purify the receptor resulted in its partial characterization on the biochemical level, but not in its molecular identification. On the other hand, several authors have shown for mammalian cells that the Ca\(^{2+}\) channel involved in NAADP action is the RyR (Figure 2); techniques involved in these experiments were single-channel recordings using reconstituted RyR in lipid planar bilayers [36], specific inhibitors of RyR [35,37,38], and knock down of RyR [37,38]. RyR as target of NAADP may also involve an additional NAADP-binding protein (Figure 2).

Similar to cADPR, administration of NAADP into intact cells activates Ca\(^{2+}\) entry [38,39]. This may proceed via the capacitative Ca\(^{2+}\) entry system (Figure 2), as discussed above for cADPR or via activation of TRPM2 by NAADP [40]. However, very high concentrations of NAADP (EC\(_{50}\) = 730 µM) were necessary to activate TRPM2 [40].

Importantly, it has recently been shown that NAADP can be taken up by cells, probably in a similar way as discussed above for cADPR, opening the possibility for paracrine functions of NAADP [41].
poly(ADPR) residues on different proteins [44]; and (iii) hydrolysis of cADPR (reviewed in [7]).

A signalling function of ADPR has become an attractive hypothesis [45], although no direct experimental evidence was available for this model until recently. Pharmacological evidence points towards an elevation of cytosolic ADPR levels upon challenging cells by oxidative stress [46]. Although most investigators demonstrated an indirect action of H₂O₂ by stimulating ADPR formation, also a direct action of H₂O₂ on TRPM2 was proposed (Figure 3). The first direct demonstration of an increase in the cytosolic ADPR concentration induced by extracellular stimulation was recently obtained in human T-cells [47], suggesting a second messenger function of ADPR.

The regulation of TRPM2 gating by different ligands is quite complex: e.g. the EC₅₀ for activation of TRPM2 by ADPR in patch–clamp experiments largely depends on the concentration of free Ca²⁺ present in the pipette solution: 130 µM under complete chelation of Ca²⁺ [8] versus 12 µM without Ca²⁺ buffering [48]. Binding of Ca²⁺/calmodulin sensitizes TRPM2 for the activation by ADPR via a positive feedback mechanism. However, Ca²⁺ is just one ligand modulating the sensitivity of TRPM2 for ADPR: high concentrations of cADPR directly activate TRPM2 in the absence of ADPR [48]. Lower concentrations, which are more in the physiological range, positively modulate TRPM2 gating by ADPR [48]. Recently, dependence of TRPM2 gating on elevated temperatures was shown by Togashi et al. [49]: temperatures above 35 °C directly activated TRPM2 and positively modulated TRPM2 gating by ADPR, cADPR and NAD⁺. In contrast, the ADPR breakdown product AMP possibly competes with ADPR for binding to the NUDT9 homology region, thereby negatively modulating TRPM2 function [48]. Recently, Beck et al. [40] reported the activation of TRPM2 by NAADP. However, the NAADP concentrations used in that study (EC₅₀ = 730 µM) were several orders of magnitude above the physiological NAADP concentrations reported in different cell systems, which are in the low-nanomolar range [30,32,33]. Although an increase in cellular NAADP levels upon stimulation was revealed recently [30,32], a direct activation of TRPM2 by NAADP seems unlikely. However, since these experiments were performed in the whole cell patch–clamp mode, it cannot be excluded that cytosolic components necessary for the interaction between NAADP and TRPM2 have been lost during the experiment, resulting in the need for much higher NAADP concentrations, as compared with the physiological situation.

The physiological function of TRPM2 has just begun to emerge. Early results demonstrating the activation of TRPM2 resulting in a massive Ca²⁺ overload induced by oxidative stress, e.g. H₂O₂, supported a model connecting oxidative stress, Ca²⁺ influx via TRPM2 and apoptosis [45]. In the last few years, these results were confirmed in a more physiological context, e.g. upon stimulation of insulinoma cells by TNF-α (tumour necrosis factor α), granulocytes, microglia cells or cardiomyocytes by H₂O₂, primary striatal cells by amyloid β-peptide, or T-cells by concanavalin A.

Taken together, adenine-based, Ca²⁺-regulating second messengers are rapidly stepping into the focus of cellular signalling research. These novel messengers significantly enlarge the Ca²⁺ signalling toolkit previously known. Their close metabolic relationships indicate that conversion between these nucleotides may be used as a powerful tool for the fine-tuning of cell function.

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