Cyclic ADP-ribose is a second messenger in the lipopolysaccharide-stimulated proliferation of human peripheral blood mononuclear cells

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Cyclic ADP-ribose (cADPR), a universal calcium mobilizer from intracellular stores, was recently demonstrated to stimulate proliferation of various cell types. The role of cADPR in a specific process of monocyte- and plasma-mediated activation of T-lymphocytes by lipopolysaccharide (LPS) was addressed using human mononuclear cells from peripheral blood (PBMCs). Incubation of PBMCs with 0.1 μg/ml of LPS for 24 h provided a doubling in the intracellular levels of cADPR as compared with unstimulated PBMCs. The cADPR increase was abolished either by prior removal of monocytes or by pre-incubating a whole PBMC population with a monoclonal antibody against the monocyte marker CD14. The increased concentrations of intracellular cADPR elicited by LPS stimulation were paralleled by significant increases in NAD^+ levels and in the activities of ectocellular and membrane-bound fractions of ADP-ribosyl cyclase/cADPR hydrolase activities. A cytosolic ADP-ribosyl cyclase was also detectable in PBMCs and its activity was comparably enhanced by LPS stimulation. This soluble cyclase is distinguished from the membrane-bound cyclase by both substrate and inhibitor sensitivities. LPS-stimulated PBMCs showed 2–3-fold increases of intracellular calcium ($[Ca^{2+}]_i$), and these changes were prevented completely by the cADPR antagonist 8-Br-cADPR and by ryanodine. Both compounds, and the cyclase inhibitor nicotinamide, significantly inhibited the T-lymphocyte proliferation induced by LPS in PBMCs. These results demonstrate that cADPR plays a role of second messenger in the adaptive immune recognition process of LPS-stimulated proliferation of PBMCs.

Key words: ADP-ribosyl cyclase, cyclic ADP-ribose, cyclic ADP-ribose hydrolase, lipopolysaccharide, nicotinamide–adenine dinucleotide (NAD^+), peripheral blood mononuclear cell.

INTRODUCTION

The NAD^+ metabolite cyclic ADP-ribose (cADPR) is a universal signalling molecule that mobilizes calcium from intracellular stores and regulates accordingly a number of calcium-related cell processes in organisms ranging from simple protozoa to humans [1–3]. cADPR-dependent cell functions in mammals include proliferation, contraction, vasodilation and secretion [1–8]. The mechanism whereby cADPR enhances cell proliferation is related to shortening of the S phase of the cell cycle in murine 3T3 fibroblasts [9]. Moreover, cADPR has been demonstrated to behave as a new haemopoietic cytokine since it stimulates the proliferation of human haemopoietic progenitors both *in vitro* [10,11] and *in vivo* [12]. Finally, cADPR proved to be required for the calcium-related triggering of activation/proliferation of T-lymphocytes that follows stimulation of the T-cell receptor–CD3 complex [13].

A well known, potent inducer of B- and T-lymphocyte proliferation is lipopolysaccharide (LPS), the main component of the cell wall of Gram-negative bacteria [14,15]. Specifically, while both murine B- and T-lymphocytes have been documented to be responsive to LPS [16–18], human T-lymphocytes, but not B-lymphocytes, proved to exhibit responses to LPS in terms of enhanced proliferation and cytokine production, and such stimulation appears to be mediated by accessory cells including monocytes and $CD34^+$ haemopoietic stem cells [19].

The intracellular mechanisms underlying monocyte- and stem-cell-mediated activation of T-lymphocytes by LPS are still little understood, although direct cell-to-cell interactions (e.g. between CD28 and B7) and release of soluble cytokines [e.g. interleukin (IL)-12] are involved as extracellular triggering signals [19]. Specifically, no information is currently available on the intracellular signalling pathways that result in the response of human T-lymphocytes to LPS. Chien et al. [20] reported an activation of PKC (protein kinase C) without any elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) in LPS-stimulated isolated human peripheral T-lymphocytes. On the other hand, stimulation of the T-cell receptor (TCR)–CD3 complex by the agonistic anti-CD3 monoclonal antibody (mAb) OKT3 in human Jurkat T-lymphocytes induced an enhanced ADP-ribosyl cyclase activity, which resulted in increases in intracellular cADPR levels and in $[Ca^{2+}]_i$. In the same study, cADPR proved to have a role in calcium-dependent proliferation of T-lymphocytes purified from human blood [13]. The current uncertainties about the intracellular mechanisms of monocyte-mediated T-cell proliferation triggered by LPS, which represents a paradigmatic process of innate (adaptive) immunity, prompted us to address the possibility of a role of cADPR in this activation process. The results obtained in

Abbreviations used: BST-1, bone marrow stromal cell antigen 1; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; cADPR, cyclic ADP-ribose; IL, interleukin; LBP, LPS-binding protein; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PCA, perchloric acid; PKC, protein kinase C; TCA, trichloroacetic acid; TCR, T-cell receptor; TdR, thymidine; TLR, Toll-like receptor.

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the present study demonstrate the occurrence of such a role for cADPR, which proved to behave as a second messenger in the cascade of events initiated by LPS-primed monocytes and eventually leading to enhanced proliferation of T-lymphocytes.

MATERIALS AND METHODS

PBMC (peripheral blood mononuclear cell) isolation and culture conditions

Buffy coats, prepared from blood of healthy volunteer human donors and obtained from the Red Cross, St. Paul, MN, U.S.A. and from Galliera Hospital, Genova, Italy, were diluted with equal volumes of PBS or RPMI 1640 medium. PBMCs were isolated by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences AB, Uppsala, Sweden), and contaminating erythrocytes were lysed by resuspending cells for 5 min in the lysing buffer (0.3 M NH_4Cl and 20 mM $KHCO_3$). PBMCs were then resuspended with the recovered autologous plasma (upper layer of the centrifugation gradient), supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin. In the experiments designed to establish the role of autologous plasma, PBMCs were alternatively resuspended in RPMI 1640 medium, supplemented with 10 % foetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The almost complete absence of granulocytes (< 3 %) in the isolated PBMCs was verified by May-Grunwald–Giemsa staining.

PBMCs were cultured at a concentration of 3×10^6 cells/ml and were either unstimulated or stimulated for various times (1–48 h) with LPS (serotype 0111:B4 or serotype 055:B5; Sigma) at concentrations ranging from 0.001 to 10 μg/ml. In experiments aimed at investigating the monocyte-dependent cADPR increases, PBMCs were cultured for 2 h in plastic flasks, before LPS stimulation, and then non-adherent (i.e. monocyte-depleted) cells were recovered by gentle pipetting. Adherent cells (mainly represented by monocytes [21,22]) were either discarded or LPS-primed to explore their effect on cADPR increases. For CD14-dependent regulation, PBMCs were first cultured with an anti-CD14 blocking mAb (10 μg/ml biG10, IgG1; Biometec, Greifswald, Germany) for 1 h. The anti-CD14 mAb was present in the medium throughout the entire incubation period. An isotype-matched mAb (anti-Cx26; catalogue no. 13-1800, Zymed Laboratories, San Francisco, CA, U.S.A.) was used in these experiments as a negative control.

Determination of intracellular cADPR and NAD^+ concentrations

PBMCs were cultured as described above for various times (1, 2, 9, 24 and 48 h). At each time point, 9.0 ml of non-adherent cells were withdrawn in triplicate and were centrifuged for 10 min at 1000 ***g***. Cell pellets were lysed in 0.5 ml of 0.6 M perchloric acid (PCA) at 4 °C, cADPR and NAD^+ contents were determined by the cycling assay as described [23], and their levels were expressed as pmol/10^6 cells and nmol/10^6 respectively. Briefly, PCA was removed by mixing the aqueous sample with 4 vol. of a solution containing 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine. After 1 min of vortex mixing, samples were centrifuged for 10 min at 1500 ***g***, and the aqueous layer, containing cADPR, was recovered. Samples were then subjected to an overnight treatment at 37 °C with a mixture of enzymes (nucleotide pyrophosphatase, alkaline phosphatase and NAD^+-glycohydrolase), to remove all contaminating nucleotides. Enzymes were removed by filtration with a multiscreen vacuum manifold using Immobilon-P membrane plates (Millipore, Bedford, MA, U.S.A.), and reactions for cADPR determination were then carried out in 96-well plates. A reagent (50 μl) containing 30 mM nicotinamide, 100 mM sodium phosphate and 0.1 μg/ml ADP-ribosyl cyclase (Sigma, St. Louis, MO, U.S.A.) was added to 0.1 ml of sample. In parallel, the same reagent without ADP-ribosyl cyclase was added to each sample. Finally, 0.1 ml of the cycling reagent [2 % (v/v) ethanol, 100 μg/ml alcohol dehydrogenase, 20 μM resazurin, 5 μg/ml diaphorase, 10 μM FMN, 10 mM nicotinamide and 100 mM sodium phosphate, pH 8.0] were added to each well. The increase in resorufin fluorescence (544 nm excitation, 590 nm emission) was measured every 3 min over a 12 h period, using a fluorescence plate reader (Fluostar Optima, BMG Labtechnologies GmbH, Offenburg, Germany). To determine endogenous levels of NAD^+, the acidic cell extracts were diluted 200-fold in 100 mM sodium phosphate buffer, pH 8.0. The cycling reagent (0.1 ml) was added to 0.1 ml of each sample. Increases in resorufin fluorescence were acquired every 1 min, as described above. A cADPR or NAD^+ standard curve was always run in parallel in each assay.

Assay of ADP-ribosyl cyclase, GDP-ribosyl cyclase and cADPR-hydrolase activities in monocyte-depleted PBMCs

PBMCs (3×10^6 cells/ml) were either unstimulated or stimulated with 0.1 μg/ml LPS for 24 or 48 h. Non-adherent PBMCs (7.0 ml) were then recovered, washed twice with 10 ml of PBS and resuspended in 3.0 ml of PBS containing 10 mM glucose and protease inhibitors [0.1 mM aprotinin, 0.1 mM leupeptin, 10 μM pepstatin, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride and 1 mM PMSF] (PBS–glu). A 0.5 ml aliquot of cell suspension was used to measure enzymic activities on intact cells, whereas the other part (2.5 ml) was lysed by sonication in ice for 1 min at 3 W (Heat-System Ultrasonics, W-380, New York, NY, U.S.A.). A 0.5 ml aliquot was used for assay of enzymic activities in the total cell lysate. The remaining part was centrifuged at 1000 ***g*** for 5 min, to discard cell debris, and the supernatant was centrifuged further at 100 000 ***g*** for 2 h at 4 °C. The resulting supernatant (S100) was collected and the pellet (P100) was resuspended in 0.5 ml PBS–glu.

ADP-ribosyl cyclase activity was measured by adding 0.1 mM NAD^+ (final concentration) to intact cells, total cell lysates, or to S100 and P100 fractions. After NAD^+ supply, 100 μl aliquots were withdrawn at various times (0, 5, 10 and 20 min). Enzymic reactions were stopped by the addition of 220 μl of 0.9 M PCA to each aliquot, and cADPR and NAD^+ concentrations were measured by the cycling assay [23].

GDP-ribosyl cyclase activity was determined by adding 0.4 mM NGD^+ to intact cells, total cell lysates, or to S100 and P100 fractions. At various times (0, 15, 60 and 120 min), 100 μl aliquots were withdrawn and trichloroacetic acid (TCA)-deproteinized. cADPR-hydrolase activity was measured by incubating total cell lysates with 0.2 mM cADPR for 0, 60 and 180 min. Aliquots were withdrawn at each time point and were TCA-deproteinized. HPLC analyses of nucleotides (to measure the production of cGDPR and ADPR respectively) were performed as described [24].

Aliquots collected from incubations with intact cells to measure ectocellular enzyme activities were first centrifuged at 1000 ***g*** for 5 min at 4 °C to remove cells and either PCA or TCA was added to the supernatant, as described above. All incubations were carried out at 37 °C and protein determination in each sample was performed according to the method of Bradford [25].

Fluorimetric determination of $[Ca^{2+}]_i$

PBMCs (3×10^6 cells/ml) were pre-incubated in duplicate in the presence of 50 μM 8-Br-cADPR or 50 μM ryanodine, or without

any addition. After 1 h, cells were either unstimulated or were stimulated with 0.1 μg/ml LPS and incubated for 24 or 48 h. Non-adherent PBMCs (2.0 ml) were then recovered, incubated with 5 μM Fura 2 acetoxymethyl ester for 40 min at 37 °C, washed with a calcium-free solution (135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$ and 5 mM Hepes, pH 7.4) and resuspended in the same solution at 1.5×10^6 cells/ml. Calcium measurements were performed at 37 °C in a 2 ml cuvette, under continuous stirring. $[Ca^{2+}]_i$ was calculated as previously described [9].

Incorporation of [^{3}H]TdR ([^{3}H]thymidine)

After 1 h of pre-incubation in the presence of 50 μM 8-Br-cADPR, 20 mM nicotinamide or 50 μM ryanodine, or without any addition (control), PBMCs were stimulated with 0.1 μg/ml LPS and were cultured for 72 h. This proved to be the optimum time, among several times investigated in parallel experiments, to record significant effects of the different molecules used. 8-Br-cADPR (50 μM), nicotinamide (20 mM) or ryanodine (50 μM) was present in the medium throughout the entire incubation period. For the last 8 h of incubation, non-adherent cells were recovered and were labelled with [^{3}H]TdR (specific activity of 48 Ci/mmol, 0.2 μCi/culture; Amersham Biosciences) and harvested on glass fibre filters (Whatman, Maidstone, U.K.) for measurements of incorporated radioactivity.

RESULTS

Intracellular cADPR concentrations in LPS-stimulated PBMCs

Freshly isolated human PBMCs, cultured as described in the Materials and methods section, were stimulated for various time intervals with 0.1 μg/ml LPS (serotype 0111:B4) and the intracellular cADPR content was then measured in non-adherent cells. After 1, 2 and 9 h of incubation, no changes of intracellular cADPR concentrations were detectable (results not shown). However, as illustrated in Figure 1(A), after 24 h of stimulation with LPS, the cADPR content of control cells (0.049 ± 0.010 pmol/10^6 cells; $n = 20$) was significantly enhanced (188 ± 21 % of control value; $n = 9$; $P < 0.0001$). The increase was no longer observed when LPS-stimulated PBMCs were resuspended in RPMI 1640 medium, in the absence of autologous plasma (Figure 1A). Occasionally (in two out of 11 experiments), we found a much greater enhancement of cADPR concentration, accounting for approx. 7-fold increases over the control value. Comparably high intracellular levels of the cyclic nucleotide were still present at 48 h of incubation (Figure 1A). When autologous plasma was diluted 1:1 with PBS or with RPMI 1640 medium, identical results were obtained.

cADPR is synthesized from NAD^+ by ADP-ribosyl cyclase activities (see below). Thus we also measured the intracellular NAD^+ concentration in PBMCs stimulated with LPS under the same conditions reported in the legend to Figure 1. PBMCs were resuspended in the presence of autologous plasma and were treated for 24 or 48 h with LPS. Non-adherent PBMCs were collected and the NAD^+ content was determined: the intracellular NAD^+ concentration of control cells (0.066 ± 0.021 nmol/10^6 cells; $n = 11$) was significantly increased in LPS-stimulated cells (212 ± 45 %, $n = 7$, and 253 ± 70 %, $n = 4$, of control values at 24 and 48 h respectively).

Our PBMC preparations proved to contain less than 3 % granulocytes. Nevertheless, in an attempt to exclude that the cADPR increase could in fact occur only in a small contaminating fraction of granulocytes, we isolated (with a Ficoll-Paque Plus centrifugation method followed by removal of erythrocytes with the lysing buffer, see the Materials and methods section) and stimulated them with LPS for 24 h. No increase in cADPR concentration was detectable in the granulocyte fraction challenged with LPS (results not shown).

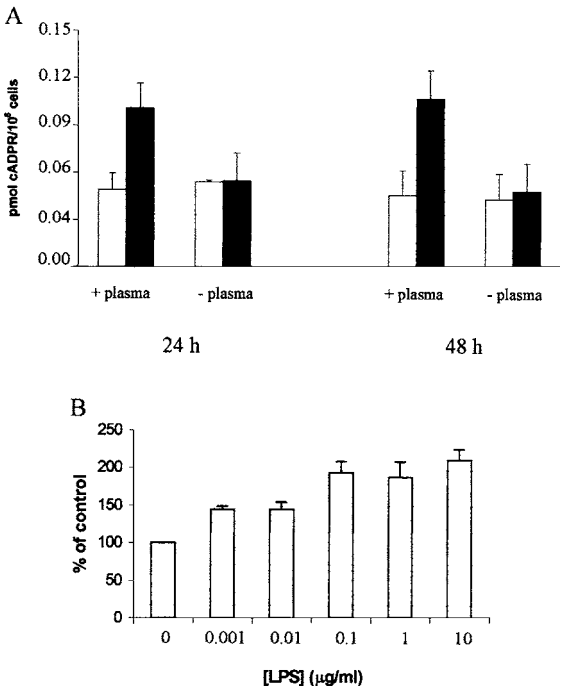

Figure 1 Intracellular cADPR increases in LPS-stimulated PBMCs

PBMCs were isolated and cultured at 3×10^6 cells/ml as described in the Materials and methods section. (**A**) After 24 or 48 h of incubation in the absence (white bars) or in the presence of 0.1 μg/ml LPS (black bars), non-adherent PBMCs were collected. Results are means $\pm$ S.D. of nine experiments on nine different donors. Each measurement was performed in triplicate. (**B**) Cells were stimulated for 24 h with LPS at the concentrations indicated and non-adherent PBMCs were collected. Intracellular cADPR content in the recovered PBMCs was determined by the cycling assay procedure [23]. Results are expressed as percentage values of cADPR content in control, non-stimulated, PBMCs (0.045 ± 0.09 pmol/10^6 cells).

Next, PBMCs were treated with different amounts of LPS, in order to explore the concentration-dependence of the cADPR increases. However, as shown in Figure 1(B), an LPS concentration as low as 1 ng/ml was sufficient to trigger a significant ($P < 0.01$) increase of intracellular cADPR. Such increases were found to level off from 0.1 to 10 μg/ml (Figure 1B). Similarly, intracellular NAD^+ content was affected by low concentrations of LPS (1 ng/ml), and the same extent of increase was observed for concentrations from 0.1 to 10 μg/ml. Comparable results for both cADPR and NAD^+ contents were obtained using a different LPS serotype (055:B5).

Role of monocytes and CD14 in LPS-induced increase of intracellular cADPR

Since the LPS-dependent cellular response of PBMCs is known to be regulated by the expression of CD14 in monocytes [26–31], a number of experiments were designed to investigate the role of these cells on the cADPR increases elicited by LPS stimulation. First, after removing adherent PBMCs (a common way to remove monocytes, as routinely tested in our experiments [21,22]), we recovered and stimulated the non-adherent cells with LPS for 24 h. Under these conditions, the cADPR increase was markedly

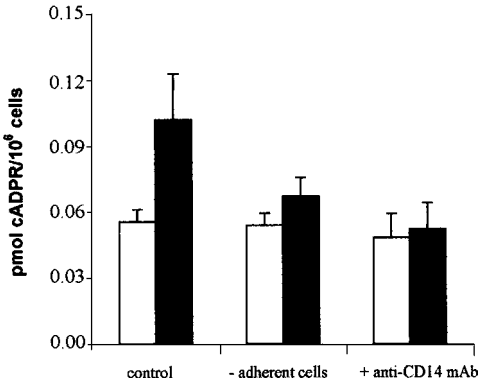

Figure 2 Effect of monocyte depletion and of anti-CD14 antibody on the LPS-induced cADPR increases in PBMCs

PBMCs were isolated, cultured at 3×10^6 cells/ml in the presence of autologous plasma and incubated for 1 h in the absence (control) or presence of 10 μg/ml anti-CD14 mAb. In the last experiments, an isotype-matched mAb was used in the same conditions, yielding results superimposable on those of the controls (results not shown). PBMCs were then stimulated with 0.1 μg/ml LPS for 24 h. In parallel, isolated PBMCs were cultured for 2 h in plastic flasks and then non-adherent PBMCs were carefully recovered and stimulated with 0.1 μg/ml LPS for 24 h. Intracellular cADPR content was determined by the cycling assay [23] on non-adherent PBMCs at the end of incubation: white bars, unstimulated cells; black bars, LPS-stimulated cells. Results are means $\pm$ S.D. of four experiments on four different donors. Each measurement was performed in triplicate.

restrained as compared with that recorded in the presence of monocytes (Figure 2). The small increase of cADPR content in LPS-stimulated non-adherent PBMC over the corresponding unstimulated control PBMCs was not statistically significant ($P = 0.26$). On the contrary, the cADPR content of LPS-stimulated non-adherent PBMCs was significantly higher ($P < 0.05$) when stimulation was performed on the complete PBMC population (control conditions) than was observed in equally LPS-primed, monocyte-depleted PBMCs (Figure 2).

In another set of experiments, we evaluated the cADPR concentrations in PBMCs after LPS stimulation in the presence of a specific mAb directed against the monocyte marker CD14 [31]. Co-incubation with the neutralizing anti-CD14 mAb, but not with an isotype-matched mAb (see the Materials and methods section) inhibited the LPS-induced cADPR increases in non-adherent PBMCs almost completely (Figure 2).

Next, the intracellular cADPR concentrations were measured in non-adherent PBMCs co-incubated with separately LPS-primed monocytes. Adherent PBMCs were first incubated for various times (0.5, 1 and 4 h) with LPS. Then, unbound LPS was removed by washing the adherent cells, and non-adherent cells were replaced and co-incubated for a further 24 h. As a positive control for the stimulatory effect of primed monocytes, cADPR levels were also measured in unfractionated PBMCs, following their incubation with LPS for 24 h. As shown in Figure 3(A), LPS priming of the monocyte fraction for 30 min elicited the same extent of cADPR increase in the PBMCs as in the complete mixture. Since a 9 h incubation of unfractionated PBMCs with LPS did not result in any increase in cADPR levels in non-adherent cells (see above), but 30 min of monocyte priming was sufficient to enhance intracellular cADPR in the co-incubated non-adherent cells, it is possible that the briefly primed monocytes need to interact for as long as 24 h with the non-adherent cells to cause a cADPR increase therein. Thus we investigated the time-course of cADPR levels in non-adherent cells following their co-incubation with monocytes stimulated identically for 30 min with LPS. As illustrated in Figure 3(B), cADPR content of non-adherent cells was found to be unchanged after 1 and 9 h of co-

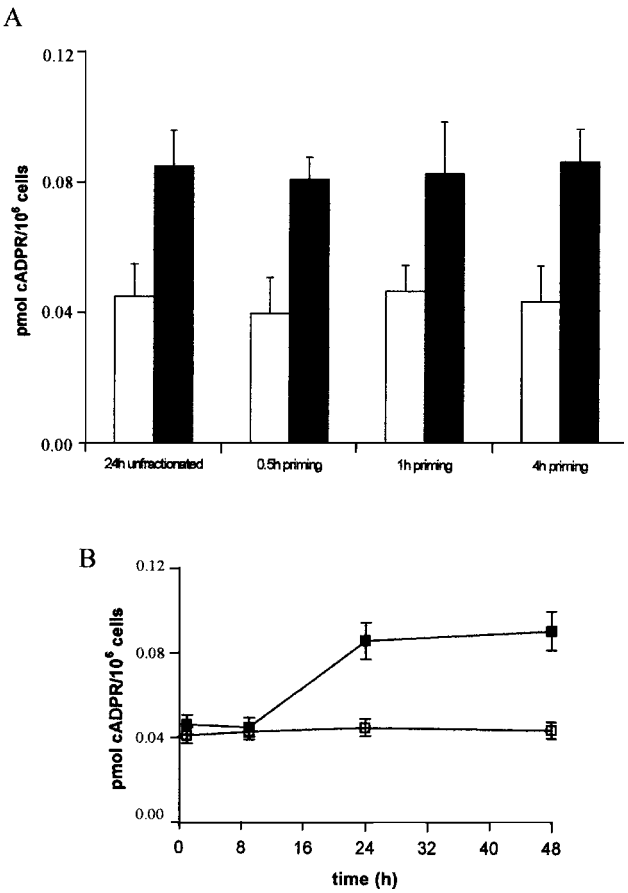

Figure 3 Relationship between LPS priming of isolated monocytes and cADPR increases in non-adherent PBMCs

Isolated PBMCs were cultured (3×10^6 cells/ml) for 2 h in plastic flasks. (**A**) Adherent cells were incubated for 0.5, 1 and 4 h with (black bars) or without (white bars) 0.1 μg/ml LPS. Unbound LPS was washed out, non-adherent cells were added to the adherent cells and were co-incubated for a further 24 h. Unfractionated cells were cultured in parallel and stimulated with LPS for 24 h as described in the Materials and methods section. Intracellular cADPR levels were determined by the cycling assay [23] in non-adherent PBMCs at the end of incubation. (**B**) Adherent cells were incubated for 30 min in the absence (control) or presence of 0.1 μg/ml LPS. Unbound LPS was removed by washing, and non-adherent cells were replaced and co-incubated for 1, 9 and 24 h. At the end of each time point, non-adherent cells were recovered and their intracellular cADPR content was determined by the cycling assay [23]. □, control; ■, incubated with LPS-primed monocytes. Results are means $\pm$ S.D. ($n = 3$).

incubation with LPS-primed monocytes, whereas it was significantly increased at 24 h and kept comparably high after 48 h.

ADP-ribosyl cyclase, GDP-ribosyl cyclase and cADPR-hydrolase activities in LPS-stimulated PBMCs

In several cell types, cADPR is synthesized from NAD^+ by a number of ADP-ribosyl cyclases, some of which [e.g. those associated with CD38 and BST-1 (bone marrow stromal cell antigen 1)] feature also cADPR hydrolase activity [3,32]. Thus we investigated whether or not the increases of intracellular cADPR could be due to a higher level of ADP-ribosyl cyclase activity in LPS-stimulated PMBCs than in unstimulated PBMCs. Indeed, the ADP-ribosyl cyclase activity, measured upon adding NAD^+ as a substrate to total lysates from non-adherent PBMCs, was almost 2-fold higher in the PBMCs incubated with LPS for 24 h, as compared with unstimulated control cells (Table 1). This LPS-induced increase of ADP-ribosyl cyclase activity was abolished

Table 1 Effects of LPS stimulation and of CD14 neutralization on ADP-ribosyl cyclase and cADPR hydrolase activities of PBMCs

ADP-ribosyl cyclase and cADPR hydrolase activities were determined on total cell lysates, as described in the Materials and methods section. Results are means ± S.D. ($n = 3$).

PBMCs	ADP-ribosyl cyclase (pmol of cADPR/min per mg)	cADPR hydrolase (nmol of ADPR/min per mg)
Unstimulated	38.21 ± 1.37	0.540 ± 0.070
LPS-stimulated	71.07 ± 5.03 ($P < 0.001$)	0.964 ± 0.205
+ anti-CD14 mAb	38.94 ± 1.40	0.562 ± 0.053
+ anti-CD14 mAb + LPS	40.80 ± 2.89	0.621 ± 0.081
+ non-specific mAb	41.44 ± 3.15	0.561 ± 0.055
+ non-specific mAb + LPS	67.43 ± 6.09 ($P < 0.01$)	0.915 ± 0.111

by co-incubation of PBMCs with the blocking anti-CD14 antibody (Table 1), in agreement with data shown in Figure 2. Bifunctionality of CD38 and BST-1 prompted us to measure cADPR hydrolase activity in lysates from control and LPS-stimulated cells as well. The cADPR-degrading activity of lysates from LPS-treated PBMCs was enhanced up to approx. 180 % of control values, in accordance with the corresponding increase of the cyclase activity, and the increase was likewise blocked by the anti-CD14 mAb (Table 1). A non-specific mAb, used as negative control (see the Materials and methods section), did not affect the cyclase and the hydrolase activities of control cells and did not inhibit the LPS-induced increase of both enzymic activities (Table 1).

In addition to membrane-bound ADP-ribosyl cyclases (CD38 and BST-1), a soluble cyclase, featuring activity on NAD^+, but not on the substrate analogue NGD^+, has been reported in Jurkat cells [13]. This suggested a comparative investigation of the substrate specificity of ADP-ribosyl cyclase(s) in intact cells (i.e. the ectocellular activity related to CD38 and BST-1), in the cytosol (S100) and in the total membrane fraction (P100) from both unstimulated (control) and LPS-stimulated PBMCs. As shown in Table 2, the membrane-bound ADP-ribosyl cyclase activity was enhanced in LPS-stimulated cells compared with unstimulated cells. This was demonstrated by assays on intact cells (from 29.55 ± 4.42 to 48.30 ± 9.66 pmol of cADPR/min per mg of protein) and on the isolated membrane fraction as well (from 265.59 ± 48.18 to 395.68 ± 46.92 pmol of cADPR/min per mg of protein). Moreover, we were able to detect and measure an ADP-ribosyl cyclase activity also in the cytosolic fraction (10.51 ± 1.37 pmol of cADPR/min per mg of protein) that increased upon LPS stimulation (up to 15.32 ± 1.73 pmol of cADPR/min per mg of protein; $P < 0.001$). This LPS-dependent enhancement of cytosolic ADP-ribosyl cyclase activity was completely prevented by co-incubating the total PBMCs with the blocking anti-CD14 mAb, but not with the isotype-matched non-specific mAb (results not shown), as previously observed in total cell lysates (Table 1). The cytosolic cyclase did not recognize NGD^+ as a substrate (Table 2). Interestingly, GDP-ribosyl cyclase activity, measured on intact cells, on total cell lysates, and on membrane fractions as well, was remarkably enhanced in PBMCs following LPS stimulation (Table 2), in agreement with corresponding variations of ADP-ribosyl cyclase activity. The failure to detect a cytosolic GDP-ribosyl cyclase activity (see above) rules out any possible membrane contamination as being responsible for the cytosolic ADP-ribosyl cyclase, which therefore proves to be an endogenous and specific enzyme activity, as reported for Jurkat cells [13]. Moreover, absence of measurable cGDPR production from NGD^+ in the cytosolic fraction is not due to a limited sensitivity of our HPLC analyses: indeed, if cGDPR were produced at the same level as cADPR in the cytosolic fraction in comparison with the membrane fraction, cGDPR would be easily detectable. Since the cytosolic ADP-ribosyl cyclase is devoid of any activity on NGD^+, we investigated whether this dinucleotide has any effect on the cytosolic cyclase activity on NAD^+. Thus we performed experiments of NAD^+ saturation in the presence of NGD^+ on both membrane and cytosolic fractions. Rather surprisingly, NGD^+ also inhibited the cytosolic ADP-ribosyl cyclase, but according to a different mechanism. As shown in Figure 4, NGD^+ behaved as a competitive inhibitor of the membrane-bound cyclase ($K_i = 5.4 \pm 0.4\ \mu M$) and as a non-competitive inhibitor of the cytosolic activity ($K_i = 2.0 \pm 0.3\ \mu M$).

High levels of ADP-ribosyl and GDP-ribosyl cyclase activities, comparable in extent with those found after 24 h of treatment with LPS, were still maintained at 48 h of incubation with LPS (results not shown), in agreement with the correspondingly delayed persistence of high levels of intracellular cADPR (Figure 1A).

Increased $[Ca^{2+}]_i$ in LPS-stimulated PBMCs

The LPS-induced increases of intracellular cADPR concentration in non-adherent PBMCs prompted us to analyse $[Ca^{2+}]_i$ in the same experimental conditions. As shown in Table 3, stimulation of PBMCs with LPS resulted in mean $[Ca^{2+}]_i$ increases from 32 ± 3 to 72 ± 5 nM (24 h), and from 28 ± 4 to 60 ± 7 (48 h). These $[Ca^{2+}]_i$ in control cells are comparable with those measured previously in similar cell preparations (cord blood-derived mononuclear cells [10,11]). In an earlier and independent study [33], a slightly lower $[Ca^{2+}]_i$ increase had been reported to occur in LPS-stimulated (12 h) PBMCs.

That the enhanced $[Ca^{2+}]_i$ following stimulation with LPS was due to Ca^{2+} release from intracellular stores was demonstrated

Table 2 Substrate specificity and activity levels of cyclases in various cell compartments

ADP-ribosyl and GDP-ribosyl cyclase activities were determined on intact cells, on total cell lysates and on membrane (P100) and cytosolic (S100) fractions as described in the Materials and methods section. Results are means ± S.D. ($n = 7$).

Cells or cell fractions	ADP-ribosyl cyclase (pmol of cADPR/min per mg)		GDP-ribosyl cyclase (nmol of cGDPR/min per mg)	
	Unstimulated	LPS-stimulated	Unstimulated	LPS-stimulated
Intact cells	29.55 ± 4.42	48.30 ± 9.66*	1.01 ± 0.11	1.88 ± 0.15†
Total cell lysate	42.86 ± 9.02	72.86 ± 13.93*	1.10 ± 0.24	1.89 ± 0.31*
Membranes	265.59 ± 48.18	395.68 ± 46.92*	3.41 ± 0.52	6.04 ± 1.18*
Cytosol	10.51 ± 1.37	15.32 ± 1.73†	ND‡	ND‡

* $P < 0.01$.
† $P < 0.001$.
‡ ND, not detectable.

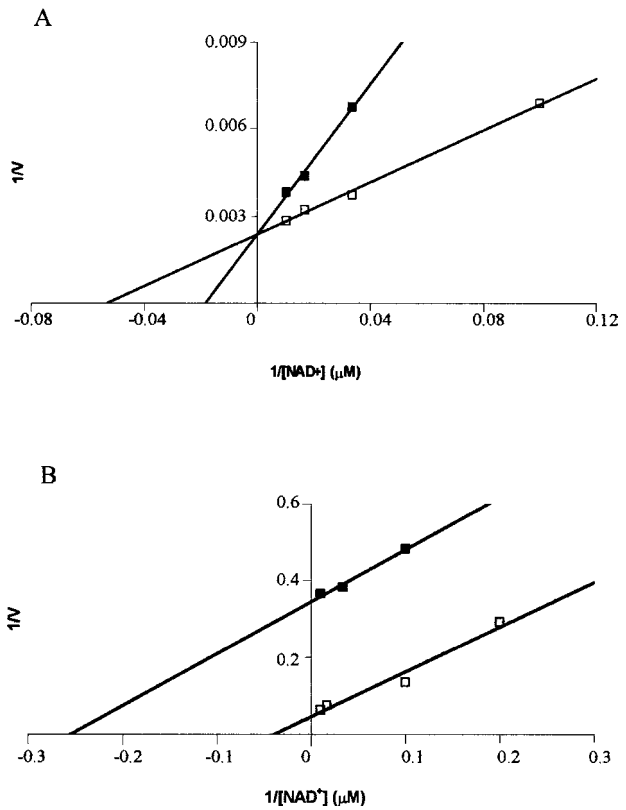

Figure 4 Distinctive mechanisms of inhibition of membrane-bound and cytosolic ADP-ribosyl cyclase activities by NGD$^+$

NAD^+ saturation curves were generated with membrane-bound cyclase activity (P100; see the Materials and methods section) (**A**) and with cytosolic cyclase activity (S100) (**B**), in the presence (■) or absence (□) of 10 μM NGD^+. Results are means ± S.D. ($n = 3$).

Table 3 Effects of 8-Br-cADPR and of ryanodine on $[Ca^{2+}]_i$ of LPS-stimulated PBMCs

$[Ca^{2+}]_i$ was determined as described in the Materials and methods section. Results are means ± S.D. ($n = 3$).

	$[Ca^{2+}]_i$ (nM)	
PBMCs incubated for . . .	24 h	48 h
Unstimulated	32 ± 3	28 ± 4
LPS-stimulated	72 ± 5 ($P < 0.01$)	60 ± 7
+ 8-Br-cADPR	25 ± 3	24 ± 4
+ 8-Br-cADPR + LPS	26 ± 3	29 ± 4
+ ryanodine	14 ± 3	12 ± 2
+ ryanodine + LPS	22 ± 4	25 ± 3

by experiments with thapsigargin (Figure 5). Thus the LPS-stimulated PBMCs showed diminished increases in $[Ca^{2+}]_i$ induced by 10 μM thapsigargin, in comparison with the unstimulated cells.

In an attempt to correlate the changes in $[Ca^{2+}]_i$ with the increased concentrations of intracellular cADPR, we incubated cells in the presence or absence of 50 μM 8-Br-cADPR, a well-known cADPR antagonist [34], and in the presence or absence of 50 μM ryanodine, a concentration that shows inhibitory effects on cADPR-sensitive calcium stores [3]. Both 8-Br-cADPR and

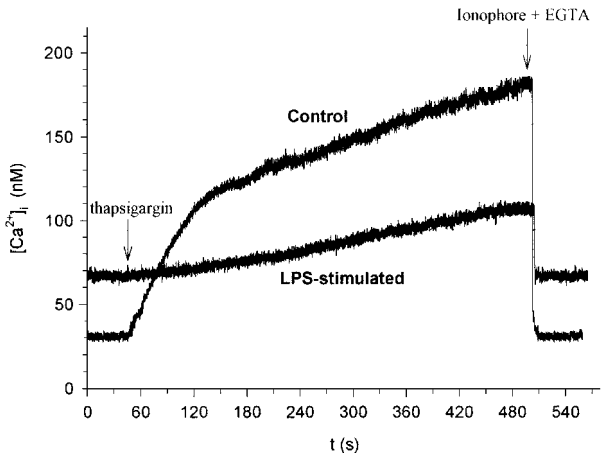

Figure 5 Thapsigargin-releasable Ca^{2+} in LPS-stimulated PBMCs

PBMCs were isolated and cultured at 3×10^6 cells/ml in the presence of autologous plasma, with or without 0.1 μg/ml LPS. After 24 h, cells were loaded with Fura 2 acetoxymethyl ester. Control and LPS-stimulated cells were challenged with 10 μM thapsigargin in a calcium-free solution (see the Materials and methods section) as indicated by the first arrow. The second arrow indicates the addition of 5 μM ionophore (A23187) and 5 mM EGTA. Traces are representative of three different experiments.

ryanodine completely prevented the LPS-induced $[Ca^{2+}]_i$ increase (Table 3), indicating a causal role of cADPR in up-regulating $[Ca^{2+}]_i$ following stimulation of PBMCs with LPS.

Role of cADPR in LPS-induced PBMC proliferation

Stimulation of PBMCs with LPS has been reported to result in cell proliferation. Only T-lymphocytes were reported to represent the proliferating cell population after stimulation with LPS, whereas B-lymphocytes, natural killer (NK) cells and monocytes were unresponsive to LPS stimulation [19,35]. Moreover, a role for cADPR in the proliferation of T-cells has been demonstrated following stimulation of the TCR–CD3 complex [13]. Therefore, in an attempt to correlate PBMC proliferation with the recorded increases in cADPR concentrations and in $[Ca^{2+}]_i$ that follow priming with LPS, we performed experiments of [^{3}H]TdR incorporation in the presence or absence of 8-Br-cADPR, in the presence and absence of nicotinamide, an inhibitor of the membrane-bound ADP- and GDP-ribosyl cyclase activities [36], and in the presence and absence of ryanodine. Before these experiments, the effect of nicotinamide on the cytosolic ADP-ribosyl cyclase activity was also investigated: with 10 mM nicotinamide, the soluble ADP-ribosyl cyclase activity was inhibited by 90 % (from 10.06 ± 0.99 to 0.89 ± 0.21 pmol of cADPR/min per mg of protein). After 72 h of incubation with LPS, [^{3}H]TdR incorporation was decreased to approx. 30 % by 50 μM 8-Br-cADPR as compared with the control (Table 4). Incubation in the presence of either 20 mM nicotinamide or 50 μM ryanodine completely prevented the effect of LPS on [^{3}H]TdR incorporation (Table 4).

DISCUSSION

LPS released from Gram-negative bacteria is responsible for many adverse reactions including fever, hypotension, tachypnoea, agranulocytaemia, disseminated intravascular coagulation and failure of several parenchymatous organs [37]. The heterogeneity

Table 4 Effects of 8-Br-cADPR, nicotinamide and ryanodine on LPS-induced proliferation of PBMCs

Proliferation of PBMCs was determined by means of [^{3}H]TdR incorporation as described in the Materials and methods section. Results, expressed as c.p.m., are means ± S.D. ($n = 4$).

PBMCs	Incorporation of [^{3}H]TdR (72 h)
Unstimulated	1288 ± 100
LPS-stimulated	2146 ± 132 ($P < 0.005$)
+ 8-Br-cADPR	1200 ± 100
+ 8-Br-cADPR + LPS	1500 ± 191
+ nicotinamide	1266 ± 91
+ nicotinamide + LPS	1207 ± 29
+ ryanodine	1390 ± 62
+ ryanodine + LPS	1236 ± 174

of these effects stems from the many cell types that are responsive to LPS either directly or indirectly. In the latter case, multiple autocrine and paracrine loops have been identified where distinct cytokines and contact mechanisms contribute to a wide range of effects [19,30,31,38–42]. Dissecting the mechanisms that ultimately result in many pathophysiological reactions to LPS is a major goal of molecular studies on infectious diseases.

More recently, a new complex process has been reported whereby LPS is able to induce human T-lymphocyte proliferation and cytokine production [19]. LPS is non-mitogenic on purified human T-cells nor is it competent to affect $[Ca^{2+}]_i$ in these cells [20]. This specific activation of T-lymphocytes is in fact mediated by accessory cells including monocytes and $CD34^+$ haematopoietic stem cells [19]. In the case of monocyte–lymphocyte interactions, LPS triggers both direct cell-to-cell contact, e.g. CD28/B7 binding, and release of soluble cytokines, e.g. IL-12 [27]. Several lines of evidence indicate that this major histocompatibility complex (MHC)-unrestricted activation of T-lymphocytes, which qualifies as an antimicrobial innate immune response [43], occurs via mechanisms other than those elicited by mitogens, superantigens and antigens.

The findings of enhanced calcium-mediated cell proliferation afforded by cADPR in different cell types [9–11], including T-lymphocytes [13], suggested an investigation of whether or not this cyclic nucleotide is involved in the monocyte-mediated activation of T-lymphocytes by LPS. This proved to be the case because human PBMC preparations were found to respond to LPS concentrations as low as 1 ng/ml, with significant increases in intracellular cADPR taking place after 24 h. These increases were paralleled by two changes in the same direction that are closely related to cADPR metabolism: (i) remarkably higher intracellular concentrations of NAD^+, suggesting regulation of cADPR synthesis at the substrate level, as previously shown in different cell systems [44]; and (ii) significantly higher levels of ADP-ribosyl cyclase activity, consistent with its involvement in up-regulating intracellular cADPR generation (see below). Thus enhanced cADPR synthesis as a consequence of LPS stimulation of monocytes probably reflects corresponding variations of both ADP-ribosyl cyclase activity and its substrate.

The increases of intracellular cADPR in PBMCs following their stimulation with LPS proved to be responsible for the observed up-regulation of $[Ca^{2+}]_i$ that occurs in this condition (Table 3). This was also indicated by the remarkably diminished size of thapsigargin-sensitive calcium stores that characterizes the LPS-stimulated PBMCs as compared with the unstimulated control cells (Figure 5). Persistence of these cADPR-mediated increases in $[Ca^{2+}]_i$ over many hours is in agreement with the earlier observation that *de novo* appearance of intracellular cADPR in CD38-transfected 3T3 fibroblasts and HeLa cells resulted in the stable doubling of $[Ca^{2+}]_i$ [9]. Moreover, the increase of intracellular cADPR in cord blood-derived mononuclear cells, either obtained by pre-loading with extracellular cADPR or by co-culture over cADPR-generating stromal cell lines, resulted in long-lasting increases in $[Ca^{2+}]_i$ [10,11]. Therefore, a causal correlation between the up-regulation of ADP-ribosyl cyclase, increased intracellular cADPR concentrations and enhanced $[Ca^{2+}]_i$, all changes occurring on a time-scale of hours/days, is a well-documented finding that received further confirmation in the present study.

The effects of LPS priming of monocytes on metabolism of cADPR in PBMCs require the presence of autologous plasma. Interestingly, LPS-binding proteins (LBPs) present in plasma have been shown to favour the interaction of LPS with the monocyte marker CD14 and LBPs proved to be necessary at LPS concentrations below 0.1 μg/ml [45]. Also, since most of the effects reported in the present study require culturing PBMCs for at least a short period of time, serum is necessary to preserve cell viability [46].

The cADPR increases in LPS-stimulated non-adherent PBMCs were monocyte- and CD14-dependent (Figure 2), as previously reported for the activation of T-lymphocytes by LPS [26–31]. Another characteristic feature of this system was that a 30 min priming of monocytes with LPS was sufficient to elicit the observed increases in cADPR concentration. Mattern et al. [35,47] reported that LPS stimulation of PBMCs was highly donor-dependent: only 50 % of the healthy donors they tested being responsive in terms of T-cell proliferation. This was not the case for the LPS-induced increases in intracellular cADPR in PBMCs, which were actually recorded in all of our experiments. Accordingly, the variability observed by Mattern et al. [35,47] seems to be due to some step(s) downstream of cADPR generation occurring in the effector T-lymphocytes as a consequence of their interaction with the LPS-primed monocytes.

It is worth noting that LPS binding and its consequent signalling effects are exceedingly complex [43]. Evidence has emerged that the glycosylphosphoinositide-anchored protein CD14 must interact with the transmembrane receptors triggering signal transduction, e.g. integrins CD11c, Toll-like receptors (TLRs, and especially TLR4), CD55 [43,48]. A comparable extent of complexity characterizes the intracellular interactions of the cytoplasmic domain of the TLRs with proteins including MyD88, IL-1 receptor-associated kinase family members and Toll-interacting protein, with the resulting activation of a unique signalling module [49]. Subsequently, other signalling pathways are activated downstream, including NF-κB (nuclear factor κB), c-Jun N-terminal kinase and p38 MAPKs (mitogen-activated protein kinases) [43,49].

Whatever the mechanisms, the present results demonstrate that cADPR in T-lymphocytes has a role of second messenger in the signalling cascade initiated by the LPS–monocyte interaction, involving enhanced $[Ca^{2+}]_i$ and eventually up-regulating T-cell proliferation.

The long time interval (24 h) required for the observed effects to take place is suggestive of some variations in the turnover of ADP-ribosyl cyclase(s). A clear correlation between ADP-ribosyl cyclase expression and intracellular cADPR concentration has already been demonstrated in different cell lines: Zocchi et al. [9] showed that CD38 transfection in $CD38^-$ cells leads to the appearance of intracellular cADPR. Munshi et al. [36] demonstrated an unequivocal correlation between the extent of CD38 expression in retinoic-acid-stimulated HL60 cells and intracellular cADPR levels. Moreover, Guse et al. [13] reported that an enhancement, upon stimulation of the TCR–CD3 complex, of ADP-ribosyl

cyclase activity in Jurkat cells is followed by an increased amount of intracellular cADPR. As far as ADP-ribosyl cyclase activities in PBMCs are concerned, it is worth noting that, in addition to membrane-bound cyclase activity, a cytosolic, nicotinamide-inhibitable ADP-ribosyl cyclase devoid of any activity on the substrate analogue NGD^+ was measurable in non-adherent PBMCs. This finding confirms the earlier report of a soluble ADP-ribosyl cyclase in Jurkat cells [13].

Both the membrane-bound and the cytosolic ADP-ribosyl cyclase activities of non-adherent PBMCs were increased significantly following interaction with LPS-stimulated monocytes in the presence of plasma (Table 2). Therefore both enzyme forms might in principle be responsible for the observed increases of intracellular cADPR levels. Recently, the functioning of vesicle-bound CD38 in generating functionally active, intracellular cADPR has been challenged and shown to be feedback-inhibited by high cytosolic $[Ca^{2+}]_i$ via a PKC-mediated phosphorylation of connexin 43 hemichannels [50]. The consequent closure of these specific hemichannels, which are competent for equilibrative NAD^+ transport [51], restricts the availability of NAD^+ to intravesicular CD38, thereby down-regulating subcellular conversion of NAD^+ into cADPR. The cytosolic ADP-ribosyl cyclase detected in T-lymphocytes, although potentially regulated via other mechanisms, is certainly not restrained by these compartmentalization processes. Accordingly, the soluble enzyme form is a reasonable candidate for synthesizing higher amounts of intracellular cADPR as a response to the interaction with LPS-primed monocytes.

The last step of the signalling cascade initiated by LPS-stimulated monocytes is T-lymphocyte proliferation. Susceptibility of enhanced PBMC proliferation to inhibition by the cADPR antagonist 8-Br-cADPR [34], by the cyclase inhibitor nicotinamide and by ryanodine (Table 4) clearly indicates the role of second messenger played by cADPR in this cascade of events. The possibility of 8-Br-cADPR counteracting the effects of LPS stimulation within monocytes rather than in T-lymphocytes seems to be quite unlikely. In fact, LPS does not affect CD38 expression in monocytes [52], nor is any evidence available for an effect of cADPR in LPS stimulation on monocytes.

Our present findings are in agreement with the previous report of a causal role of cADPR in the T-cell activation elicited by the agonistic anti-CD3 antibody, OKT3 [13]. Thus cADPR turns out to represent a second messenger for calcium-mediated up-regulation of human T-cell proliferation whose intracellular generation is activated by more than one agonistic stimulus.

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