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Modulation by cADPr of Ca^{2+} mobilization and oxidative response in dimethylsulfoxide- or retinoic acid-differentiated HL-60 cells

S. Bréchard, A. Brunello, J.-L. Bueb, E.J. Tschirhart*

Laboratoire de Biologie et Physiologie Intégrée. Faculté des Sciences, de la Technologie et de la Communication. Université du Luxembourg, Luxembourg

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Abstract

In human phagocytic cells, reactive oxygen species (ROS) generation in response to N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLF) is largely dependent on cytosolic free calcium concentration ($[Ca^{2+}]_i$). Cyclic ADP-ribose (cADPr) is able to regulate Ca^{2+} release from intracellular stores through the ryanodine receptor but its potential role in biological responses has so far not been determined. In this study, we examined whether extracellular and intracellular cADPr is required in fMLF-induced $[Ca^{2+}]_i$ rise and consequently in the oxidative response in human neutrophil-like HL-60 cells differentiated with dimethylsulfoxide or all-trans-retinoic acid (ATRA). We establish that extracellular cADPr cannot elicit $[Ca^{2+}]_i$ elevation. Furthermore, we demonstrate that 8-Br-cADPr, a functional antagonist of cADPr, inhibits Ca^{2+} entry into HL-60 cells differentiated with ATRA and stimulated with fMLF (95±4 and 148±5 nM respectively, $n=3$). Finally, we show that this partial inhibition of Ca^{2+} mobilization is unrelated to ROS production (10.0±0.3 vs. 9.6±0.5 A.U., $n=3$). In conclusion, we showed that cADPr can control fMLF-induced Ca^{2+} influx but is unable to regulate a Ca^{2+}-dependent biological response, i.e. H_2O_2 production.

Keywords: Ca^{2+} mobilization; cADPr; fMLF; H_2O_2 production; HL-60 cells

1. Introduction

Inositol 1,4,5-trisphosphate (IP_3) has been identified as a calcium (Ca^{2+}) mobilizing second messenger, transducing external signals into intracellular responses [1]. In addition to the IP_3 pathway, internal Ca^{2+} stores can be mobilized by at least two other molecules, cyclic nicotinic acid adenine dinucleotide phosphate [2] and cyclic ADP-ribose (cADPR) [3], by distinct mechanisms. The cyclic metabolite cADPr is a potent Ca^{2+} mobilizer identified in sea urchin eggs [3]; subsequently, it has been proposed that cADPr has effects on Ca^{2+} signalling in a variety of cell types [3,4]. Its formation is catalyzed by an ADP-ribosyl cyclase expressed at the outer surface of the cell and known as CD38 [5]. In contrast to neutrophils, resting human promyelocytic leukaemia HL-60 cells do not express CD38. The differentiation of these cells into granulocytic cells by all-trans-retinoic acid (ATRA) permits the induction of CD38 [6] while it is only scarcely expressed in cells treated with dimethylsulfoxide (DMSO) [7,8].

The main function of cADPr is the modulation of Ca^{2+}-induced Ca^{2+} release, a major mechanism of Ca^{2+} mobilization, most likely via ryanodine receptor (RyR) regulated stores [9], which are distinct from those controlled by IP_3 receptors [10]. However, information is scarce about the mechanism of cADPr-induced Ca^{2+} release; by contrast, IP_3 is able to mediate an elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) predominantly through the store depletion-dependent Ca^{2+} channels called store-operated Ca^{2+} channels (SOC) [11]. cADPr has previously been shown to control extracellular Ca^{2+} influx in T cells [12], suggesting that cADPr may regulate Ca^{2+} entry through a SOC mechanism similarly to that reported for IP_3 [1].

Abbreviations: Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; ATRA, all-trans-retinoic acid; $[Ca^{2+}]_i$, cytosolic free calcium concentration; cADPR, cyclic ADP-ribose; DMSO, dimethylsulfoxide; fMLF, N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine; fura-2/AM, fura-2 acetoxymethyl ester; HRP, horseradish peroxidase; IP_3, inositol 1,4,5-trisphosphate; ≠HL-60 cells, neutrophil-like differentiated HL-60 cells; ROS, reactive oxygen species; RyR, ryanodine receptor; SOC, store-operated calcium channels

* Corresponding author. Fax: +352 46 66 44 6435.
E-mail address: eric.tschirhart@uni.lu (E.J. Tschirhart).

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This hypothesis has been supported by a recent study, which underlines that cADPr controls Ca^{2+} release from intracellular stores and extracellular Ca^{2+} influx in murine neutrophils stimulated by N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLF) [13]. Consequently, cADPr may regulate extracellular Ca^{2+} entry in response to G protein-coupled chemoattractants (fMLF). This release of intracellular Ca^{2+} by cADPr via RyR may activate SOC, allowing a sustained Ca^{2+} influx in response to fMLF [13].

Finally, cADPr may be an essential modulator of capacitative Ca^{2+} entry; however, its mechanism of action remains elusive. A possible link between cADPr elevation, extracellular Ca^{2+} influx and reactive oxygen species (ROS) production has not been investigated in human neutrophils. However, it has been established that increased levels of oxidative response, exerting microbicidal functions, are largely dependent on $[Ca^{2+}]_i$ elevation [14,15].

In order to characterize the role of cADPr in Ca^{2+} mobilization and its implication in activation of ROS generation, we have determined the effect of extracellular cADPr and 8-Br-cADPr, a functional cADPr antagonist, on $[Ca^{2+}]_i$ as well as on H_2O_2 production of neutrophil-like DMSO- or ATRA-differentiated HL-60 cells (DMSO- or ATRA-≠HL-60 cells).

Our results suggest that cADPr regulates $[Ca^{2+}]_i$ mobilization in ATRA-≠HL-60 cells that have been stimulated with fMLF, indicating that cADPr may be implicated in extracellular Ca^{2+} entry in addition to its action on internal Ca^{2+} stores.

2. Materials and methods

2.1. Chemicals

RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were obtained from Cambrex BioSciences (Verviers, Belgium). N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine, thapsigargin, ethylene glycol-bis (beta-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ionomycin, horseradish peroxidase (HRP) type II, all-trans-retinoic acid, cyclic adenosine diphosphate-ribose, 8-Br-cyclic ADP-ribose were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Fura-2 acetoxymethylester (fura-2/AM), 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) were obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany). The physiological salt solution (PSS) used throughout this study had the following composition (mM): NaCl 115, KCl 5, KH_2PO_4 1, glucose 10, $MgSO_4$ 1, $CaCl_2$ 1.25, Hepes-Na 25, supplemented with bovine serum albumin (BSA) 0.1%, pH 7.4. Where indicated, $CaCl_2$ was omitted in the physiological salt solution.

2.2. Cell culture

The human promyelocytic cell line HL-60 was grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), streptomycin (100 μg/mL) and penicillin (100 units/mL). The cells were cultured at 37 °C, in a humidified atmosphere of 5% CO_2. Cells were passaged twice weekly. The differentiation towards neutrophil-like cells was induced by addition of dimethylsulfoxyde (final concentration 1.3%) for 4 days [16] or all-trans-retinoic acid (1 μM) for 5 days [17].

2.3. Fluorometric determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by the use of a double labelling fluorescent technique [18] simultaneously with H_2O_2 production. ≠HL-60 cells (5×10^5/mL, 2 mL) were loaded with 2.5 μM fura-2/AM in PSS for 30 min at 37 °C. Thereafter, the cells were washed three times by centrifugation in PSS at 4 °C to remove the extracellular dye; cells were resuspended in PSS with or without Ca^{2+} (at a density of 5×10^5/mL, 2 mL). Fura-2 fluorescence was measured with a Quantamaster Model QM-8/2003 spectrofluorometry system (PTI, Lawrenceville, NJ, USA) in a thermostated cuvette (37 °C) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. During measurement, fMLF, thapsigargin or cADPr was injected into the cell suspension. An internal calibration was performed for each cell sample by addition of 10 μM ionomycin (to measure Ca^{2+}-saturated fura-2) and 12.5 mM EGTA (to measure Ca^{2+}-free fura-2). $[Ca^{2+}]_i$ was calculated according to Grynkiewics et al. [19] using a K_d of 224 nM. Results are expressed in $[Ca^{2+}]_i$ augmentation ($\Delta[Ca^{2+}]_i$). Fura-2 leakage was trivial and had no influence on $[Ca^{2+}]_i$ calculations (data not shown).

2.4. Fluorometric determination of H_2O_2 production

H_2O_2 production was measured on fura-2 loaded cells with Amplex Red [18], a cell impermeant reagent used to detect the release of H_2O_2 from activated neutrophils. In the presence of HRP, Amplex Red reacts with H_2O_2 to produce highly fluorescent resorufin (1:1 stoichiometry) allowing a direct monitoring of H_2O_2. ≠HL-60 cells (loaded with fura-2, washed and resuspended in PSS) were preincubated for 10 min at 37 °C and during this time, 30 μM of Amplex Red and 1 unit/mL of HRP were added. The generation of resorufin was quantified by fluorescence measurements at 587 nm after excitation at 563 nm, concomitantly with the $[Ca^{2+}]_i$. Basal and stimulated H_2O_2 productions were determined by calculating the initial and maximal slopes of the tangents (dFluo/dt) to the H_2O_2 production time course curve, before and after stimulation of the cells with fMLF, thapsigargin or cADPr. Net production of H_2O_2, expressed in arbitrary units (A.U.), was finally calculated by subtracting basal from maximal fMLF-stimulated production of H_2O_2.

2.5. 8-Br-cADP-ribose addition

≠HL-60 cells were incubated at 37 °C during different times with 8-Br-cADPr before loading with 2.5 μM fura-2/AM.

2.6. Experimental design and statistics

Values obtained from different cell batches are expressed as mean±standard error of the mean (S.E.M.), $n=3$. Data were analyzed by the ANOVA test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Ca^{2+} mobilization and oxidative response in DMSO and ATRA-≠HL-60 cells stimulated by extracellular cADPr

As illustrated in Fig. 1a (right panel), DMSO-≠HL-60 cells neither showed any significant increase of $[Ca^{2+}]_i$ when stimulated by extracellular cADPr, nor an elevation of H_2O_2 production. The basal $[Ca^{2+}]_i$ level in DMSO-≠HL-60 cells increased slowly and steadily during the time of measurements (Fig. 1a; left panel) and it reached 121±29 nM ($n=3$) at 4500 s. In ATRA-≠HL-60 cells, kinetics of $[Ca^{2+}]_i$ and H_2O_2 basal production were similar to those obtained with DMSO-≠HL-60 cells (Fig. 1b, left panel).

Addition of cADPr did not permit $[Ca^{2+}]_i$ rise and did not affect H_2O_2 production in DMSO-≠HL-60 cells (Fig. 1a, right panel). Further concentration-response studies confirmed these results, indicating that cADPr neither authorized $[Ca^{2+}]_i$ elevation (134±3 nM, $n=3$, 4500 s after addition of 10 μM cADPr for example; Fig. 1c, left panel), nor H_2O_2 production

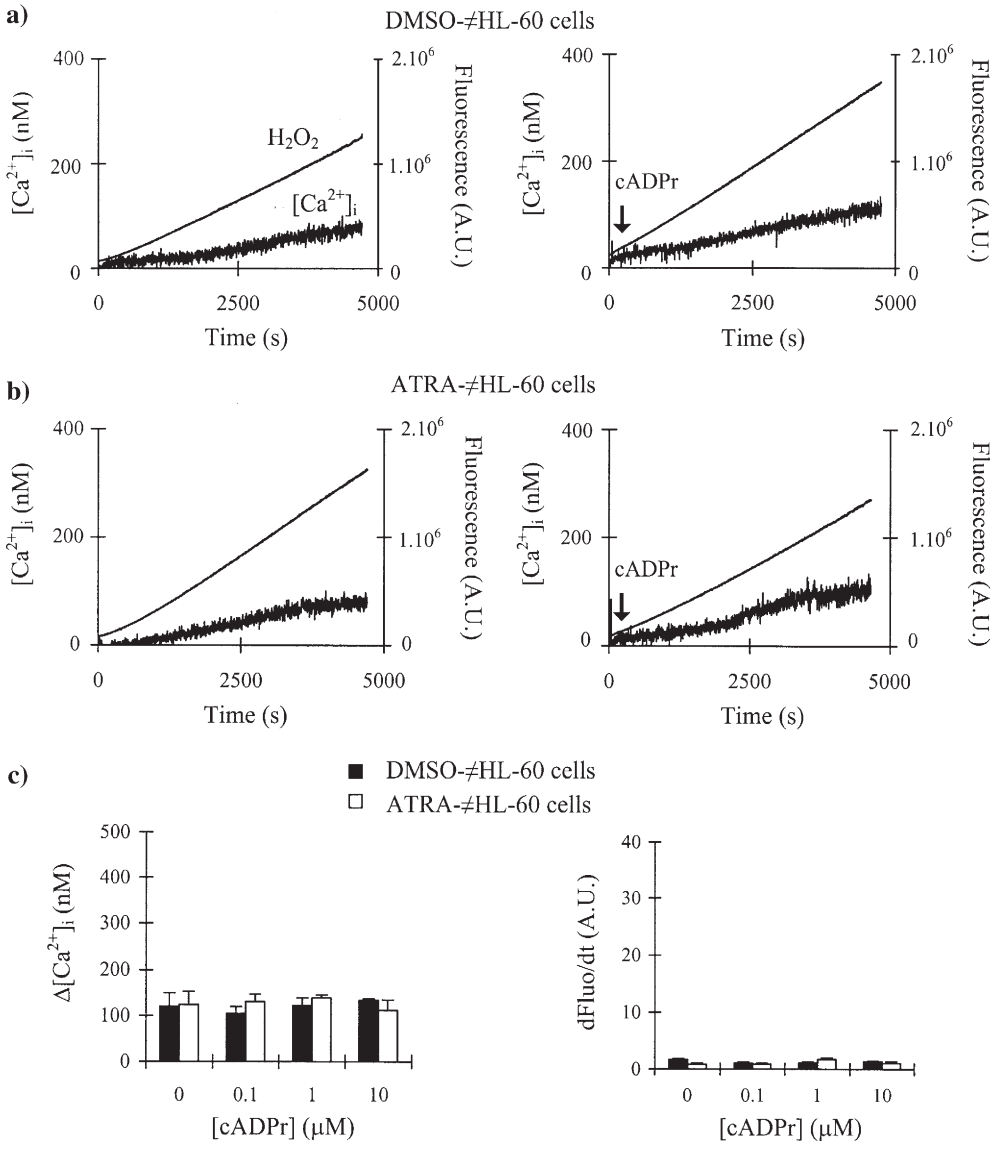

Fig. 1. Effects of extracellular cADPr on $[Ca^{2+}]_i$ rise and H_2O_2 production in DMSO or ATRA-≠HL-60 cells. (a) DMSO or (b) ATRA-≠HL-60 cells were assayed for $[Ca^{2+}]_i$ and H_2O_2 production in basal (left panels) or cADPr-stimulated (right panels) conditions, as described in Materials and methods. 10 μM cADPr was added (right panel) or not (left panel) at the time indicated by the arrow. Tracings representative out of three experiments are shown. (c) Cells were stimulated or not by different concentrations of cADPr from 0.1 μM to 10 μM. $[Ca^{2+}]_i$ elevation and H_2O_2 production were monitored at 4500 s. Results are expressed as mean±S.E.M. of three separate experiments. *Significantly different from control (without cADPr).

(1.3±0.9 vs. 1.4±0.1 A.U., $n=3$, at 10 μM cADPr for example; Fig. 1c, right panel) in DMSO-≠HL-60 cells. The same feature was observed in ATRA-≠HL-60 cells. Increasing concentrations of cADPr did not stimulate $[Ca^{2+}]_i$ elevation (112±23 at 10 μM cADPr for example, vs. 124±28 nM without cADPr, $n=3$, 4500 s after cADPr addition; Fig. 1c, left panel) or H_2O_2 production in comparison to unstimulated cells (10 μM cADPr, 1.0±0.3 vs. 0.8±0.2 A.U., without cADPr, $n=3$; Fig. 1c, right panel).

3.2. cADPr can regulate fMLF-induced $[Ca^{2+}]_i$ in ATRA-≠HL-60 cells

In response to a submaximal concentration of fMLF (1 μM), DMSO or ATRA-≠HL-60 cells generated $[Ca^{2+}]_i$ elevations and H_2O_2 productions to different degrees. DMSO-≠HL-60 cells showed the most pronounced responses ($[Ca^{2+}]_i$ elevation, 365±17 nM and H_2O_2 production, 28.4±3.1 A.U., $n=3$; Fig. 4a). In comparison, both responses were strongly attenuated (approximately 60%) in ATRA-≠HL-60 cells ($[Ca^{2+}]_i$ elevation, 148±5 nM and H_2O_2 production, 9.6±0.5 A.U., $n=3$; Fig. 4a).

Concentration–response curves for 8-Br-cADPr, a purported membrane-permeant antagonist of cADPr [20], for inhibiting fMLF-induced $[Ca^{2+}]_i$ elevation and H_2O_2 production were constructed (Fig. 2). 8-Br-cADPr (1–100 μM) inhibited $[Ca^{2+}]_i$ elevation in ATRA-≠HL-60 cells and this inhibition appeared maximal at 100 μM (47% inhibition) (Fig. 2a). However, 100 μM 8-Br-cADPr did not further inhibit $[Ca^{2+}]_i$ elevation in comparison to 30 μM 8-Br-cADPr (Fig. 2a). No reduction of H_2O_2 production was observed in ATRA-≠HL-60 cells treated with increasing concentrations of 8-Br-cADPr (Fig. 2b).

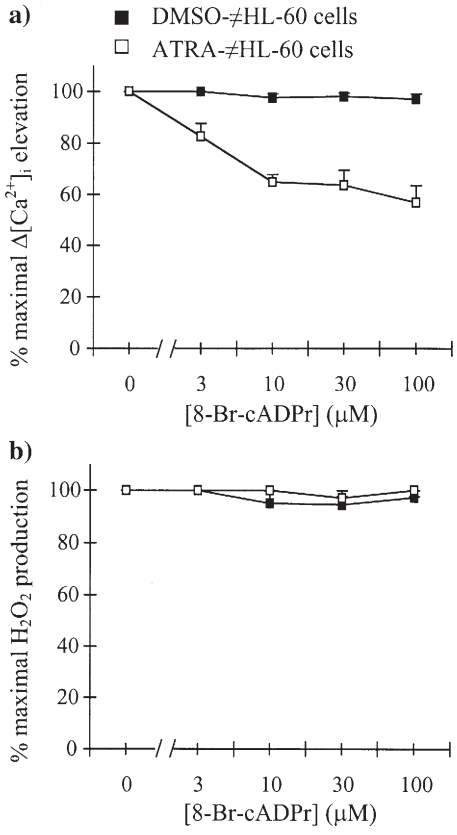

Fig. 2. Effects of 8-Br-cADPr on a) $[Ca^{2+}]_i$ elevation and b) H_2O_2 production in fMLF-stimulated DMSO-≠HL-60 and ATRA-≠HL-60 cells. DMSO or ATRA-≠HL-60 cells were incubated for 2 h with varying concentrations of 8-Br-cADPr then stimulated by 1 μM fMLF. The filled squares represent experiments with DMSO-≠HL-60 cells, the open squares, experiments with ATRA-≠HL-60 cells.

Maximal level of $[Ca^{2+}]_i$ (148±5 nM, n=3; Fig. 4a) observed after fMLF stimulation seemed more rapidly reached (20 s) in untreated ATRA-≠HL-60 cells (Fig. 3), whereas it was slightly delayed (30 s) and lower (95±4 nM, n=3; Fig. 4a) in preincubating ATRA-≠HL-60 cells with 10 μM 8-Br-cADPr (Fig. 3). Proportion differences of the early peaks of $[Ca^{2+}]_i$, between cells preincubated or not with 8-Br-cADPr (Fig. 3), suggest an inhibition of the internal Ca^{2+} mobilization by 8-Br-cADPr. Removal of extracellular Ca^{2+} reduced fMLF-induced $[Ca^{2+}]_i$ rise in ATRA-≠HL-60 cells (148±5 nM, n=3) to a value of 109±9 nM (n=3; Fig. 4a) and 8-Br-cADPr was able to increase this inhibition (approximately of 15%) of fMLF-induced $[Ca^{2+}]_i$ elevation (88±5 vs. 109±9 nM, n=3; Fig. 4a). fMLF-mediated H_2O_2 production (9.6±0.5 A.U., n=3) was comparatively inhibited in the absence of added extracellular Ca^{2+} with 8-Br-cADPr (4.3±1.1 A.U., n=3) or without 8-Br-cADPr (3.2±0.5 A.U., n=3; Fig. 4a).

$[Ca^{2+}]_i$ elevation appeared more prolonged in untreated cells than in 8-Br-cADPr-treated cells (Fig. 3). Also, in ATRA-≠HL-60 cells exposed to 8-Br-cADPr, $[Ca^{2+}]_i$ returned to its baseline value, whereas in control cells, post-fMLF baseline value was higher than before fMLF stimulation (Fig. 3). Moreover, $[Ca^{2+}]_i$ elevation was slightly more important in 8-Br-cADPr-untreated ATRA-≠HL-60 cells resuspended in $[Ca^{2+}]_i$-free media (75±4%, n=3) than in cells preincubated with 8-Br-cADPr (65±4%, n=3; Fig. 4b).

In DMSO-≠HL-60 cells, pretreatment of these cells with 1–100 μM 8-Br-cADPr did not result in any inhibition of fMLF-induced $[Ca^{2+}]_i$ elevation (Fig. 2a) although a very slight H_2O_2 production decrease was observed with 10 μM 8-Br-cADPr (Fig. 2b, Fig. 4a).

3.3. cADPr is implicated in fMLF-stimulated H_2O_2 production, not in fMLF-stimulated $[Ca^{2+}]_i$ increase in DMSO-≠HL-60 cells

Addition of 8-Br-cADPr to DMSO-≠HL-60 cells (up to 4 h, data not shown) prior to stimulation by 1 μM fMLF had no effect on $[Ca^{2+}]_i$ elevation. In contrast, fMLF-induced H_2O_2 production was significantly reduced already after 2 h of preincubation with the antagonist (23.2±1.4 vs. 28.4±3.1 A.U., n=3; Fig. 4a). In this case, oxidative response was not dependent on $[Ca^{2+}]_i$. The reduction in H_2O_2 production by approximately 20% induced by lower concentrations of fMLF (0.1 μM) (Fig. 5b) was not accompanied by a decrease of fMLF-induced $[Ca^{2+}]_i$ (395±36 nM, n=3) elevation after preincubation with 10 μM 8-Br-cADPr (336±47 nM, n=3; Fig. 5a).

8-Br-cADPr did not further reduce the 1 μM fMLF-induced $[Ca^{2+}]_i$ rise (141±14 vs. 130±21 nM, n=3) and H_2O_2 production (11.8±0.9 vs. 13.1±0.4 A.U., n=3) observed in the absence of added extracellular Ca^{2+} (Fig. 4a).

3.4. cADPr has no effect on thapsigargin-stimulated increase in $[Ca^{2+}]_i$ in ATRA-≠HL-60 cells

ATRA and DMSO-≠HL-60 cells responded differently when stimulated by 1 μM thapsigargin, an inhibitor of the Ca^{2+}-ATPase of intracellular Ca^{2+} stores causing a release of Ca^{2+} from endoplasmic Ca^{2+} stores and a subsequent activation of Ca^{2+} store-operated influx [22]. $[Ca^{2+}]_i$ variation obtained in

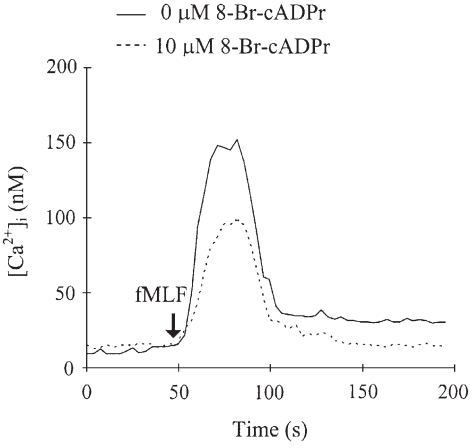

Fig. 3. 8-Br-cADPr inhibits fMLF-induced $[Ca^{2+}]_i$ elevation in ATRA-≠HL-60 cells. ATRA-≠HL-60 cells were incubated or not for 2 h with 10 μM 8-Br-cADPr and then stimulated by 1 μM fMLF. Tracings representative out of three experiments are shown.

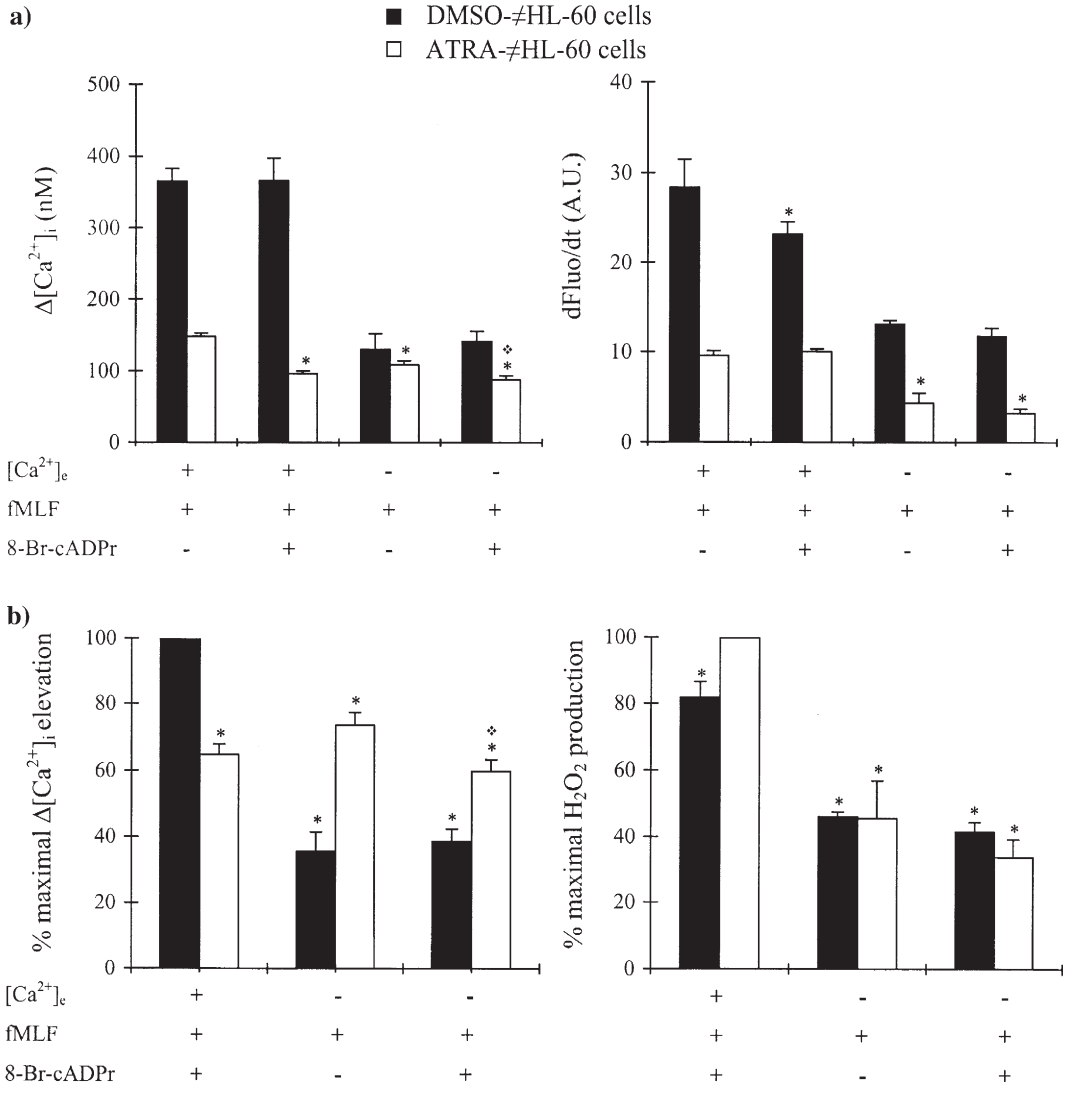

Fig. 4. Effects of 8-Br-cADPr on fMLF-induced $[Ca^{2+}]_i$ elevation and H_2O_2 production in DMSO or ATRA-≠HL-60 cells. DMSO or ATRA-≠HL-60 cells were incubated or not for 2 h with 10 μM 8-Br-cADPr and then stimulated by 1 μM fMLF. (a) The histograms show maximal $[Ca^{2+}]_i$ elevation (left) and H_2O_2 production (right), in the presence or in the absence of extracellular Ca^{2+}. (b) The histograms indicate the percentage of maximal $[Ca^{2+}]_i$ elevation (left) and the percentage of maximal H_2O_2 production (right) in the presence or in the absence of extracellular Ca^{2+}. Maximal responses correspond to those obtained when ≠HL-60 cells were stimulated by fMLF in the presence of extracellular Ca^{2+}. Results are expressed as mean±S.E.M. of three separate experiments. *Significantly different from control (fMLF alone in the presence of extracellular Ca^{2+}). ❖Significantly different from control (fMLF alone in the absence of added extracellular Ca^{2+}).

ATRA-≠HL-60 cells (222±20 nM, n=3) was approximately 2-fold lower than the observed $[Ca^{2+}]_i$ rise in DMSO-≠HL-60 cells (467±8 nM, n=3; Fig. 6a). Removal of extracellular Ca^{2+} was able to strongly reduce thapsigargin-induced $[Ca^{2+}]_i$ elevation in both conditions (for example, in ATRA-≠HL-60 cells, 94±7 vs. 222±20 nM, n=3; Fig. 6a).

ATRA-≠HL-60 cells pretreatment (2 h) with 10 μM of 8-Br-cADPr, prior to thapsigargin (1 μM) stimulation, did not reveal a blockade of Ca^{2+} influx induced by thapsigargin (221±5 vs. 222±20 nM, n=3; Fig. 6a). Moreover, 8-Br-cADPr was not able to potentiate the reduction of thapsigargin-induced $[Ca^{2+}]_i$ increase observed in the absence of added extracellular Ca^{2+} (102±6 vs. 94±7 nM, n=3). No effect of this antagonist has been seen on $[Ca^{2+}]_i$ mobilization in DMSO-≠HL-60 cells stimulated by thapsigargin (443±33 vs. 467±8 nM, n=3; Fig. 6a). In the absence of extracellular Ca^{2+}, 8-Br-cADPr did not inhibit the slight $[Ca^{2+}]_i$ elevation observed in DMSO-≠HL-60 cells (134±9 vs. 146±19 nM, n=3; Fig. 6a).

Under both conditions of differentiation (ATRA and DMSO), no H_2O_2 production was observed (Fig. 6b).

4. Discussion

In response to fMLF, ≠HL-60 cells are able to produce a large amount of ROS dependent on the influx of extracellular Ca^{2+} [14,15]. In comparison to DMSO-≠HL-60 cells, ATRA-≠HL-60 cells respond to fMLF by a reduced Ca^{2+} mobilization, correlated to a lower level of oxidative response. Similar results were observed with thapsigargin, known to deplete intracellular Ca^{2+} stores resulting in the activation of store-operated Ca^{2+} influx [21], $[Ca^{2+}]_i$ elevation being strongly attenuated in ATRA-≠HL-60 cells compared to DMSO-≠HL-60 cells. The

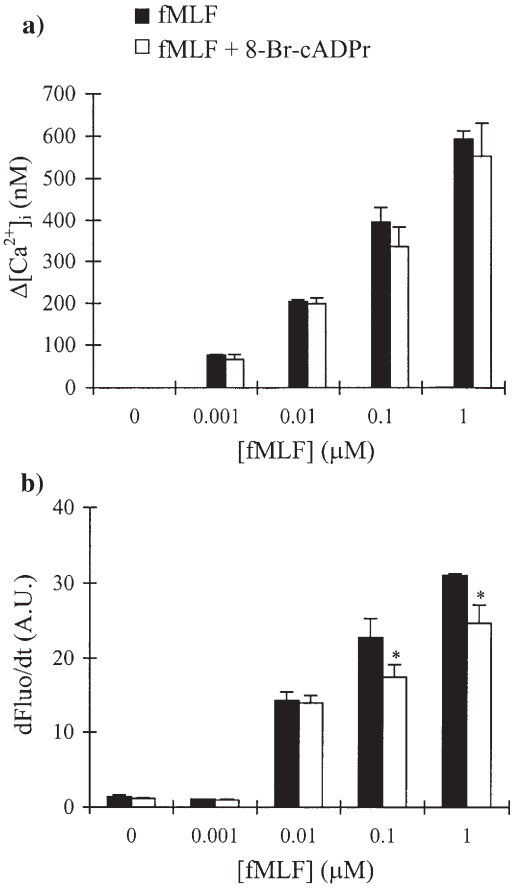

Fig. 5. Effects of 8-Br-cADPr on $[Ca^{2+}]_i$ elevation and H_2O_2 production induced by increasing concentrations of fMLF in DMSO-≠HL-60 cells. DMSO-≠HL-60 cells were incubated or not for 2 h with 10 μM 8-Br-cADPr and then stimulated with different concentrations (0.001–1 μM) of fMLF. The histograms show (a) $[Ca^{2+}]_i$ elevation and (b) H_2O_2 production after fMLF stimulation. Results are expressed as mean±S.E.M. from three separate experiments. *Significantly different from control (fMLF alone).

weak responses obtained in ATRA-≠HL-60 cells may however be caused by a reduced expression of store-operated channels under these differentiation conditions.

In mammals, CD38 catalyzes the synthesis of ectocellular cADPr and is located on the cell surface. Several possibilities have been proposed to explain how extracellular generated cADPr can exert its intracellular Ca^{2+} signalling function. Franco et al. [22] have suggested that CD38 is able to act as a catalytically active transporter of cADPr into cells. Alternatively, CD38 may be internalized triggering cADPr production in the cytosol upon stimulation by the ligand NAD^+ [23]. Recently, cADPr has also been shown to cross the plasma membrane and to be internalized by HL-60 cells through nucleoside transporters [24], allowing a cADPr influx, which mediates $[Ca^{2+}]_i$ elevation in DMSO-≠HL-60 cells lacking CD38. Munshi et al. [25] have proposed that CD38 is expressed not solely on the cell surface but also intracellularly in HL-60 cells. This hypothesis is further supported by Sun et al. [26] who suggested that a full cytosolic Ca^{2+} response can be triggered through the expression of functional intracellular CD38. Our results show that extracellular cADPr, on its own, has no effect on $[Ca^{2+}]_i$ elevation and H_2O_2 production in ATRA-≠HL-60 cells suggesting that ATRA-≠HL-60 cells are not permeable to cADPr. Identical results were obtained when HL-60 cells were differentiated with DMSO. Similarly, in T-lymphocytes, no relation between ectocellular cADPr synthesis by CD38 and cADPR-mediated $[Ca^{2+}]_i$ increase could be established, suggesting that ectocellular cADPr did not accumulate intracellularly [27]. Accordingly, we observed that under both conditions of differentiation, extracellular cADPr had a very limited role in Ca^{2+} mobilization, which may have been due to cellular cADPr efflux. Therefore, extracellular cADPr does not seem to be actively transported into the cells.

In accordance with previous results in murine neutrophils [13], we observed that fMLF-induced $[Ca^{2+}]_i$ elevation in ATRA-≠HL-60 cells was partly abolished by a preincubation with 8-Br-cADPr which was not accompanied by a reduction of fMLF-induced H_2O_2 production. This feature may document the concept of a very high functional sensitivity to intracellular

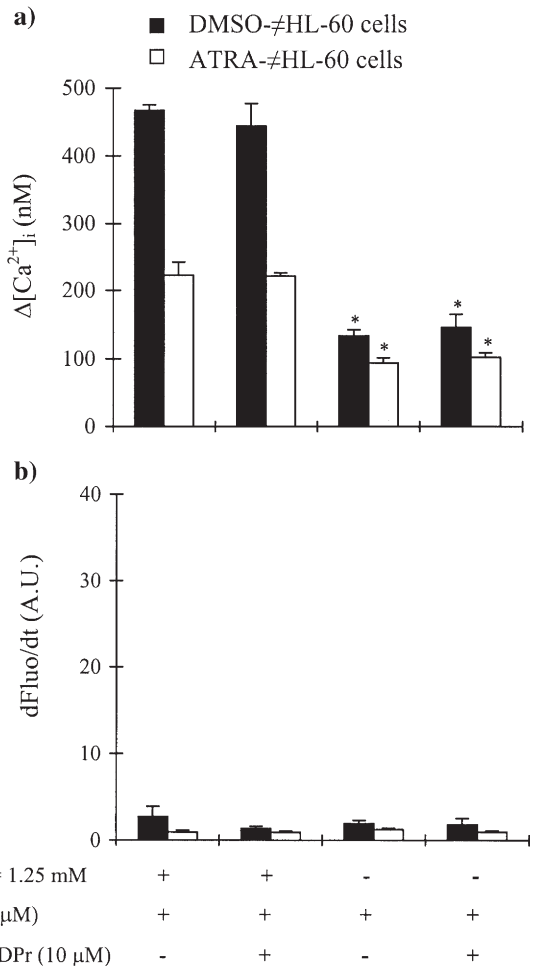

Fig. 6. Effects of 8-Br-cADPr on thapsigargin-induced $[Ca^{2+}]_i$ elevation in DMSO or ATRA-≠HL-60 cells. DMSO or ATRA-≠HL-60 cells were incubated or not for 2 h with 10 μM 8-Br-cADPr then stimulated by 1 μM thapsigargin. The histograms show a) $[Ca^{2+}]_i$ elevation and b) H_2O_2 production after addition of thapsigargin. Results are expressed as mean±S.E.M. of three separate experiments. *Significantly different from control (thapsigargin alone in the presence of extracellular Ca^{2+}).

calcium levels of the H_2O_2 producing enzyme complex [28]; in our experimental cell system, cADPr would therefore be ineffective in modulating the release of H_2O_2 in view of the high levels of $[Ca^{2+}]_i$ reached. This phenomenon does not occur in DMSO-≠HL-60 cells, confirming the probable lack of CD38 expression in these differentiated cells [7,8] despite an unexplained slight decrease in H_2O_2 production. Under our experimental conditions, reduction of fMLF-induced $[Ca^{2+}]_i$ elevation by 8-Br-cADPr in ATRA-≠HL-60 cells remained weak (35%) even at relatively high concentrations of the antagonist. These data suggest that cADPr is necessary to obtain an optimal fMLF-induced $[Ca^{2+}]_i$ elevation but that its role is maybe minor. According to Partida-Sanchez et al. [29], fMLF may induce Ca^{2+} mobilization independently of cADPr in human neutrophils. Human myeloid cells express a low affinity receptor related to the fMLF receptor [30] and both receptors can trigger distinct Ca^{2+} responses. They suggest that related fMLF receptor-dependent $[Ca^{2+}]_i$ elevation is primarily mediated by extracellular Ca^{2+} influx and is further regulated by cADPr opposed to the fMLF receptor, which induces a cADPr-independent Ca^{2+} response through intracellular Ca^{2+} release [29]. However, our data establish a role for cADPr in Ca^{2+} mobilization in response to fMLF. Also, 8-Br-cADPr was able to reduce $[Ca^{2+}]_i$ elevation in the absence of extracellular Ca^{2+} in fMLF-stimulated ATRA-≠HL-60 cells suggesting that cADPr may regulate intracellular Ca^{2+} stores release in response to fMLF.

In addition to its action on internal Ca^{2+} stores, cADPr could control extracellular Ca^{2+} entry. ADPr can activate TRPM2, a non-selective cation channel expressed in neutrophils and HL-60 cells [31]. Kolisek et al. [32] have shown that cADPr can also activate TRPM2 at very high concentrations (EC_{50} = 700 μM), a concentration probably not present in cells [12]. At a possible physiological concentration [12], cADPr is able to decrease the EC_{50} of ADPr from 1200 to 90 nM indicating that cADPr could be a coregulator of Ca^{2+} influx mediated by TRPM2 activation. Other studies proposed a role for cADPr in Ca^{2+} entry in different cell types and notably in neutrophils. Partida-Sanchez et al. [13] demonstrated that cADPr is required for sustained extracellular Ca^{2+} influx in neutrophils stimulated by fMLF. Our findings seem to confirm that cADPr participates to this Ca^{2+} entry mediated by a non-capacitative mechanism. Indeed, $[Ca^{2+}]_i$ elevation was slightly more inhibited in ATRA-≠HL-60 cells preincubated with 8-Br-cADPr in comparison to control cells resuspended in $[Ca^{2+}]_i$-free media. These results suggest that cADPr may regulate extracellular Ca^{2+} entry, in addition to capacitative Ca^{2+} entry mechanism. The difference of tracings in $[Ca^{2+}]_i$ observed between fMLF-stimulated ATRA-≠HL-60 cells with and without 8-Br-cADPr seems to confirm cADPr implication in Ca^{2+} influx, and thus its involvement in the regulation of membrane cation channel permeability. Also, stimulation by thapsigargin elicited a $[Ca^{2+}]_i$ elevation in ATRA-≠HL-60 cells, which was not abolished by 8-Br-cADPr, while cADPr had no effect on thapsigargin-stimulated $[Ca^{2+}]_i$ increase. Both results seem to exclude a direct involvement of cADPr in a Ca^{2+} influx mediated by store-operated channel. In accordance with Kolisek et al. [32], cADPr could synergize with ADPr by causing a large activation of a cation channel independently of cADPr, and activating TRPM2 to authorize a maximal extracellular Ca^{2+} entry in physiological conditions.

In conclusion, the HL-60 cell line appears to be an insufficient cell system for studying the role of cADPr role in the Ca^{2+} signalling pathway in human neutrophils. Although the differentiation of the cells with ATRA results in CD38 expression, only a weak response to fMLF or thapsigargin was detected. However, our results clearly illustrate that cADPr can act as messenger in fMLF-induced Ca^{2+} mobilization by a yet unknown mechanism whereas it appears not implicated in fMLF-induced oxidative response.

In fact, cADPr could have a dual role: (1) a role in the capacitative mechanism in which it could support intracellular Ca^{2+} release authorizing store-operated channel activation in cells stimulated by fMLF; (2) a role in extracellular Ca^{2+} entry in supporting non store-operated cation channel activation.

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