Extracellular NAD\(^{+}\) regulates intracellular free calcium concentration in human monocytes

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Ca\(^{2+}\) ions play a critical role in the biochemical cascade of signal transduction pathways, leading to the activation of immune cells. In the present study, we show that the exposure of freshly isolated human monocytes to NAD\(^{+}\) results in a rapid concentration-dependent elevation of [Ca\(^{2+}\)]\(_{i}\), (intracellular free Ca\(^{2+}\) concentration) caused by the influx of extracellular Ca\(^{2+}\). NAD\(^{+}\) derivatives containing a modified adenine or nicotinamide ring failed to trigger a Ca\(^{2+}\) increase. Treating monocytes with ADPR (ADP-ribose), a major degradation product of NAD\(^{+}\), also resulted in a rise in [Ca\(^{2+}\)]\(_{i}\). Selective inhibition of CD38, an NAD-glycohydrolase that generates free ADPR from NAD\(^{+}\), does not abolish the effect of NAD\(^{+}\), excluding the possibility that NAD\(^{+}\) might act via ADPR. The NAD\(^{+}\)-induced Ca\(^{2+}\) response was prevented by the prior addition of ADPR and vice versa, indicating that both compounds share some mechanisms mediating the rise in [Ca\(^{2+}\)]\(_{i}\). NAD\(^{+}\), as well as ADPR, were ineffective when applied following ATP, suggesting that ATP controls events that intersect with NAD\(^{+}\) and ADPR signalling.

Key words: ADP-ribose, ATP, calcium, CD38, monocyte, NAD\(^{+}\).

INTRODUCTION

NAD\(^{+}\) and its metabolites are mainly known as important regulators of numerous intracellular processes. Only lately, has NAD\(^{+}\) gained much attention as an extracellular molecule that is involved in cellular signalling [1–4]. The ability to carry out such dual functions is not restricted to NAD\(^{+}\). It is shared by ATP, which has been established as an important molecule in signalling pathways [5–10], besides its well-known function in energy transduction. The relatively recent identification of extracellular membrane-bound NAD\(^{+}\)-metabolizing enzymes, including CD38 [11], a 45 kDa type II integral transmembrane glycoprotein, and ARTs (mono-ADP-ribosyltransferases) [12] led to the suggestion that NAD\(^{+}\) as a substrate of these enzymes has regulatory activities.

ARTs catalyse the transfer of the ADP-ribose moiety from NAD\(^{+}\) to specific amino acids; e.g. arginine or cysteine of target proteins [13,14], thereby usually modifying their function. In T-cells, ADP-ribosylation of cell-surface molecules has been reported to inhibit association of receptors into a signal-transmitting cluster [15] and to induce apoptosis [2,3]. NAD\(^{+}\) is also used for the synthesis of cADPR (cyclic ADP-ribose) generated by an ADP-ribosyl cyclase and degraded by a cADPR hydrolase [16]. In mammals, both activities are expressed at the outer surface of the cell by CD38. CD38 also functions as an NAD\(^{+}\)-glycohydrolase (NADase), which cleaves, similarly to ARTs, NAD\(^{+}\) at the adenosine diphosphoribosyl–nicotinamide linkage and thus generates free ADPR (ADP-ribose), which has been shown to attach covalently to proteins [17]. cADPR is a potent Ca\(^{2+}\)-mobilizing agent [18] that activates a family of Ca\(^{2+}\)-release channels known as ryanodine receptors that are expressed in eukaryotic cell microsomal membranes [19]. The observed increase in [Ca\(^{2+}\)]\(_{i}\), (intracellular free Ca\(^{2+}\) concentration) in response to extracellular NAD\(^{+}\) has been ascribed to a CD38-dependent release of Ca\(^{2+}\) from intracellular stores. This mode of Ca\(^{2+}\) entry clearly differs from the capacitative Ca\(^{2+}\) entry, which, triggered by depletion of Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores, is a major component of agonist-induced Ca\(^{2+}\) signalling in non-excitable cells [27].

The mechanism whereby NAD\(^{+}\) and ADPR induce a Ca\(^{2+}\) response is unknown; however, according to our data, it may interfere with ATP signalling. Our findings suggest that NAD\(^{+}\) and ADPR induce the activation of a Ca\(^{2+}\) channel through a pathway that involves Ca\(^{2+}\) influx, but not depletion of Ca\(^{2+}\) stores. This mode of Ca\(^{2+}\) entry clearly differs from the capacitative Ca\(^{2+}\) entry, which, triggered by depletion of Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores, is a major component of agonist-induced Ca\(^{2+}\) signalling in non-excitable cells [27].

EXPERIMENTAL

Reagents

LPS (lipopolysaccharide; from Escherichia coli 055:B5), ADPR, ATP, 1,1′-etheno-NAD\(^{+}\), α-NAD\(^{+}\) and nicotinamide were purchased from Sigma–Aldrich (Taufkirchen, Germany). AMP, ADP and MnCl\(_2\) were from AppliChem GmbH (Darmstadt, Germany).

Abbreviations used: ADPR, ADP-ribose; cADPR, cyclic ADP-ribose; β-araF-NAD, 2′-deoxy-2′-fluoroorabinoside NAD; ART, mono-ADP-ribosyltransferase; [Ca\(^{2+}\)]\(_{i}\), intracellular free Ca\(^{2+}\) concentration; fMLP, N-formylmethionyl-leucylphenylalanine; fura 2/AM, fura 2 acetoxymethyl ester; NADase, NAD\(^{+}\)-glycohydrolase; β-riboNAD, 2′-deoxy-2′-F-ribose-NAD; TRP, transient receptor potential.

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NAD\(^+\) and NADP\(^+\) were from Roche Diagnostics GmbH (Mannheim, Germany), and β-araF-NAD (2'-deoxy-2'-fluoro-arabinoside NAD) and β-riboF-NAD (2'-deoxy-2'-fluoro-ribose-NAD) have been described previously [28,29].

**Cell separation and cell culture**

Human PBMCs (peripheral blood mononuclear cells) from healthy donors were obtained by centrifugation at 700 g for 40 min at 20 °C over a Ficoll-Isopaque (Amersham Biosciences, Freiburg, Germany) density gradient. After repeated washing (500 g for 10 min at 4 °C) in PBS containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-6B elutriation system (Beckman Instruments, Palo Alto, CA, U.S.A.), as described previously [30]. The purity of the monocyte preparation was >90 %, as assessed by morphological screening and immunofluorescence staining with a monoclonal antibody against CD14 (BL-M/G14; DiaMak, Leipzig, Germany) and 1 % penicillin/streptomycin (Sermo

**Measurement of \([\text{Ca}^{2+}]\), by Ca\(^{2+}\) imaging**

A sample (300 µl) of cell suspension (2 × 10⁶/ml) was seeded on to 30-mm-diameter sterile glass coverslips (Marienfeld Laboratory Glassware, Bad Mergentheim, Germany) incubated for 30 min at 37 °C and in 5 % CO\(_2\). Monocytes that adhered to coverslips were incubated with 10 µM fura 2/AM (fura 2 acetoxymethyl ester) (TEF Labs, Austin, TX, U.S.A.) and 0.0125 % Pluronic® F-127 (TEF Labs) in 1 ml of standard Ca\(^2+\)-free solution [125 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 10 mM Hepes and 7.5 mM glucose (adjusted to pH 7.4 with NaOH)] at room temperature (21 °C) for 30 min in the dark.

The coverslips were placed in a recording chamber and were continuously perfused at room temperature at a rate of 2 ml/min. Solutions were removed by a vacuum pump. Experiments were performed on a Zeiss Axiovert 135 microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with Axiovert 135 UV transparent optics (Carl Zeiss Jena GmbH). Dye-excitation illumination was provided by a dual-wavelength illuminator system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) consisting of a xenon arc lamp, a variable-speed reflective optic chopper and two monochromators both under computer control. The excitation wavelengths used were 340 and 380 nm. Emitted fluorescence filtered at 510 nm was collected by a photomultiplier tube and wavelengths used were 340 and 380 nm.

**Mn\(^{2+}\)-uptake assay**

Cells were loaded with fura 2/AM in Ca\(^{2+}\)-free solution [125 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 6 mM Hepes, 1 mM EGTA, 10 mM Hepes and 7.5 mM glucose (adjusted to pH 7.4 with NaOH)], in the presence and absence of thapsigargin (0.3 µM). Before the addition of NAD\(^+\) (200 µM) or NADP\(^+\) and thapsigargin, the cells were incubated with 0.6 mM MnCl\(_2\) for 20 s. Fluorescence was monitored as described above at a wavelength of 360 nm. Data were normalized using 360 nm values obtained immediately before the addition of NAD\(^+\).

**Determination of NADase activity**

The assay was carried out according to Stoeckler et al. [31] with minor modifications. Monocytes (4 × 10⁶/ml) were pre-incubated in the absence and presence of β-araF-NAD (500 nM) in PBS supplemented with 20 mM Hepes (pH 7.2) for 30 min before the addition of 0.6 mM NAD\(^+\). Controls were run in the absence of cells. The samples were incubated under constant shaking at 37 °C. After 60 min, the reaction mixture was stored on ice for 5 min and then centrifuged at 3000 g for 2 min at 4 °C. The supernatant (0.2 ml) was mixed with 0.6 ml of 1.33 M KCN and incubated for 5 min at room temperature. The cyanide complex of NAD\(^+\) was measured spectrophotometrically at 325 nm (Ultrascope 2000; Amersham Biosciences, Uppsala, Sweden). The mean difference in absorbance between reactions stopped at 0 and 60 min was used as an arbitrary unit of NADase activity.

**Analysis of P2X\(_7\)-dependent pore formation by flow cytometry**

A sample (300 µl) of human monocytes (2 × 10⁶/ml) was resuspended in medium containing 25 mM Hepes, 130 mM potassium glutamate, 5 mM KCl, 0.5 mM CaCl\(_2\), 10 mM glucose, 0.5 % BSA (adjusted to pH 7.4 with KOH) and 2.5 mM ethidium bromide. The cells were incubated with ATP (2 mM), NAD\(^+\) (2 mM) or ADPR (2 mM) for 15 min at 37 °C, washed twice and measured by FACS analysis (Becton Dickinson, Heidelberg, Germany) using 488 nm excitation and 585 nm emission filters.

**RESULTS**

**NAD\(^+\) induces a rise in \([\text{Ca}^{2+}]\)**

Incubating fura 2-loaded human monocytes with 200 µM NAD\(^+\) results in a rapid increase in \([\text{Ca}^{2+}]\), reaching peak values within 10–20 s and declining thereafter to baseline levels (Figure 1A). The increase was concentration-dependent in the range 25–400 µM, and reached a maximum at 400 µM (Figure 1B). All further experiments were performed at 200 µM NAD\(^+\).

To determine if extracellular Ca\(^{2+}\) was required for the observed intracellular Ca\(^{2+}\) increase, NAD\(^+\) was added to monocytes in Ca\(^{2+}\)-free medium (Ca\(^{2+}\)-free solution plus 0.5 mM EGTA). As seen in Figure 2(A), in the absence of extracellular Ca\(^{2+}\), NAD\(^+\)

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**Figure 1 NAD\(^+\) induces a rise in cytosolic Ca\(^{2+}\) in human monocytes**

(A) Representative trace of an increase in intracellular Ca\(^{2+}\) following application of NAD\(^+\) (200 µM, open bar) (n = 31). Shown is the 340 nm/380 nm emission ratio from one out of 20 experiments. (B) Dose-dependent effect of NAD\(^+\) on \([\text{Ca}^{2+}]\), measured as the change in the 340 nm/380 nm emission ratio. Results are means ± S.E.M. (n = 15–39 cells) of one out of three experiments.

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failed to induce an increase in \([\text{Ca}^{2+}]\). To test the involvement of \(\text{Ca}^{2+}\)-channels in the \(\text{NAD}^+\)-induced elevation of \([\text{Ca}^{2+}]\), the cells were pre-treated with the \(\text{Ca}^{2+}\)-channel-blockers \(\text{LaCl}_3\) or \(\text{BaCl}_2\), before \(\text{NAD}^+\) was added. Both inhibitors prevented \(\text{Ca}^{2+}\) mobilization (Figure 2B), indicating that the elevation of \([\text{Ca}^{2+}]\), following addition of \(\text{NAD}^+\) requires extracellular \(\text{Ca}^{2+}\), the influx being mediated by \(\text{Ca}^{2+}\) channels.

To verify that the \(\text{NAD}^+\)-induced increase in \([\text{Ca}^{2+}]\), depends on \(\text{Ca}^{2+}\) influx, extracellular \(\text{Ca}^{2+}\) was replaced by \(\text{Mn}^{2+}\). \(\text{Mn}^{2+}\), which can serve as a \(\text{Ca}^{2+}\) surrogate [32,33], has the advantage that it cannot be modulated by a number of processes such as buffering, extrusion and sequestering that may interfere with the interpretation of studies on \(\text{Ca}^{2+}\) uptake. Activation of a \(\text{Ca}^{2+}\)-channel should allow \(\text{MnCl}_2\) to permeate cells and quench fura 2. \(\text{Mn}^{2+}\) has a higher affinity to fura 2 than \(\text{Ca}^{2+}\) does [34]. In the presence of 0.6 mM \(\text{MnCl}_2\), we observed a decrease in fluorescence after application of 200 \(\mu\text{M} \text{NAD}^+\) caused by the influx of \(\text{MnCl}_2\) and quenching of fura 2 fluorescence (Figure 3A).

To test whether \(\text{Ca}^{2+}\) released from endoplasmic reticulum stores may contribute to the entry of cations, cells were treated with thapsigargin to deplete intracellular stores [35] before \(\text{NAD}^+\) was applied. As seen in Figure 3(B), thapsigargin-treated monocytes react in the same way as untreated cells. A \(\text{MnCl}_2\)-induced quenching of the fluorescence could be observed in the presence and absence of thapsigargin, indicating that the \([\text{Ca}^{2+}]\) increase in response to \(\text{NAD}^+\) is due to \(\text{Ca}^{2+}\) influx and is independent of internal \(\text{Ca}^{2+}\) mobilization.

**ADPR induces a rise in \([\text{Ca}^{2+}]\)**

We described above that extracellularly added \(\text{NAD}^+\) is rapidly degraded by intact monocytes to nicotinamide and ADPR and to minor amounts of AMP, ADP and cADPR [36]. To test whether the main \(\text{NAD}^+\) metabolites had similar effects as \(\text{NAD}^+\), monocytes were incubated in the presence of ADPR or nicotinamide and the \([\text{Ca}^{2+}]\), was determined. Whereas monocytes did not respond to nicotinamide (Figure 4A), the addition of ADPR elicited a rise in \([\text{Ca}^{2+}]\), which resembled that induced by \(\text{NAD}^+\) (Figure 4C). Similarly to \(\text{NAD}^+\), ADPR had no effect in the absence of extracellular \(\text{Ca}^{2+}\) (results not shown).

Other \(\text{NAD}^+\)-degradation products such as ADP, AMP or adenosine can be excluded as putative inducers of \([\text{Ca}^{2+}]\), because the \(\text{NAD}^+\)-induced rise is too short (seconds) for these metabolites to be formed in substantial amounts. As shown in previous experiments, albeit carried out at lower \(\text{NAD}^+\) concentrations, even after 60 min of incubation, minor amounts of AMP and ADP are formed, and adenosine was not detectable at all [36]. Furthermore, AMP had hardly any effect on the \([\text{Ca}^{2+}]\), and, although ADP induced a rapid rise in \([\text{Ca}^{2+}]\), it only declined gradually over the next 2 min to baseline levels and thus differs from the \(\text{NAD}^+\)-induced increase. An increase in \([\text{Ca}^{2+}]\), was also observed when \(\text{NADP}^+\) was used instead of \(\text{NAD}^+\) (Figure 4D). The effect of the reduced forms of the nucleotides could not be measured because the high fluorescence of the compounds disturbed the interpretation of the fura 2 fluorescence data.

**\(\text{NAD}^+\) does not act via its degradation product ADPR**

Because ADPR was equally effective as \(\text{NAD}^+\), we raised the question of whether \(\text{NAD}^+\) itself and/or its degradation product ADPR was responsible for the observed \(\text{Ca}^{2+}\) mobilization. Monocytes were pre-incubated with the \(\text{NAD}^+\) analogue, \(\beta\)-araF-\(\text{NAD}\) for 30 min before \(\text{NAD}^+\) was administered.
β-araF-NAD is one of a series of potent, slow-binding and selective inhibitors of CD38 [37] and has a $K_i$ for human CD38 of 1.7 nM [38]. NAD$^+$-glycohydrolases, like CD38, generate free ADPR by cleaving NAD$^+$ at the adenosine diphosphoribosyl-nicotinamide linkage. The data presented in Figure 5(A) demonstrate that β-araF-NAD inhibited NADase activity completely, but had no effect on the NAD$^+$-induced increase in [Ca$^{2+}$]i. (Figure 5B). These results establish that degradation of NAD$^+$ to ADPR is very likely to be mediated by CD38, and that this reaction is not a prerequisite for NAD$^+$ to induce changes in [Ca$^{2+}$]i. Adding β-riboF-NAD (200 µM), which is resistant to hydrolysis by CD38 [29], to monocytes also results in an increase in [Ca$^{2+}$]i, (Figure 5C). The response is slightly delayed compared with that induced by NAD$^+$.

In further experiments, we studied the ability of NAD$^+$ derivatives to mobilize intracellular Ca$^{2+}$. Instead of NAD$^+$, we treated the cells with 1,6-etheno-NAD$^+$ or α-NAD$^+$ that contain a modified adenine or nicotinamide ring respectively. Both derivatives failed to trigger a Ca$^{2+}$ release (results not shown), suggesting that, for NAD$^+$ to induce a Ca$^{2+}$ response, its structural properties seem to play an important role.

**NAD$^+$ prevents the ADPR-induced rise in [Ca$^{2+}$]i and vice versa**

Having shown that NAD$^+$ and ADPR both induce an influx in Ca$^{2+}$, we tested whether the compounds interfered with each other. We therefore treated monocytes with ADPR before the addition of NAD$^+$. Figure 6(A) shows that application of NAD$^+$ prevented monocytes from responding to the subsequent application of ADPR. This desensitization was also observed when the stimuli were applied in reversed order (Figure 6B). In contrast, when cells were treated with NAD$^+$ or ADPR first and then re-stimulated with fMLP (N-formylmethionyl-leucylphenylalanine), they responded with a large increase in [Ca$^{2+}$]i, (Figures 6C and 6D). These data indicate that NAD$^+$ and ADPR may share common components or pathways to induce a Ca$^{2+}$ response, while fMLP exerts its effects via different mechanisms.

**ATP prevents the NAD$^+$ - and ADPR-induced rise in [Ca$^{2+}$]i and, in contrast with NAD$^+$ and ADPR, triggers pore formation**

We next tested in how far extracellular ATP has any influence on NAD$^+$- and ADPR-mediated Ca$^{2+}$-influx, and whether it shares functional events with ADPR and NAD$^+$. As seen in Figure 7, when monocytes were treated with ATP before the addition of NAD$^+$ (Figure 7A) or ADPR (Figure 7B), ATP rendered the cells insensitive to both compounds. The inhibitory action of ATP might be consistent with several mechanisms. ATP may occupy binding sites also used by NAD$^+$ and ADPR. It may interfere with NAD$^+$-/ADPR-mediated downstream effects.
Figure 6  NAD\(^+\) (followed by ADPR) treatment prevents stimulation with ADPR (NAD\(^+\)), but not with fMLP

Monocytes were treated with NAD\(^+\) (200 \(\mu\)M, open bar) (A and C) or ADPR (200 \(\mu\)M, grey bar) (B and D) before the addition of ADPR (200 \(\mu\)M, grey bar) (A) or NAD\(^+\) (200 \(\mu\)M, open bar) (B) or fMLP (1 \(\mu\)M, black bar) (C and D). Intracellular Ca\(^{2+}\) levels were measured as the 340 nm/380 nm emission ratio. Representative traces (\(n = 40–45\) cells) from one out of three experiments are shown.

or modulate signalling events that may prevent ADPR and NAD\(^+\) from causing an influx in Ca\(^{2+}\).

On the other hand, if NAD\(^+\) (Figure 7C) and ADPR (Figure 7D) were applied before ATP, the cells were fully responsive. This would imply that putative binding sites occupied by NAD\(^+\) or ADPR only represent a minor part of receptors that have been described to mediate ATP-induced Ca\(^{2+}\) signalling [39]. Among the ATP receptors the P2X\(_7\) receptor has been examined extensively [40].

Upon brief exposure to ATP, it triggers depolarization, Ca\(^{2+}\) influx and rapid equilibration of Na\(^+\) and K\(^+\) gradients, whereas sustained activation of P2X\(_7\) results in the formation of a non-selective pore that permits the passage of solutes of molecular mass as large as 800 Da [41]. The molecular mechanism underlying pore formation remains to be established. It is not clear whether P2X\(_7\) receptors themselves form pores or induce pore formation by binding to other proteins. In contrast with human macrophages, human monocytes, which are P2X\(_7\)-receptor-positive [42], are resistant to pore formation by ATP when stimulated in standard NaCl-based salines [43,44]. However, when changing the ionic composition of the extracellular medium to a high K\(^+\) and low Na\(^+\) solution wherein organic anions substitute for Cl\(^-\) [45], the cells become responsive to ATP, confirming previous studies that showed that the activation of the P2X\(_7\) receptor by ATP is extremely sensitive to the surrounding ionic composition [41,46,47].

To test whether NAD\(^+\) and ADPR share the ability of ATP to induce non-selective pore formation, monocytes were suspended in potassium glutamate basic salt solution in the presence of ethidium bromide before they were incubated in the presence and absence of 2 mM ATP, NAD\(^+\) or ADPR at 37 °C for 15 min.
Monocytes were suspended in potassium glutamate basic salt solution in the presence of 2.5 \( \mu \)M ethidium bromide. The cells were incubated with 2 mM ATP (A), 2 mM NAD\(^{+}\) (B) or 2 mM ADPR (C) for 15 min at 37 °C, and washed twice with PBS. Ethidium bromide uptake was measured by FACS analysis. Traces from one out of two independent experiments are shown.

Under these experimental conditions, which have been described as optimal for pore formation [45], ATP triggers the uptake of ethidium bromide, whereas neither NAD\(^{+}\) nor ADPR had any effect on the uptake during the test incubation (Figure 8). Thus NAD\(^{+}\) and ADPR, unlike ATP, lack the ability to induce pore formation in human monocytes.

### DISCUSSION

The potential of extracellular NAD\(^{+}\) to participate in the regulation of immune cells has gained much interest in recent years [2–4,15]. In the present paper, we show that extracellular NAD\(^{+}\) induces a rapid (seconds) transient rise in cytosolic Ca\(^{2+}\) levels in human monocytes. A similar immediate response to NAD\(^{+}\) has been detected in various cells, including astrocytes [48], MC3T3-E1 [23], CD38-transfected HeLa [20] and NIH3T3 cells [23]. In those cells, cADPR generated from CD38 catalysis has been implicated to play a critical role in mediating the Ca\(^{2+}\) increase via the release of Ca\(^{2+}\) from internal stores. Our data indicate that the action of NAD\(^{+}\) in monocytes appears to be mediated via influx of extracellular Ca\(^{2+}\), since removal of extracellular Ca\(^{2+}\) from the external medium completely blocked the response.

The ability of NAD\(^{+}\) to induce an increase in [Ca\(^{2+}\)]\(_i\), which is shared by ADPR, is not due to the CD38-catalysed formation of ADPR, as established by the use of the CD38 inhibitor \( \beta \)-araF-NAD.

Based on these results, the central question is how NAD\(^{+}\) triggers the rapid rise in [Ca\(^{2+}\)]\(_i\), if the CD38/ADPR system is not responsible for it. There is some evidence that cell membranes are permeable to pyridine nucleotides [23], including NAD\(^{+}\), NADH, NADP\(^+\) and NADPH [22]. According to Zocchi et al. [22], the NAD\(^{+}\) flux depends on a concentration gradient and does not require any energy stores. The transport system is characterized by low dinucleotide specificity, and transportation is supposed to occur across a channel rather than involving a transporter. Under physiological conditions, the extracellular NAD\(^{+}\) concentration is too low to allow the entry of NAD\(^{+}\) into cells; however, high enough concentrations may be reached under specific conditions in which the intracellular content of cells is released, such as cell death or by the regulated release from specific cells such as fibroblasts and epithelial cells [21]. How far this may also apply to ADPR is not yet known. Intracellular ADPR and NAD\(^{+}\) may directly enable Ca\(^{2+}\) influx as suggested by Sano et al. [49] who observed that both compounds activate a member of the TRP (transient receptor potential) channel family (LTRPC2), which functions as a Ca\(^{2+}\)-permeant non-selective cation channel in immunocytes. Only recently, Heiner et al. [50] provided evidence for a prominent role of the LTRPC2 as a Na\(^{+}\) and Ca\(^{2+}\) entry pathway in neutrophil granulocytes regulated by intracellular ADPR and NAD\(^{+}\), and Campo et al. [51] reported on a non-selective ionic current, which is activated by sustained depolarization, intracellular ADPR and NAD\(^{+}\) in rat peritoneal macrophages.

For LTRPC2 to operate as described [49–51], one would have to postulate the uptake of NAD\(^{+}\) and ADPR across the cell membrane, a process which is difficult to envisage. Furthermore the fact that extracellular NAD\(^{+}\) induces a rise in [Ca\(^{2+}\)]\(_i\), whereas it fails to activate ion currents when applied into the cell [51], suggests that, if LTRPC2 were engaged, they might have different properties.

How far NAD\(^{+}\) and ADPR act via similar mechanisms is not known. However, they do share some properties which may point to some similar mode of action. Both compounds induce an extracellular Ca\(^{2+}\)-dependent rise in [Ca\(^{2+}\)]\(_i\), and NAD\(^{+}\) when applied to the cells before ADPR renders the cells insensitive to the second stimulus and vice versa. However, a challenge with fMLP following the application of NAD\(^{+}\) and ADPR always results in a Ca\(^{2+}\) response. It is conceivable that NAD\(^{+}\) and ADPR use similar binding components and/or share signal transduction pathways that are distinct from those described for the chemotactic peptide fMLP. fMLP mediates its effect by binding to a G-protein-coupled receptor. Signalling through the receptor results in mobilization of Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores, activation of plasma membrane influx pathways and stimulation of diacylglycerol-dependent protein kinase C enzymes [52].

Desensitization was also observed when ATP was applied before NAD\(^{+}\) or ADPR. Speculations might include that purinergic receptors are involved in NAD\(^{+}\) and ADPR-induced Ca\(^{2+}\) signalling or that ATP controls a signalling event that intersects with NAD\(^{+}\) and ADPR signalling. Rapid desensitization of ATP receptors, compared with other types of receptors, has been reported previously [53]. ATP can activate cells via P2 receptors of which there are two classes [54]. P2Y receptors are coupled to a G-protein which activates phospholipase C, leading to the production of Ins(1,4,5)P\(_3\) and Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release [55]. Among the P2X receptors, which are ligand-gated ion channels, the P2X receptor is of special interest, since, in the presence of ATP, it converts into a pore that allows passage of molecules as large as 800 Da [56]. In the present paper, we show that in monocytes, which express various purinergic receptors [57,58], including P2X receptors, ATP triggers pore formation, whereas NAD\(^{+}\) and ADPR are unable to activate non-selective pores. This implies that ATP-induced pore formation involves mechanisms that are distinct from the ones that interfere with NAD\(^{+}/\)ADPR-mediated Ca\(^{2+}\) signalling.

When considering the mechanism whereby NAD\(^{+}\) induces a rise in [Ca\(^{2+}\)]\(_i\), it cannot be excluded that, as a substrate for ARTs, NAD\(^{+}\) modifies signalling events by stimulating ADP-ribosylation of proteins. Thus exposure of mouse T-cells to NAD\(^{+}\) has been reported to result in cell death by activating the P2X receptor via ART-2-catalysed ADP-ribosylation [59]. Similarly to NAD\(^{+}\), the highly reactive molecule ADPR has the potential to modify proteins by being non-enzymically transferred to specific amino acids of a target protein [60]. The fact that extracellular NAD\(^{+}\) induces Ca\(^{2+}\) mobilization in human monocytes raises the question of how effective concentrations (\( \mu \)M) of extracellular NAD\(^{+}\) can be reached in vivo.

Under physiological conditions, the concentration of NAD\(^{+}\) in extracellular body fluids is in the submicromolar range, whereas cytosolic NAD\(^{+}\) can reach 1 mM [22,61]. An increase could occur...
under specific conditions and in selective tissues. One possibility is that NAD$^+$ is released into the extracellular compartment from cells as a consequence of lysis during tissue injury and inflammatory immune reactions. ADPR could then be formed by degradation of NAD$^+$. As cell damage would also cause the efflux of other nucleotides such as ATP, a well-described immune modulator [62], it is likely that these purine nucleotides and their degradation products at sites of inflammation influence the outcome of an immune response in a concerted manner.

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NAD$^+$-induced Ca$^{2+}$ response in monocytes

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