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ORIGINAL ARTICLE

Involvement of P2X receptors in the NAD^+-induced rise in $[Ca^{2+}]_i$ in human monocytes

Anja Grahnert · Carina Klein · Sunna Hauschildt

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Abstract In the present study, we show that the extracellular addition of nicotinamide adenine dinucleotide (NAD^+) induces a transient rise in $[Ca^{2+}]_i$ in human monocytes caused by an influx of extracellular calcium. The NAD^+-induced Ca^{2+} response was prevented by adenosine triphosphate (ATP), suggesting the involvement of ATP receptors. Of the two subtypes of ATP receptors (P2X and P2Y), the P2X receptors were considered the most likely candidates. By the use of subtype preferential agonists and antagonists, we identified $P2X_1$, $P2X_4$, and $P2X_7$ receptors being engaged in the NAD^+-induced rise in $[Ca^{2+}]_i$. Among the P2X receptor subtypes, the $P2X_7$ receptor is unique in facilitating the induction of nonselective pores that allow entry of ethidium upon stimulation with ATP. In monocytes, opening of $P2X_7$ receptor-dependent pores strongly depends on specific ionic conditions. Measuring pore formation in response to NAD^+, we found that NAD^+ unlike ATP lacks the ability to induce this pore-forming response. Whereas as little as 100 μM ATP was sufficient to activate the nonselective pore, NAD^+ at concentrations up to 2 mM had no effect. Taken together, these data indicate that despite similarities in the action of extracellular NAD^+ and ATP there are nucleotide-specific variations. So far, common and distinct features of the two nucleotides are only beginning to be understood.

A. Grahnert · C. Klein · S. Hauschildt (✉)
Department of Immunobiology, Institute of Biology II,
University of Leipzig,
Talstrasse 33,
04103 Leipzig, Germany
e-mail: shaus@rz.uni-leipzig.de

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Introduction

There is increasing evidence that nicotinamide adenine dinucleotide (NAD^+), in addition to its intracellular role, also functions as an extracellular signaling molecule [1]. NAD^+ can serve as a substrate for mono-ADP-ribosyltransferases (ARTs) that catalyze the transfer of the ADP-ribose moiety from NAD^+ to target proteins [2], or it can be used by CD38 and CD157, also called BST-1, for the synthesis of metabolically active degradation products involved in Ca^{2+} signaling. Both enzymes possess NAD^+ glycohydrolase and adenosine diphosphate (ADP)-ribose cyclase activity, generating ADP-ribose (ADPR) and cyclic ADP-ribose (cADPR) [3]. Furthermore, they catalyze hydrolysis of cADPR to ADPR and formation of nicotinic acid adenine dinucleotide phosphate (NAADP) by a base-exchange reaction [4]. cADPR, NAADP, and ADPR are involved in the regulation of cellular Ca^{2+} homeostasis. While cADPR and NAADP trigger Ca^{2+}-release from intracellular stores [5–7], ADPR controls calcium entry through the plasma membrane channel TRPM2 [8, 9]. It is difficult to envision how cADPR and NAADP can function inside the cell if they are generated by ectoenzymes. Several studies suggest that cADPR and NAADP are directly transported by CD38 or more likely by nucleotide transporters into the cytosol [10–14]. So far, in many cell types, responding to extracellular NAD^+ with an increase in $[Ca^{2+}]_i$, conversion of NAD^+ to the Ca^{2+} mobilizer cADPR [14–18] has been implicated as the principal mechanism leading to the rise in $[Ca^{2+}]_i$.

In previous studies, we demonstrated that the exposure of freshly isolated monocytes to NAD^+ results in a rapid,

concentration-dependent elevation of $[Ca^{2+}]_i$, caused by an influx of extracellular Ca^{2+} independent from internal Ca^{2+} mobilization [19]. By the use of a selective inhibitor of CD38 and a stable NAD^+ analogue, we could exclude that NAD^+ might act via a degradation product.

Given that NAD^+ is a nucleotide and that the NAD^+-induced rise in $[Ca^{2+}]_i$ was prevented when the cells were incubated with adenosine triphosphate (ATP), here we focused on the possibility that the signaling promoted by NAD^+ was the result of interfering with receptors also used by ATP. ATP elicits direct effects on cell function via its ability to activate P2 receptors, which are divided into two categories: the ion channel receptors (P2X) and the G-protein-coupled receptors (P2Y).

P2X receptors are ligand-gated ion channels which are highly permeable to calcium, whereas activation of most P2Y receptors, which belong to the G-protein-coupled receptor family, results in the release of calcium from intracellular stores. The signaling properties of the P2X receptors and the fact that in monocytes the NAD^+-induced $[Ca^{2+}]_i$ increase solely depends on the influx of extracellular calcium suggested that of the two P2 receptor subtypes, the P2X receptors, play a role in NAD^+ signaling. In the present study, we could indeed demonstrate by the use of P2X receptor agonists and antagonists the involvement of P2X receptors in the NAD^+-induced Ca^{2+} signaling in human monocytes.

Experimental procedures

Reagents and antibodies

ATP, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt (BzATP), KN62, 2-MeSATP, suramin, oxidized ATP (oxATP), and ivermectin (IVM) were purchased from Sigma-Aldrich (Taufkirchen, Germany). NF449 was from TOCRIS Bioscience (Bristol, UK). NAD^+ was from Roche Diagnostics GmbH (Mannheim, Germany).

The polyclonal goat anti-human $P2X_1$ receptor Ab (A-15, sc-31489), the goat IgG isotype (sc-2028), and the FITC-labeled donkey-anti-goat antibody (sc-2024) were from Santa Cruz Biotechnology (Heidelberg, Germany); the rabbit-anti-human $P2X_4$ receptor was from Alomone Labs (Jerusalem, Israel); the FITC-labeled goat anti-mouse antibody was from DiaMak (Leipzig, Germany); the IgG2b isotype, the anti-β-actin antibody (clone AC-74), and the POD-conjugated goat anti-mouse antibody were from Sigma-Aldrich (Taufkirchen, Germany); and the monoclonal mouse anti-human $P2X_7$ receptor Ab was kindly provided by GlaxoSmithKline (Harlow, UK).

Cell separation and cell culture

Human peripheral blood mononuclear cells (PBMCs) 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 phosphate-buffered saline (PBS) containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-6B elutriation system (Beckman Instruments, Palo Alto, CA, USA), as described previously [20]. The purity of the cell preparation was >90% as assessed by morphological screening and immunofluorescence staining with a monoclonal antibody against CD14 (BL-M/G14, DiaMak, Leipzig, Germany).

Monocytes (2×10^6/ml) were suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (*v*/*v*) fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Seromed® Biochrom KG).

Measurement of $[Ca^{2+}]_i$ by Ca^{2+} imaging

Measurements were performed essentially as described previously [19]. Freshly isolated monocytes (6×10^5/300 μl) were seeded onto 30-mm diameter sterile glass coverslips (Marienfeld Laboratory Glassware, Bad Mergentheim, Germany) and incubated for 30 min at room temperature. Monocytes that adhered to coverslips were incubated with 5 μM FURA-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy) ethane-*N,N,N′,N′*-tetraacetic acid pentaacetoxymethyl ester; TEFLABS, Austin, TX, USA) and 0.0125% Pluronic® F-127 (TEFLABS) in 1 ml standard Ca^{2+} solution (Ringer solution) or Ca^{2+}-free solution at room temperature for 25 min in the dark. Ringer solution contained NaCl (125 mM), KCl (5 mM), $CaCl_2$ (2 mM), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES; 10 mM), and glucose (7.5 mM; adjusted to pH 7.4 with NaOH), and the Ca^{2+}-free solution contained NaCl (125 mM), KCl (5 mM), $MgCl_2{\cdot}6H_2O$ (2 mM), EGTA (1 mM), HEPES (10 mM), and glucose (7.5 mM; adjusted to pH 7.4 with NaOH). Coverslips were placed in a recording chamber and perfused at room temperature at a rate of 1 ml/min. Solutions were removed by a vacuum pump.

Experiments were performed on a ZEISS microscope (Axiovert 135, Carl Zeiss Jena GmbH, Jena, Germany) equipped with UV transparent optics (Neofluar, Carl Zeiss Jena GmbH, Jena, Germany). 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 a monochromator (Polychrome V, T.I.L.L. Photonics GmbH, Gräfelfing, Germany) both under computer control.

The excitation and emission wavelengths used were 340 and 380 nm, respectively. Emitted fluorescence filtered at 510 nm was collected by a photomultiplier tube and a charge-coupled device camera.

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were expressed as the ratio (*R*) of dye fluorescence at 340 and 380 nm. Fluorescence intensities for both excitation wavelengths were acquired in intervals of 2 s. Ca^{2+} measurements were performed on fields containing 50–200 cells. Δ ratio is the difference between the basal 340/380 ratio and the maximum peak 340/380 ratio.

Analysis of pore formation by flow cytometry

A sample (300 μl) of human monocytes (2×10^6/ml) was resuspended in potassium glutamate basic salt solution containing HEPES (25 mM), potassium glutamate (130 mM), KCl (5 mM), $CaCl_2$ (0.5 mM), glucose (10 mM), BSA (0.5%; adjusted to pH 7.4 with KOH), and 2.5 μM ethidium bromide. The monocytes were incubated with ATP or NAD^+ at concentrations from 0.1 to 3 mM for 15 min at 37°C. After incubation, the cells were washed twice with PBS and measured by florescence-activated cell sorting (FACS) analysis (Becton Dickinson, San Jose, CA, USA).

Immunofluorescence analysis of the $P2X_1$ and the $P2X_7$ receptor expression

Freshly isolated monocytes (2×10^6/ml) were incubated at 4°C with 1% goat serum ($P2X_1$) or 10% human AB serum ($P2X_7$) to saturate Fc receptors and block unspecific bindings. After 20 min, the goat anti-human $P2X_1$ Ab (1:50 dilution) and the respective goat IgG isotype or the mouse anti-human $P2X_7$ Ab (5 μg/ml) and the respective IgG2b isotype were added and incubated for 45 min at 4°C. Cells were then washed in PBS containing 10% Haemaccel® (Hoechst, Frankfurt, Germany) and 0.1% sodium azide and further incubated for 30 min at 4°C with FITC-labeled donkey anti-goat antibody ($P2X_1$) or FITC-labeled goat anti-mouse antibody ($P2X_7$). After washing and fixation in 1% formaldehyde, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Western Blot analysis of the $P2X_4$ receptor

Western blot analysis was carried out as described previously [21]. Cells (1.0×10^7/ml) were suspended in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% (*v*/*v*) NP-40, 0.5% (*w*/*v*) deoxycholate, 0.1% sodium dodecyl sulfate (SDS; *w*/*v*) and cOmplete protease inhibitor cocktail (Roche, Mannheim, Germany)) and sonicated. Samples (30 μg) were run on a 10% SDS-polyacrylamide gel (MiniProtean II, BioRad GmbH) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Munich, Germany). Membranes were probed with an anti-$P2X_4$ receptor antibody (4 μg/ml) or anti-β-actin antibody (1:2,000 dilution) and detected with a POD-conjugated goat anti-rabbit (1:60,000 dilution) or goat anti-mouse (1:20,000 dilution) secondary antibody, respectively, using the Western blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) detection system. The control peptide antigen was used in a concentration of 0.5 μg/ml.

Results

In previous studies, we have shown that exposure of freshly isolated human monocytes to NAD^+ results in a transient elevation of $[Ca^{2+}]_i$ and that treating the cells with ATP before the addition of NAD^+ renders the cells insensitive to NAD^+ [19]. However, if NAD^+ was applied before ATP, the cells were fully responsive. To determine how far ATP receptors participate in the NAD^+-induced increase in $[Ca^{2+}]_i$, we pretreated the cells with PPADS, a selective P2 receptor antagonist [22], before adding NAD^+. Figure 1a shows that PPADS leads to a significant decrease in the NAD^+-mediated increase in $[Ca^{2+}]_i$.

To address the type of P2 receptors that may interact with NAD^+, we started to focus on P2X receptors. P2X receptors are likely candidates since activation of the receptors results in an influx of extracellular calcium [23] and the NAD^+-induced rise in $[Ca^{2+}]_i$ solely depends on Ca^{2+} influx [19]. As seen in Fig. 1b, when incubating monocytes in Ca^{2+}-free solution prior to the addition of NAD^+, NAD^+ fails to induce an increase in $[Ca^{2+}]_i$. The cells are still responsive to ATP when added subsequent to NAD^+, indicating that the lack to respond is not due to depletion of intracellular Ca^{2+} stores. In contrast to NAD^+, ATP induced a Ca^{2+} response which consisted of a peak phase followed by a plateau phase. Under Ca^{2+}-free conditions, the peak phase hardly changed, whereas the plateau phase was diminished. Since ATP is a well-known agonist at both P2X and P2Y receptors, the initial phase is likely due to the release of Ca^{2+} from intracellular stores following P2Y receptor activation. These data clearly show that P2Y receptors are involved in the ATP but not in the NAD^+-induced Ca^{2+} response.

Of the seven P2X receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$) currently known, human monocytes express $P2X_1$, $P2X_4$, and $P2X_7$ receptors as revealed at the mRNA level by reverse transcription PCR analysis [24]. Measuring surface expression of the receptor proteins by FACS analysis revealed expression of both $P2X_1$

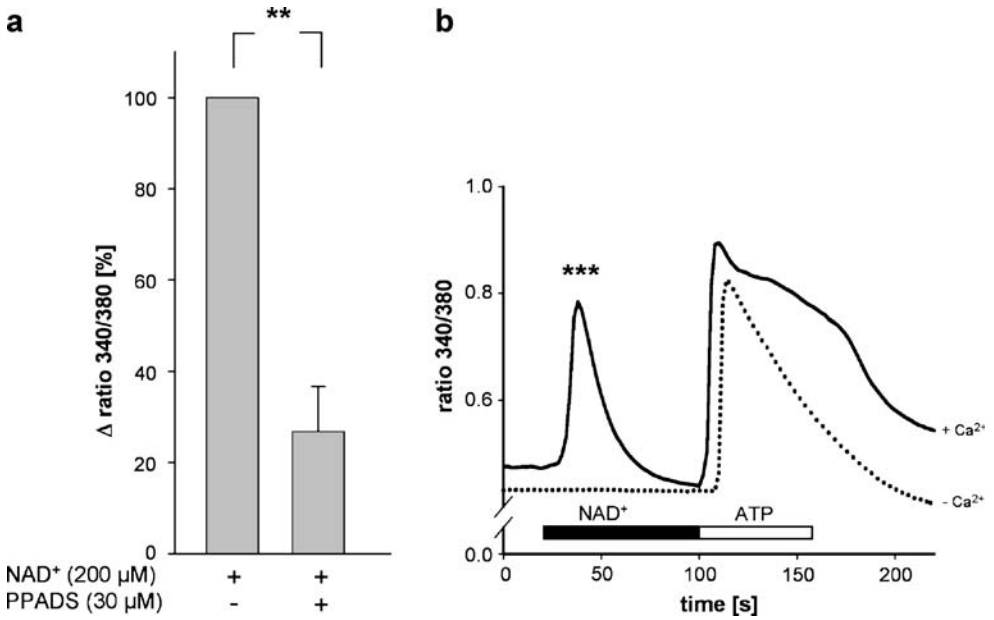


Fig. 1 ATP receptors are involved in the NAD^+-induced increase in $[Ca^{2+}]_i$. Monocytes were preincubated with PPADS (30 μM) for 25 min and continuously perfused with PPADS during the addition of NAD^+ (200 μM, 80 s; **a**). Intracellular Ca^{2+} levels were measured as the change in the 340 nm/380 nm emission ratio. Values obtained in the presence of NAD^+ were set as the 100% reference. Results are means ± SEM of six measurements. ***$P<0.001$ (Student's t test). Monocytes were incubated with 200 μM NAD^+ (*black bar*) before 100 μM ATP (*open bar*) was added in a Ca^{2+}-containing (*solid line*) or Ca^{2+}-free (*dotted line*) solution (**b**). Shown is the 340 nm/380 nm emission ratio from one representative measurement. ***$P<0.001$ (Student's t test) Δ ratio 340/380 $NAD^+ + Ca^{2+}$ versus $NAD^+ - Ca^{2+}$ ($n=3$)

(Fig. 2a) and $P2X_7$ receptors (Fig. 2b). $P2X_4$ receptor expression was detected by Western blot analysis (Fig. 2c). Activation of each receptor subtype leads to a rapid increase in cytosolic Ca^{2+} levels [25]. To determine the involvement of the receptor subtypes in the NAD^+-induced rise in $[Ca^{2+}]_i$, the P2 receptor agonists BzATP and 2-MeSATP were used. The agonists were added to monocytes before the addition of NAD^+ and in reversed order. When monocytes were treated with BzATP (Fig. 3a), a potent activator at both the $P2X_1$ [26] and $P2X_7$ [23] receptor, they failed to respond to the subsequent application of NAD^+. Presumably, BzATP occupies binding sites such as $P2X_1$ and/or $P2X_7$ receptors that are essential for NAD^+ to exert its effects. On the other hand, when NAD^+ was added to monocytes prior to BzATP, the BzATP-induced calcium response was only slightly reduced (Fig. 3b). These data indicate that BzATP not only acts as an agonist on receptors that interact with NAD^+ but also recognizes receptors not interfering with NAD^+.

Engagement of $P2X_1$ receptors

To distinguish between $P2X_1$ and $P2X_7$ receptors interacting with NAD^+, monocytes were treated with 2-MeSATP before NAD^+ was added. 2-MeSATP functions as a potent agonist at the $P2X_1$ receptors and exhibits modest agonist activity at the $P2X_4$ and even less activity at the $P2X_7$ receptor [26].

As seen in Fig. 3, 100 μM 2-MeSATP clearly prevented the NAD^+-induced Ca^{2+} response (c) and when applied subsequent to NAD^+ it hardly showed any activity (d). Lowering the concentration of 2-MeSATP to 10 μM led to the same result (data not shown). Obviously, the interaction between NAD^+ and $P2X_1$ and probably $P2X_4$ receptors is strong enough to prevent 2-MeSATP from exerting its effects.

Another useful tool for identifying the participation of $P2X_1$ receptors is suramin [23, 27]. Suramin is highly active as a $P2X_1$ and weak or nearly inactive as a $P2X_7$ and $P2X_4$ receptor antagonist [28]. As seen in Fig. 3e, 10 μM suramin prevented the NAD^+-induced rise in $[Ca^{2+}]_i$, confirming an essential role of $P2X_1$ receptors in the interaction with NAD^+.

The fact that the selective $P2X_1$ antagonist NF449 [29] inhibited the increase in $[Ca^{2+}]_i$ when added prior to the addition of NAD^+ (Fig. 3f) further demonstrates the involvement of $P2X_1$ receptors in the NAD^+-mediated Ca^{2+} response.

Engagement of $P2X_7$ receptors

To define the role of $P2X_7$ receptors in the NAD^+-induced Ca^{2+} response, monocytes were treated with KN62 prior to the addition of NAD^+. The isoquinoline derivative KN62 is widely used as a potent and selective antagonist of both human and mouse $P2X_7$ receptors [30, 31]. As seen in

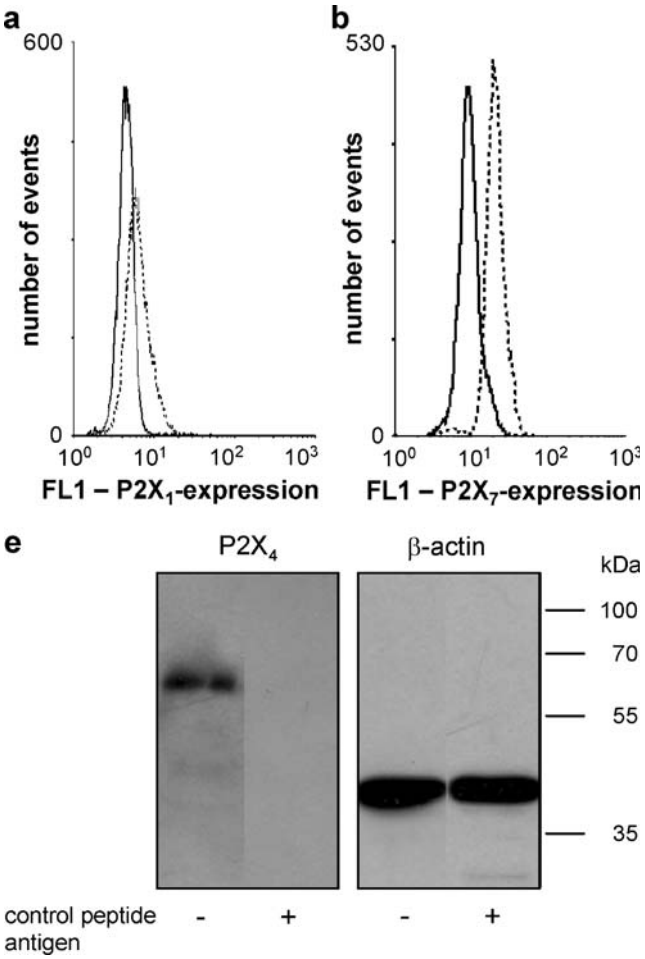


Fig. 2 P2X receptor expression on human monocytes. Freshly isolated monocytes were analyzed by FACS analysis. **a** After blocking the cells with 1% goat serum, the surface antigen expression was analyzed using an anti-$P2X_1$ Ab (*dotted line*) or the respective IgG isotype (*solid line*). Data show one representative experiment out of three. **b** The cells were blocked with 10% human AB serum and the surface antigen expression was analyzed using an anti-$P2X_7$ mAb (*dotted line*) or the respective IgG2b isotype (*solid line*). Data show one representative experiment out of 11. Cell lysates from monocytes were analyzed by Western blot analysis (**c**). The $P2X_4$ receptor expression was detected by an anti-$P2X_4$ Ab in the presence and absence of the control peptide antigen. After stripping, the blot was reprobed with a beta-actin-specific antibody

Fig. 4a, preincubation with KN62 resulted in an inhibition of the rapid NAD^+-promoted increase in $[Ca^{2+}]_i$, indicating the participation of $P2X_7$ receptors.

A similar response was observed (Fig. 4b) when applying periodate-oxATP, currently in use as an efficient $P2X_7$ receptor blocker [32]. In addition to KN62 and oxATP, we tested an anti-$P2X_7$ mAb which has been demonstrated to act as a selective antagonist of human $P2X_7$ receptors in several functional studies [33]. As seen in Fig. 4c, in the presence of the anti-$P2X_7$ mAb, the Ca^{2+} response was diminished lending further support for the involvement of $P2X_7$ receptors in the NAD^+-dependent change in $[Ca^{2+}]_i$.

When testing the effect of KN62, anti-$P2X_7$ mAb and oxATP on the cytosolic $[Ca^{2+}]$ triggered by 100 μM ATP, we also observed that all three compounds led to a decrease in $[Ca^{2+}]_i$ (Fig. 4d–f). Extracellular ATP at concentrations as low as 100 μM, similar to NAD^+, does seem to act in part via $P2X_7$ receptors. These data show that the often described use of high ATP concentrations (millimolar) to activate $P2X_7$ receptors [34] does not apply to the activation of monocytic $P2X_7$ receptors. The relatively minor contribution of $P2X_7$ receptors to the Ca^{2+} response induced by ATP (100 μM) is not surprising, given that such low concentrations of ATP activate only a small number of $P2X_7$ receptors.

The $P2X_7$ receptor subtype is distinguished from other P2X family members by its ability to trigger induction of a nonselective pore on repeated or prolonged stimulation by ATP [35]. The pore is permeable to molecules with molecular masses up to 900 Da, irrespective of the charge [36]. To measure $P2X_7$-dependent pore formation, monocytes were suspended in potassium glutamate basic salt solution [37] containing ethidium bromide before ATP and NAD^+ were added at different concentrations for 15 min at 37°C. Ethidium is a 340 Da hydrophilic molecule normally excluded by intact plasma membranes. Upon entering cells, ethidium fluorescence greatly increases with binding to RNA and DNA. As seen in Fig. 5a, application of ATP, starting at concentrations as low as 100 μM, resulted in a concentration-dependent accumulation of ethidium bromide as assayed by FACS analysis. Addition of anti-$P2X_7$ mAb, KN62, and oxATP to monocytes prior to ATP inhibited the entry of ethidium, confirming the role of $P2X_7$ receptors in pore formation (Fig. 5b). In contrast to ATP, NAD^+ at concentrations from 0.1 to 3 mM failed to induce ethidium uptake (Fig. 5a). Thus, under the experimental conditions used, NAD^+ lacks the ability to promote pore formation.

Engagement of $P2X_4$ receptors

To asses the potential role of $P2X_4$ receptors in the NAD^+-induced increase in $[Ca^{2+}]_i$, monocytes were exposed to extracellular NAD^+ in the presence of IVM. IVM, a widely used antiparasitic agent in human and veterinary medicine, has been shown to augment ATP-evoked currents through rat and human $P2X_4$ channels [38]. To test the effect of IVM on the ATP- and NAD^+-induced changes in the cytosolic $[Ca^{2+}]$, cells were preincubated with IVM prior to the addition of the nucleotides. Exposure of monocytes to IVM potentiates the ATP- (Fig. 6a) and NAD^+ (b)-induced increase in $[Ca^{2+}]_i$, supporting a role of $P2X_4$ receptors in the Ca^{2+} response to extracellular ATP and NAD^+.

Discussion

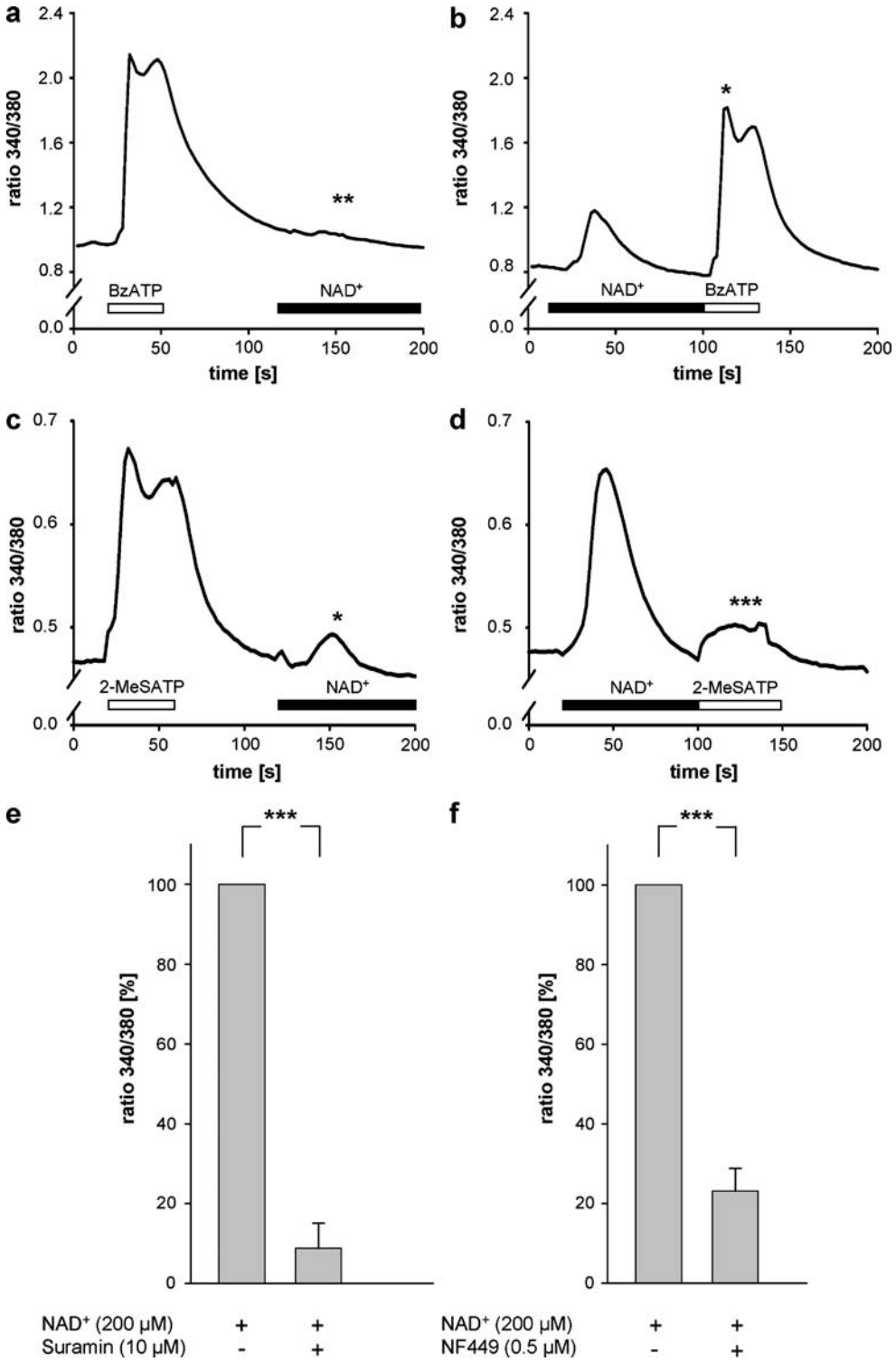
NAD^+ and ATP share many properties. Beyond their central roles in energy metabolism, they serve as substrates for extracellular enzymes, giving rise to the generation of metabolites that themselves exhibit signaling functions [39]. Well-known biologically active metabolites of ATP are AMP and adenosine, whereas degradation of NAD^+ yields cADPR and NAADP [3], two second messengers that induce release of calcium from intracellular stores [40, 41]. However, whereas ATP per se has been shown to function through the specific activation of purinergic receptors (P2X and P2Y), there is hardly any information about surface receptors triggering intracellular events induced by intact NAD^+. Moreschi et al. [42] showed that NAD^+ is an agonist of the $P2Y_{11}$ receptor subtype and that $P2Y_{11}$ is the endogenous receptor in granulocytes mediating the sustained $[Ca^{2+}]_i$ increase responsible for the functional activation in these cells. Furthermore, NAD^+ as a substrate of mono-ADP-ribosyltransferase 2 (ART2), which catalyzes the ADP-ribosylation of $P2X_7$ receptors, facilitates Ca^{2+} influx, pore formation, and cell death [43].

According to the data presented here, we postulate a role of P2 receptors in mediating NAD^+-induced responses in human monocytes. A first clue came from the finding that ATP applied prior to the addition of NAD^+ prevented the NAD^+-induced rise in $[Ca^{2+}]_i$. Possible explanations for this effect include usage of the same receptor or interactions that may lie downstream of the receptor. The finding that a selective ATP receptor antagonist (PPADS) prevented the rise in $[Ca^{2+}]_i$ triggered by NAD^+ made us favor the hypothesis of a shared receptor. Experiments showing that the rise in $[Ca^{2+}]_i$ promoted by NAD^+ solely depends on the influx of extracellular calcium and that the Ca^{2+} response is prevented by 2-MeSATP and BzATP, two receptor antagonists acting at ATP receptors including P2X receptors suggest that of the two known groups of ATP receptors, the P2X receptors, are the ones engaged in NAD^+ signaling in monocytes. To associate a specific P2X receptor subtype with an increase in $[Ca^{2+}]_i$, we used P2X receptor agonists and antagonists, being aware that these substances often lack high selectivity. The inhibition of the NAD^+-promoted change in $[Ca^{2+}]_i$ by 2-MeSATP, a potent $P2X_1$ receptor agonist [26], and by suramin, a potent $P2X_1$ blocker [28], suggested that NAD^+ interferes with the $P2X_1$ receptor. The inhibitory effect of the selective $P2X_1$ antagonist NF449 further underlined a possible role of NAD^+ as a $P2X_1$ agonist.

Assessment of $P2X_4$ as a potential receptor was hampered by the lack of any selective agonists and antagonists. At present, the use of ivermectin seems to be the most appropriate tool to study $P2X_4$ receptor activation. Recently, single channel recordings of ATP-evoked currents through human $P2X_4$ expressed in HEK293 cells suggested that ivermectin increases maximal channel currents after binding to a high affinity site (pEC_{50}=6.6) and may also bind to a low affinity site (pEC_{50}=5.7) to increase the affinity of ATP by stabilizing the open channel conformation [44]. As ivermectin induced an increase in the NAD^+-induced rise in $[Ca^{2+}]_i$, we assume that $P2X_4$ receptors are involved in the NAD^+ response.

Besides $P2X_1$ and $P2X_4$ receptors, we also postulate a role of $P2X_7$ receptors in mediating the NAD^+-induced responses in freshly isolated monocytes. The $P2X_7$ receptor shows a wide distribution including cells of the immune and hemopoietic system [27, 34, 45] and is distinguished by its low affinity for extracellular ATP and by its ability to trigger pore formation. The involvement of $P2X_7$ is supported by the finding that the increase in $[Ca^{2+}]_i$ is inhibited by KN62 [30, 31] and oxATP [32], two selective antagonist of the human $P2X_7$ receptor, and by a blocking anti-$P2X_7$ mAb which has been reported to be highly selective for human $P2X_7$ receptors but does not recognize human $P2X_1$ and $P2X_4$ receptors [33]. When exposing monocytes to similar concentrations of ATP, the inhibitory effects of KN62, oxATP, and anti-$P2X_7$ mAb were also observed, confirming the participation of $P2X_7$ receptors. Although the expression of $P2X_7$ receptors on monocytes and their contribution to the Ca^{2+} peak is rather low, one would assume that monocytes would become permeable to ethidium when stimulated with high concentrations of ATP. However as described earlier, monocytes, in contrast to monocyte-derived macrophages, do not form pores in

Fig. 3 Engagement of $P2X_1$ receptors. Monocytes were treated with 100 μM BzATP (*open bar*) before the addition of 200 μM NAD^+ (*black bar*; **a**) or with 200 μM NAD^+ (*black bar*) before the addition of 100 μM BzATP (*open bar*; **b**). Shown is the 340 nm/380 nm emission ratio from one representative measurement. $^{**}P<0.01$ (Student's *t* test) Δ ratio 340/380 NAD^+ (**a**) versus NAD^+ (**b**; $n=3$); $^{*}P<0.05$ (Student's *t* test) Δ ratio 340/380 BzATP (**a**) versus BzATP (**b**; $n=3$). Monocytes were treated with 100 μM 2-MeSATP (*open bar*) before the addition of 200 μM NAD^+ (*black bar*; **c**) or with 200 μM NAD^+ (*black bar*) before the addition of 100 μM 2-MeSATP (*open bar*; **d**). Shown is the 340 nm/380 nm emission ratio from one representative measurement. $^{*}P<0.05$ (Mann–Whitney rank sum test) Δ ratio 340/380 NAD^+ (**a**) versus NAD^+ (**b**; $n=3$); $^{***}P<0.001$ (Student's *t* test) Δ ratio 340/380 2-MeSATP (**a**) versus 2-MeSATP (**b**; $n=3$). Monocytes were preincubated with suramin (10 μM) for 25 min and continuously perfused with suramin during the addition of NAD^+ (200 μM, 80 s; **e**). Intracellular Ca^{2+} levels were measured as the change in the 340 nm/380 nm emission ratio. Values obtained in the presence of NAD^+ were set as the 100% reference. Results are means ± SEM of three measurements. $^{***}P<0.001$ (Student's *t* test). Monocytes were preincubated with NF449 (0.5 μM) for 25 min and continuously perfused with NF449 during the addition of NAD^+ (200 μM, 80 s; **f**). Intracellular Ca^{2+} levels were measured as the change in the 340 nm/380 nm emission ratio. Values obtained in the presence of NAD^+ were set as the 100% reference. Results are means ± SEM of three experiments. $^{***}P<0.001$ (Student's *t* test)



response to extracellular ATP under physiological ionic conditions [46, 47]. Considering the crucial role of ionic compositions of the extracellular medium in $P2X_7$ receptor activation by ATP [48–51], Gudipaty et al. [37] showed that replacement of extracellular Na^+ and Cl^- with K^+ and nonhalide anions strongly facilitated ATP-dependent pore formation in monocytes. In line with their data, we also observed that these ionic conditions resulted in an increased agonist affinity, such that 100 µM ATP was sufficient for the activation of a nonselective pore by $P2X_7$ receptors. Although it is not known why ATP induces a pore in macrophages and not in monocytes in normal NaCl-

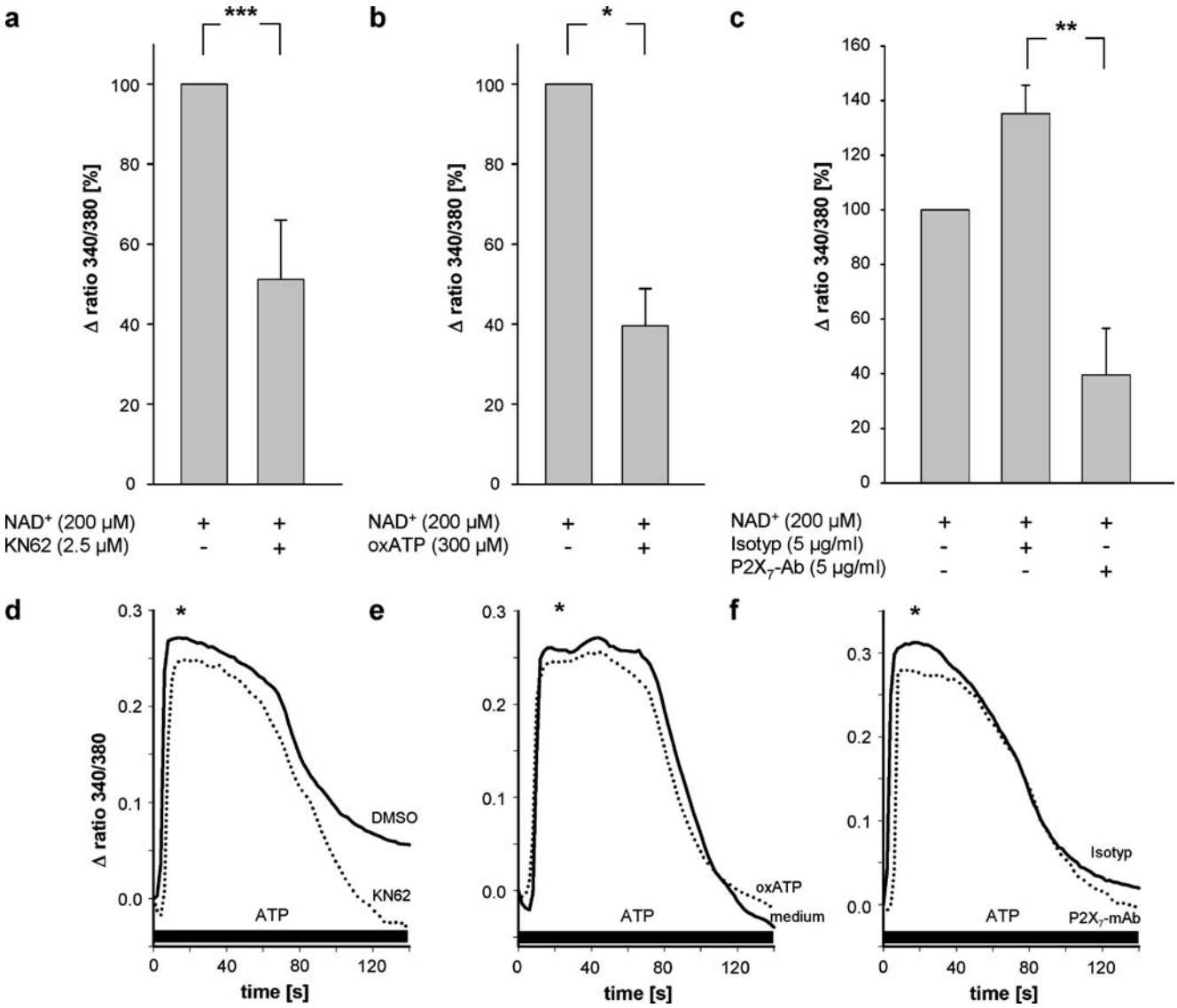


Fig. 4 Engagement of $P2X_7$ receptors. Monocytes were preincubated with KN62 (2.5 μM) or DMSO (0.5%; control; **a**), oxATP (300 μM) or medium (control; **b**), and an anti-$P2X_7$ mAb (5 μg/ml) or the respective IgG2b isotype (5 μg/ml; **c**) for 25 min before NAD^+ (200 μM) was added for 80 s. Intracellular Ca^{2+} levels were measured as the change in the 340 nm/380 nm emission ratio. Values obtained in the presence of NAD^+ were set as the 100% reference. Results are means ± SEM of seven experiments, ***$P<0.001$ (Student's t test; **a**); four experiments, *$P<0.05$ (Student's t test; **b**); or three experiments, **$P<0.01$ (Student's t test; **c**). Monocytes were preincubated with KN62 (2.5 μM) or DMSO (0.5%; control; **d**), oxATP (300 μM) or medium (control; **e**), and an anti-$P2X_7$ mAb (5 μg/ml) or the respective IgG2b isotype (5 μg/ml; **f**) for 25 min before ATP (100 μM) was added. Shown are Δ ratios 340/380 from one representative measurement. *$P<0.05$ (Mann–Whitney rank sum test) Δ ratio 340/380 ATP + DMSO versus ATP + KN62 ($n=4$); *$P<0.05$ (Mann–Whitney rank sum test) Δ ratio 340/380 ATP + medium versus ATP + oxATP ($n=4$); *$P<0.05$ (Mann–Whitney rank sum test) Δ ratio 340/380 ATP + isotype versus ATP + $P2X_7$ mAb ($n=4$)

containing medium, the number of cell surface $P2X_7$ receptors, which is significantly higher in macrophages than in monocytes, seems to play a critical role [37]. To our surprise, NAD^+, in contrast to ATP, failed to induce the nonselective pore that typifies activated $P2X_7$ receptors. The molecular mechanisms underlying selective pore formation by $P2X_7$ receptors are far from clear. It has been suggested that $P2X_7$ subunits may aggregate and that the ion channel dilated to a size sufficient to allow dye uptake [34]. However, several studies claimed that this dye uptake path is more likely to be formed by a distinct protein that is activated by $P2X_7$ receptors [34, 52–54]. Recently, Pelegrin et al. [55] identified this accessory protein as pannexin-1. Whatever the underlying mechanism, either NAD^+ lacks the ability to steer events necessary to form a pore-like structure, or alternatively the interaction between NAD^+ and $P2X_7$ receptors is too weak to allow for detectable pore formation.

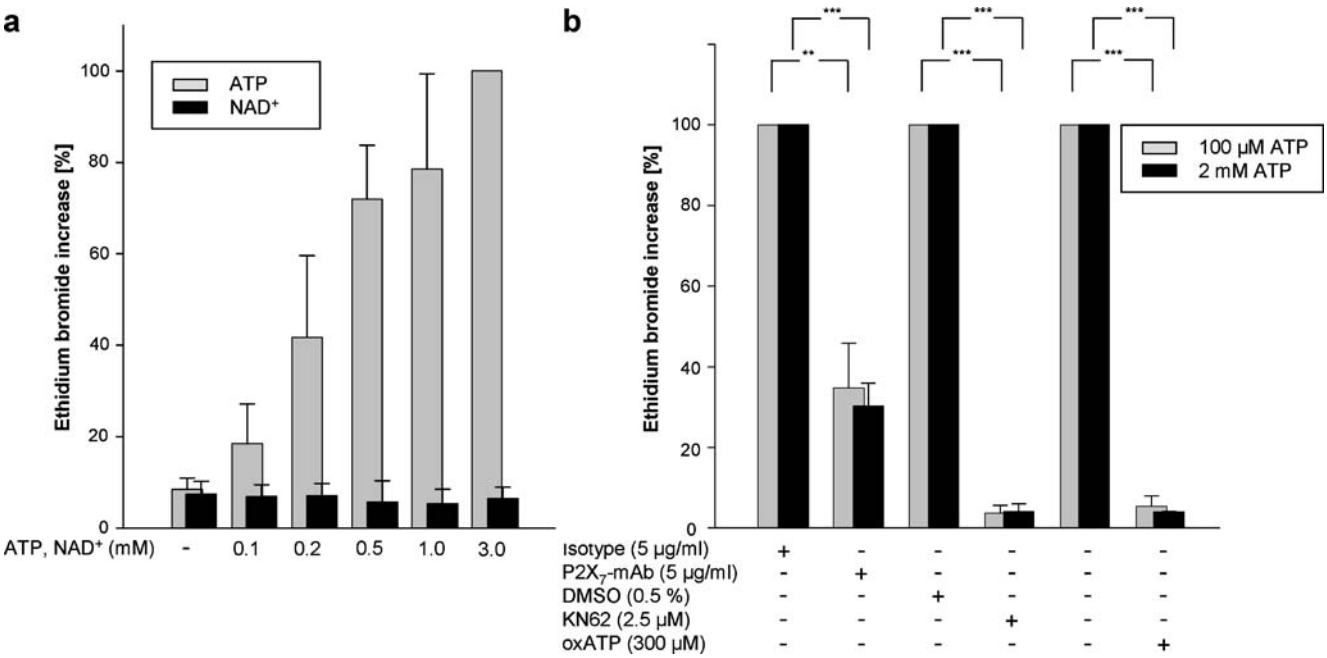


Fig. 5 ATP induces $P2X_7$-dependent pore formation. Monocytes were suspended in potassium glutamate basic salt solution in the presence of 2.5 µM ethidium bromide. The cells were incubated with ATP or NAD^+ at concentrations from 0.1 to 3 mM for 15 min at 37°C. After incubation, the monocytes were washed twice with PBS and the ethidium bromide uptake was measured by FACS analysis. Data show one representative experiment out of five (**a**). To study the engagement of the $P2X_7$ receptor in pore formation, the cells were preincubated with an anti-$P2X_7$ mAb (5 µg/ml) or the respective IgG2b isotype (5 µg/ml), KN62 (2.5 µM), or DMSO (0.5%; control) and oxATP (300 µM) or PBS (control) for 25 min at room temperature before ATP (100 µM/2 mM) was added. After 15 min at 37°C, the monocytes were washed twice with PBS and measured by FACS analysis. Results are means ± SEM of three experiments. $^{**}P<0.01$; $^{***}P<0.001$ (Student's *t* test; **b**)

Besides interfering with nucleotide receptors, NAD^+ can be cleaved by ectoenzymes. Nucleotide phosphodiesterase/pyrophosphatase I (E-NPP, CD203 family) yields AMP, which in turn can be hydrolyzed to adenosine by ecto-5′-nucleotidase (CD73). Alternatively, NAD^+ can serve as a substrate for NAD^+ hydrolases (CD38, CD157) which catalyze the formation of (c)ADP-ribose and nicotinamide. When analyzing NAD^+ degradation products generated by human monocytes, we found that NAD^+ was mainly degraded to ADP-ribose, nicotinamide, and minor amounts of AMP, ADP, and cADPR [56]. Adenosine, a potent anti-inflammatory substance acting via P1 receptors, was not detectable. All these by-products, if produced in sufficient amounts, have signaling functions mediated by different

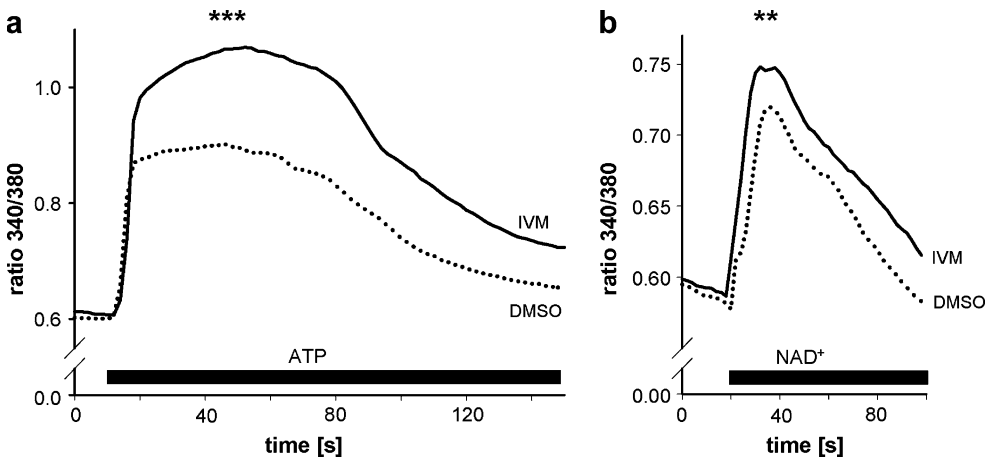


Fig. 6 Engagement of $P2X_4$ receptors. Monocytes were preincubated with ivermectin (*IVM*, 3 µM, *solid line*) or DMSO (0.03%, *dotted line*) for 25 min before 100 µM ATP (**a**) or 200 µM NAD (**b**) was added. Shown are the 340 nm/380 nm emission ratios from one representative measurement. $^{***}P<0.001$ (Mann–Whitney rank sum test) Δ ratio 340/380 ATP + DMSO versus ATP + IVM ($n=6$); $^{**}P<0.01$ (Mann–Whitney rank sum test) Δ ratio 340/380 NAD^+ + DMSO versus NAD^+ + IVM ($n=6$)

receptors. The same holds true for ATP. It serves as a substrate for ectonucleoside triphosphate diphosphohydrolase (E-NTPDase, CD39) and CD203, and its main degradation products ADP, AMP, and adenosine regulate important physiological functions by triggering purinoceptors [57]. In contrast to NAD^+ and ATP, none of their by-products except ADP have so far been described to interfere with P2X receptors. Their capacity to signal through different receptors adds to the complexity of biological reactions originating from one single nucleotide.

Taken together, our data show that NAD^+ induces a strong calcium response in human monocytes which is mediated by the engagement of P2X receptors, ATP-gated ion channels. Among P2X receptors, the subtypes $P2X_1$, $P2X_4$, and $P2X_7$ were found to be involved in NAD^+ signaling. The $P2X_1$ receptor seemed to be somewhat more effective than the $P2X_7$ receptor in mediating the Ca^{2+} response, whereas the contribution of the $P2X_4$ receptor compared to the other two receptors was relatively small. Given that NAD^+ and ATP share mechanisms leading to an increase in $[Ca^{2+}]_i$, one could speculate that ATP, which prevents the NAD^+-induced response, may control the fate of NAD^+ by making it available to other biochemical pathways.

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