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Cyclic ADP-Ribose Mediates Formyl Methionyl Leucyl Phenylalanine (fMLP)-Induced Intracellular Ca^{2+} Rise and Migration of Human Neutrophils

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Abstract. Although cyclic ADP-ribose (cADPR), a novel Ca^{2+}-mobilizing mediator, is suggested to be involved in the functions of neutrophils in rodents, its role in human neutrophils remains unclear. The present study examined the ability of cADPR to mobilize Ca^{2+} and mediate formyl methionyl leucyl phenylalanine (fMLP)-stimulated increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and migration in human neutrophils. cADPR induced Ca^{2+} release from digitonin-permeabilized neutrophils, and the release was blocked by 8Br-cADPR, an antagonist of cADPR. Immunophilin ligands, FK506 and rapamycin, but not cyclosporine A, inhibited cADPR-induced Ca^{2+} release. 8Br-cADPR partially reduced fMLP-induced $[Ca^{2+}]_i$ rise and abolished the rise in combination with 2APB, an IP_3-receptor antagonist. Anti-CD38Ab and NADase that interfere with cADPR formation, reduced the fMLP-induced $[Ca^{2+}]_i$ rise. When β-NAD^+, a substrate of ADP-ribosyl cyclase, and cADPR were added to the medium, the former gradually increased $[Ca^{2+}]_i$ and the latter potentiated the fMLP-induced $[Ca^{2+}]_i$ rise. The β-NAD^+–induced $[Ca^{2+}]_i$ rise in Ca^{2+}-free medium was inhibited by anti-CD38Ab, 8Br-cADPR, FK506, ruthenium red, and thapsigargin. mRNAs of nucleoside transporter (NT), ENT1, ENT2, CNT, and CNT3 were expressed in neutrophils; and their inhibitors, inosine, uridine, and *s*-(4-nitrobenzyl)-6-thioinosine, reduced the $[Ca^{2+}]_i$ rise induced by β-NAD^+ and fMLP. fMLP-stimulated migration was inhibited by the removal of Ca^{2+} from the medium or by the addition of 8Br-cADPR, anti-CD38Ab, NADase, and NT inhibitors. These results suggest that cADPR was synthesized extracellularly by CD38, transported into the cells through NTs, and then Ca^{2+} was mobilized by FK506-binding protein–dependent process. This process may be involved in fMLP-induced intracellular Ca^{2+} signaling and migration in human neutrophils.

Keywords: cyclic ADP-ribose (cADPR), FK506-binding protein (FKBP), cytosolic Ca^{2+} dynamics, nucleoside transporter, migration

Introduction

An increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key signal for neutrophil functions such as migration and reactive oxygen species generation. Inositol 1,4,5-trisphosphate (IP_3) is a Ca^{2+}-mobilizing second messenger, mediating transducing signals from a formyl peptide receptor, G protein–coupled receptor, for a chemoattractant ligand, formyl methionyl leucyl phenylalanine (fMLP), into cellular responses. It has been reported that neutrophils from mice deficient in phospholipase C/IP_3 function retain the $[Ca^{2+}]_i$ signal and migration activity to fMLP, and thus mechanisms other than IP_3-mediated changes in $[Ca^{2+}]_i$ are related to neutrophil functions (1).

Cyclic ADP-ribose (cADPR) is postulated as another potent Ca^{2+} mobilizer through an IP_3-insensitive, ryanodine receptor (RyR)-mediated mechanism in many

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tissues (2). Evidence shows that cADPR directly activates the RyR channel by stimulating [^{3}H]ryanodine binding (3 – 6). Others showed an indirect mode of action of cADPR with the requirement of accessory protein FK506-binding proteins (FKBPs) that bind tightly to RyRs in pancreatic islet cells (7), reconstituted RyRs from arterial smooth muscle (8), and bovine adrenal chromaffin cells (9) or without the requirement of FKBPs in sea urchin eggs and rat pancreatic acinar cells (10, 11).

cADPR induced Ca^{2+} release in digitonin-permeabilized rabbit neutrophils (12). Evidence suggests that cADPR may function as a second messenger for the regulation of $[Ca^{2+}]_i$ in response to chemoattractants in neutrophils. CD38 has a characterized mammalian ADP-ribosyl cyclase that catalyzes the conversion of β-NAD^+ into cADPR (13). Recently, Partida-Sánchez et al. (14) demonstrated that CD38-deficient mice are susceptible to bacterial infections due to the inability of neutrophils to direct migration. These results suggest that CD38/cADPR-mediated regulation of $[Ca^{2+}]_i$ is quite important for neutrophil functions; however, the role of cADPR as a mediator of intracellular Ca^{2+} signals and migration in human neutrophils is obscure, as seen in the following reports. 8Br-cADPR, a membrane permeant cADPR antagonist did not block either the Ca^{2+} signal or the chemotactic response towards formyl peptide fMLP in human neutrophils (14). Extracellular cADPR does not elicit a $[Ca^{2+}]_i$ rise, and it cannot control the fMLP-induced Ca^{2+} influx and Ca^{2+}-dependent biological response in differentiated human promyelocytic leukemia HL-60 cells that express CD38 (15).

Therefore, in order to clarify the role of cADPR in the mediation of fMLP-induced $[Ca^{2+}]_i$ rise and migration in human neutrophils, the following points were examined in this study: 1) the ability of cADPR to release Ca^{2+} from permeabilized human neutrophils and the requirement of FKBPs for the action, 2) participation of cADPR in $[Ca^{2+}]_i$ dynamics in response to fMLP in intact neutrophils, 3) the role of CD38 in extracellular formation of cADPR and transportation of cADPR through plasma membrane into cells in intact neutrophils, and 4) involvement of the CD38/cADPR system in migrations in response to fMLP.

Materials and Methods

Materials

The following reagents were obtained as indicated: aprotinine and ruthenium red were from CalbioChem Biochemicals (La Jolla, CA, USA); antimycin A and sodium azide (NaN_3) were from MERCK (Frankfurter, Germany); cyclic adenosine diphosphate-ribose (cADPR), creatine phosphate, creatine phosphokinase from bovine heart, cyclosporin A, digitonin, inosine, rapamycin, thapsigargin, and uridine were from Wako Pure Chemicals Industries (Osaka); 8Br-cADPR, benzamidine, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), NAD glycohydrolase (NADase, N 9629), monoclonal human anti-CD38 antibody clone H157 (C 1586), *s*-(4-nitrobenzyl)-6-thioinosine (NBMPR), oligomycin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA); leupeptine was from Peptide Institute, Inc., (Osaka); nicotineamide adenine dinucleotide (β-NAD^+) was from Roche Diagnostics GmbH (Germany); RPMI 1640 was from GIBCO BRL (Grand Island, NY, USA); bis[*N,N*-bis(carboxymethyl)aminomethyl] fluorescein acetoxymethyl ester (calcein-AM) fura-2 and fura-2 acetoxymetylester were from Dojindo Laboratories (Kumamoto); 2-aminoethoxydiphenylborane (2APB) was from TOCRIS Bioscience (Bristol, UK); FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka); all other chemicals were reagent grade. 2APB, thapsigargin, and NBMPR were dissolved in dimethyl sulfoxide to make stock solutions. Antimycin A, cyclosporin A, FK506, rapamycin, and oligomycin were dissolved in ethanol to make stock solutions. Dimethyl sulfoxide (0.1%) or ethanol (0.1%) at the highest final concentration used had no effect on $[Ca^{2+}]_i$ or migration, respectively (data not shown).

Neutrophil preparation

Studies were performed in compliance with the Institutional Review Board of Hiroshima University. Informed consent was obtained for blood withdrawal from healthy volunteers. Human neutrophils were isolated from the blood immediately after venipuncture, using sodium citrate as an anticoagulant. Neutrophils were isolated from whole blood by a one-step purification procedure using Polymorphoprep gradient solution (AXIS-SHIELD PoC AS, Oslo, Norway). The neutrophil fraction was collected, and the osmolarity was adjusted. Neutrophils were then washed and resuspended in PBS. Contaminating erythrocytes were separated by hypotoniclysis with 0.2% sodium chloride for 30 s on ice. The cells were resuspended in RPMI culture medium without supplements and used for experiments immediately after isolation. No neutrophil aggregation (i.e., the hallmark for neutrophil activation) was observed using this isolation procedure. Cell viability was monitored by trypan blue exclusion and purity (>98%) was verified by cytology from cytocentrifuged preparations colored by Diff-Quick staining. Cell viability was systematically evaluated before and after each treatment.

RT-PCR analysis

To analyze the expression of FKBPs, RyRs and nucleoside transporters, total RNA, and then cDNA were prepared from purified human neutrophils and spinal cord using TRIzol RNA extraction and RT-PCR kits, respectively.

The neutrophils were quickly lysed by the addition of TRIzol® reagent (Life Technologies, Rockville, MD, USA). Total RNA was isolated according to the manufacturer's instructions.

Oligo(dT)-primed first-strand cDNA synthesis was performed with a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer Mannheim) using 10 μg of total RNA as a template in a total volume of 20 μl. Reaction conditions were as described by the manufacturer. Negative controls were performed by omission of the reverse transcription step or by exclusion of the template from the PCR. The reaction solution (20 μl) contained 0.5 μM primer (each for reverse and forward primers), 2 mM $MgCl_2$, 0.2 mM dNTP (each), and 0.5 units of Taq polymerase (Ampli Taq Gold; PerkinElmer, Roche Molecular System, Branchburry, NJ, USA) in 1 × PCR buffer II supplied by the manufacturer. As a template, 2 μl of total RNA solution or 2 μl of cDNA solution was used. The primers used in these experiments are indicated in Table 1. The cycling conditions were: 92°C for 2 min, followed by 35 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were separated by migration on a 1.6% (W/V) agarose gel and visualized by staining with ethidium bromide. Products were excised and purified using a QIAQuick™ gel extraction kit (QIAGEN GmbH, Germany) and subjected to restriction digest analysis and automated sequencing. PCR products corresponding to the published cDNA sequence were obtained.

Table 1. Summary of RT-PCR primers used in this study: oligonucleotide primers for analysis of the human nucleoside transporter, ryanadine receptor, and FKBP12/12.6 gene and amplification of those cDNAs

cDNA	Primer name	Primer set sequences (5' to 3')		EMBL and GenBank with assigned accession Nos	Primer positions (5' to 3')	PCR product size
hENT1	hENT1F	acatgtcccagaatgtgtcc	20 bp	NM_004955	309 – 328	666 bp
	hENT1R	attcctctttgcctgctcttgg	22 bp		974 – 953	
hENT2	hENT2F	tcatcaactccttcagtgcag	21 bp	AF029358	410 – 430	342 bp
	hENT2R	gaatcccgttctcatcagact	21 bp		751 – 731	
hCNT1	hCNT1F	tagccttcttgatgggtgtggcgt	24 bp	U62966	1599 – 1622	532 bp
	hCNT1R	gcacagatcgtgtggttgtaaaaccg	26 bp		2130 – 2105	
hCNT2	hCNT2F	gtccattgctctgtccacagtgg	23 bp	NM_004212	83 – 105	293 bp
	hCNT2R	ccagtgccctctggaaattc	20 bp		375 – 356	
hCNT3	hCNT3F	tgtaaacggtgtgcagcaat	20 bp	AF305210	1712 – 1731	302 bp
	hCNT3R	tcaccttggttgtgtttcca	20 bp		2013 – 1994	
hRyR1	hRyR1F	ctcagctgctggggatgtgtcaggtgc	27 bp	NM_000540	13915 – 13941	497 bp
	hRyR1R	ggtttgggcttgcgctcattgtgggctg	27 bp		14411 – 14385	
hRyR2	hRyR2F	gaatcagtgaattacttggcatgg	24 bp	NM_001035	14115 – 14138	636 bp
	hRyR2R	gttggtctcttagttctccaaaagc	25 bp		14750 – 14726	
hRyR3	hRyR3F	agaagagaccaaagcagaagc	21 bp	NM_001036	13831 – 13851	271 bp
	hRyR3R	caggagaccgacagtcagaac	21 bp		14101 – 14081	
hFKBP12	hFKBP12F	gccggatccatgggagtgcaggtggaaaccatc (BamH1 site)	33 bp	X52220	31 – 54	350 bp
	hFKBP12R	ggaggccagaattctcattccagttttagaagctccac (EcoR1 site)	38 bp		357 – 334	
hFKBP12.6	hFKBP12.6F	gggggatccatgggcgtggagatcgagaccatctc (BamH1 site)	35 bp	L37086	67 – 92	347 bp
	hFKBP12.6R	ccttcgaattctcactctaagttgagcagctccac (EcoR1 site)	35 bp		393 – 370	

Measurement of Ca^{2+} release

For measurement of Ca^{2+} release from digitonin-permeabilized neutrophils, cells were washed and suspended in potassium glutamate buffer (145 mM potassium glutamate, 20 mM PIPES, and 1 mM EGTA, pH 6.6) containing an ATP generating system (2 mM Mg-ATP, 5 mM creatine phosphate, 40 unit/ml creatine phosphokinase) and protease inhibitors (2.5 mM benzamidine, 0.5 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg/ml trypsin inhibitor) and then given permeabilization by incubating cells with digitonin (4 μM) for 5 min at 25°C. The cells were washed and re-suspended (1×10^7 cells/ml) in KH medium (140 mM KCl, 10 mM NaCl, and 30 mM HEPES, pH 7.0) containing an ATP generating system, protease inhibitors, mitochondrial inhibitors (10 μg/ml antimycin A, 10 μg/ml oligomycin, and 10 mM NaN_3), and 0.025% BSA. A 1-ml aliquot of cell suspension was transferred to a fluorescence cuvette and supplemented with fura-2 (1 μM). Fluorescence was continuously monitored using a fluorescence spectrophotometer (Hitachi F-4500; Hitachi, Tokyo) at an excitation of 340/380 nm and emission of 510 nm. At the end of each run, sequential additions of 1 nmol of $CaCl_2$ were used to calibrate the fluorescence signal internally. The Ca^{2+} release (nM) represents an increased Ca^{2+} concentration in KH medium.

Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using the calcium-sensitive dye fura-2, as described previously (16). Briefly, the cells were incubated at 32°C with 1 μM fura-2 acetoxymetylester for 30 min for dye loading. Cells were then centrifuged at $100 \times g$ for 10 min and re-suspended to yield 3×10^6 cells. Cells were washed with normal medium or Ca^{2+}-deficient medium with rapid centrifugation and then re-suspended in the medium immediately before use. Fluorescence was measured with the dual-wavelength fluorescence spectrophotometer (Hitach F-4500) with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio at 340 and 380 nm using the equation of Grynkiewicz et al. (17) and a value of 224 nM for the K_d of fura-2.

Measurement of migration

Migration assays were performed using Transwell tissue-culture permeable supports with 3-μm pores (Chemotaxicell; Kurabo, Osaka). Chemoattractants were placed in the lower chamber, and neutrophils in the upper chamber. For all migration studies, neutrophils were isolated and suspended in 2 ml of phenol red-free RPMI 1640 medium containing 2% fatty acid-free bovine serum albumin (BSA). The cells were then incubated in 2 μM of calcein-acetoxymethyl ester (calcein-AM; Molecular Probes, Eugene, OR, USA) for 30 min in an CO_2 (5%) and temperature (37°C)-controlled incubator in the dark. The neutrophil concentration was then adjusted to 10^7 cells/ml. Approximately 2×10^6 cells were set aside for use in the standard curve. For each experiment under each condition, two chambers were set up; in one, the lower chamber contained 600 μl of fMLP diluted in phenol red-free RPMI 1640 medium containing 2% BSA, and the other blank contained medium (with vehicle where appropriate) only. Blanks were used to determine the random migration (chemokinesis) of an identically treated aliquot of neutrophils in the absence of chemoattractant in the lower chamber. Where various treatments were used, the same concentration was always present in both the upper and lower chambers. In cases where neutrophils were preincubated with various treatments (for 5 – 20 min at 37°C), the PMN suspension was centrifuged for 10 s at 4500 rpm. The neutrophils were then resuspended in phenol red-free RPMI 1640 medium at a concentration of 5×10^6 cells/ml before being placed in the chemotactic chamber. After each upper chamber was loaded with 100 μl of neutrophils (5×10^5 cells /chamber), the system was incubated in humidified air with 5% CO_2 at 37°C for 1 h in the dark. The upper chambers were then removed without any attempt made to dislodge any adherent neutrophil. Calcein fluorescence in the lower chamber was measured using a fluorescence spectrophotometer at an excitation of 490 nm and an emission of 512 nm. The absolute number of neutrophils migrating into the bottom chamber was then calculated from a standard curve created using reserved, calcein-labeled neutrophils. Finally, the number of neutrophils in the corresponding experimental blanks was subtracted from the total number of neutrophils migrating under experiment conditions to assess specific chemotaxis.

Statistical analyses

The significance of the differences in mean values between groups was examined using analysis of variance, followed by ANOVA. Student's *t*-test was used to evaluate statistical significance within groups.

Results

Ca^{2+} release by cADPR in digitonin-permeabilized neutrophils

The addition of cADPR to the medium caused the rapid and transient increase of Ca^{2+} release in digitonin-permeabilized human neutrophils (Fig. 1A). Heat-inacti-

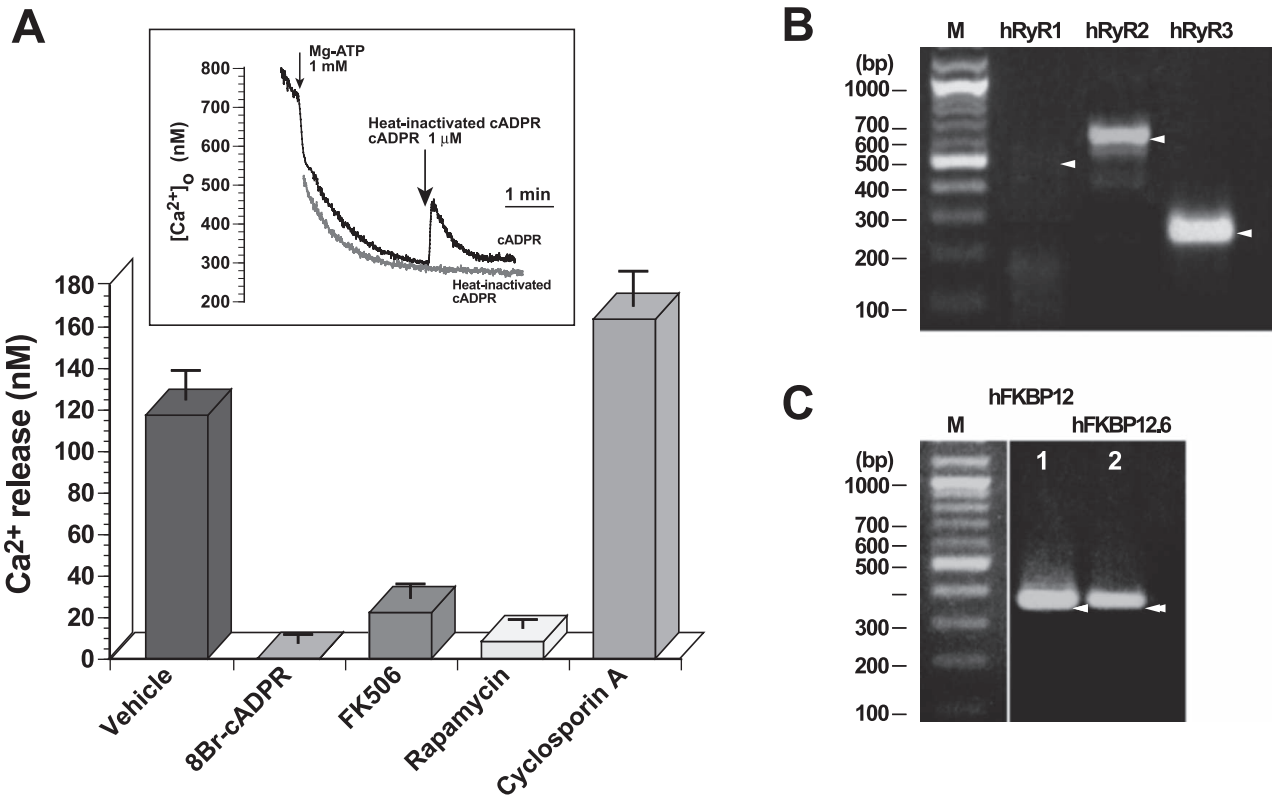

Fig. 1. cADPR-induced Ca^{2+} release from digitonin-permeabilized human neutrophils and effects of various treatments (A) and the expression profile of members of the ryanodine receptor gene family (B) and FKBP12/12.6 gene (C) in human neutrophils. A: The patterns of Ca^{2+} release induced by cADPR (insert) and the effects of 8Br-cADPR, FK506, rapamycin, and cyclosporine A on Ca^{2+} release. 8Br-cADPR (100 μM), FK506 (10 μM), rapamycin (5 μM), and cyclosporine A (10 μM) were added 1 – 3 min before the addition of cADPR (1 μM). Fluorescence was continuously monitored using a fluorometer at excitation of 340/380 nm and emission of 510 nm. Ca^{2+} released from permeabilized neutrophils was calibrated by the addition of known amounts of Ca^{2+}. Values are each the mean ± S.E.M. of the Ca^{2+} release at the time indicated (n = 3). B and C: Total RNA was prepared and subjected to RT-PCR analysis. cDNA fragments of human ryanodine receptor (B) and FKBP12/12.6 (C) were amplified from human neutrophil cDNA as described in Methods. Each panel shows a typical example of the results.

vated cADPR was ineffective. RyR1 and RyR2 are highly expressed in skeletal and cardiac muscle cells, respectively, (18) and RyR3 is more ubiquitously expressed, but at much lower levels (19). RyR functions are regulated by phosphorylation and dephosphorylation by calmodulin/calcinurin. This protein complex is attached to RyR via FKBP12 and/or FKBP12.6. The mRNAs of RyR2 and RyR3 but not RyR1 and the mRNAs of FKBP12 and FKBP12.6 were amplified by RT-PCR in human neutrophils (Fig. 1: B and C). The effects of 8Br-cADPR, a competitive inhibitor of cADPR binding and immunophilin ligands, such as FK506, rapamycin, and cyclosporin A, on Ca^{2+} release induced by cADPR were examined in digitonin-permeabilized neutrophils. Treatment with 8Br-cADPR completely inhibited cADPR-induced Ca^{2+} release. FK506 and rapamycin bind to FKBPs and dissociate them from RyR. cADPR-induced increase of Ca^{2+} release was inhibited by FK506 and rapamycin. Cyclosporin A is an inhibitor of calcinurin but does not dissociate FKBPs from RyR. Cyclosporin A did not inhibit, but rather enhanced the release by cADPR. Thus, associated FKBPs are suggested to be required for Ca^{2+} release by cADPR in human neutrophils.

Effect of 8Br-cADPR and 2APB on fMLP-induced $[Ca^{2+}]_i$ rise in intact neutrophils

fMLP induced a sharp initial $[Ca^{2+}]_i$ rise and a subsequent sustained rise in a concentration-dependent manner in intact neutrophils (Fig. 2A). Pretreatment with 8Br-cADPR, a membrane-permeable cADPR antagonist, partially reduced the fMLP-induced $[Ca^{2+}]_i$ rise (Fig. 2: B and C). The concentration of 8Br-cADPR required to exert its antagonistic effect in intact neutrophils was much higher than in digitonin-permeabilized neutrophils because of less permeability through the plasma membrane in intact cells than in leaky cells. Pretreatment of 2APB, a membrane-permeable IP_3-

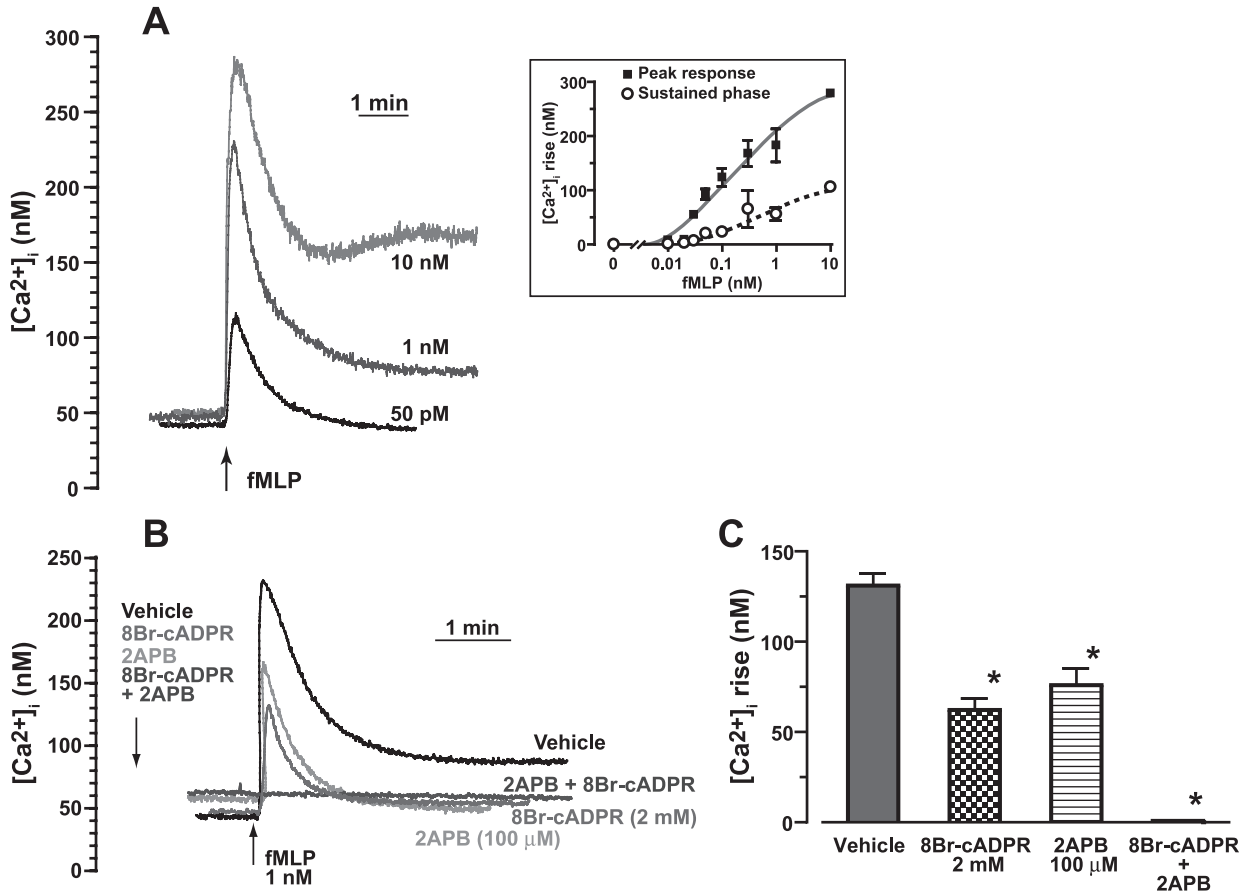

Fig. 2. fMLP-induced [Ca^{2+}]i rise and inhibition by 8Br-cADPR and 2APB in intact human neutrophils. A: Typical pattern of fMLP-induced [Ca^{2+}]$_i$ rise. Inset represents the concentration-response relationship of fMLP-induced peak and sustained [Ca^{2+}]$_i$ rise. The sustained [Ca^{2+}]$_i$ rise was the value at 3 min after the addition of fMLP. B: Typical pattern of the effect of 8Br-cADPR (2 mM) and 2APB (100 μM) on fMLP (1 nM)-induced [Ca^{2+}]$_i$ rise. 8Br-cADPR (2 mM) and 2APB (100 μM) were added 20 min before the addition of fMLP. C: Values each represent the mean ± S.E.M. of the peak [Ca^{2+}]$_i$ rise induced by fMLP in the absence or presence of 8Br-cADPR and 2APB (n = 5-16). *P<0.01, compared to the control.

receptor antagonist, also partially reduced fMLP-induced [Ca^{2+}]$_i$ rise (Fig. 2: B and C). In the presence of 8Br-cADPR and 2APB, the fMLP-induced [Ca^{2+}]$_i$ rise disappeared (Fig. 2C). These results suggest that the fMLP-induced [Ca^{2+}]$_i$ rise was mediated by both cADPR and IP_3.

Involvement of CD38 and nucleoside transporters in fMLP-induced [Ca^{2+}]$_i$ rise

CD38 is a multifunctional ectoenzyme expressed in a wide range of cell types. CD38 is a type II plasma membrane protein and the primary and best-characterized mammalian ADP-ribosyl cyclase that catalyzes the formation of cADPR from β-NAD^+. To test the involvement of CD38 in [Ca^{2+}]$_i$ rise in response to fMLP, the effect of anti-CD38Ab on fMLP-induced [Ca^{2+}]$_i$ rise was examined in intact neutrophils. Pretreatment of cells with anti-CD38Ab effectively reduced the fMLP-induced [Ca^{2+}]$_i$ rise (peak [Ca^{2+}]$_i$ rises in the absence and presence of anti-CD38Ab were 156.9 ± 10.0 and 75.7 ± 4.5 nM (n = 4), respectively, P<0.01) (Fig. 3A). To examine whether cADPR is synthesized outside the cells from β-NAD^+ and mobilizes Ca^{2+} from intracellular Ca^{2+} stores, neutrophils were preincubated with NADase to remove the substrate β-NAD^+ present outside of cells. In these neutrophils, the increase in fMLP-induced [Ca^{2+}]$_i$ rise was reduced (Fig. 3B). In contrast, when β-NAD^+ was added to the medium, [Ca^{2+}]$_i$ gradually increased with a time lag of several tens of seconds after the addition of β-NAD^+ (Fig. 4A). The [Ca^{2+}]$_i$ rise induced by β-NAD^+ was not dependent on the presence of Ca^{2+} in the medium (Fig. 4B), suggesting that the β-NAD^+–induced [Ca^{2+}]$_i$ rise is due to the release of Ca^{2+} from intracellular Ca^{2+} stores. The β-NAD^+–induced Ca^{2+} rise in Ca^{2+} deficient medium is also blocked by anti-CD38Ab (Fig. 5). 8Br-cADPR and FK506 inhibited the β-NAD^+–induced Ca^{2+} rise (Fig. 5). Ruthenium red, an inhibitor of the RyR channel, and thapsigargin, an

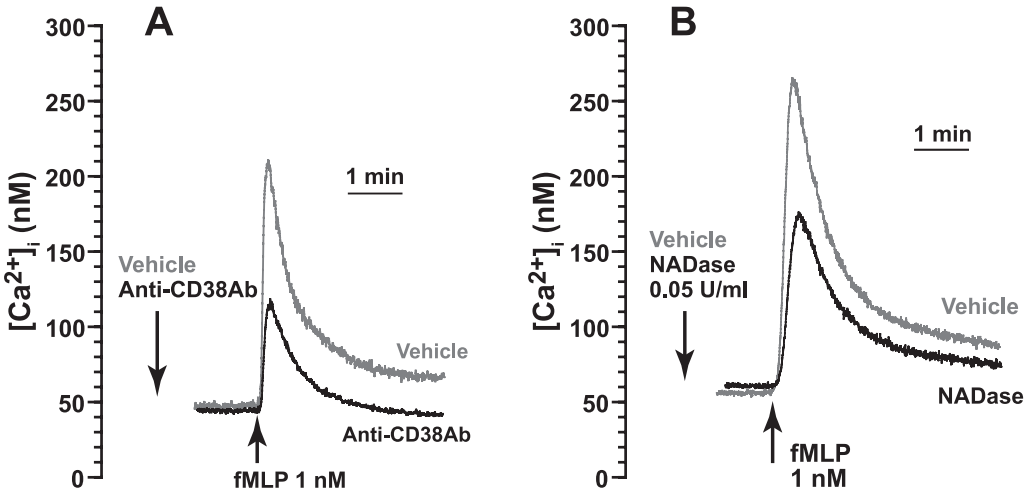

Fig. 3. Effect of anti-CD38Ab and NADase on fMLP-induced $[Ca^{2+}]_i$ rise in intact human neutrophils. Typical pattern of $[Ca^{2+}]_i$ rise induced by fMLP in the absence or presence of anti-CD38Ab (A) and NADase (B). Anti-CD38Ab (3 μg/ml) or NADase (0.05 U/ml) was added to the cells at 20 or 5 min before addition of fMLP (1 nM), respectively. Anti-CD38Ab was dialyzed before use for 3 h at 4°C against normal solution.

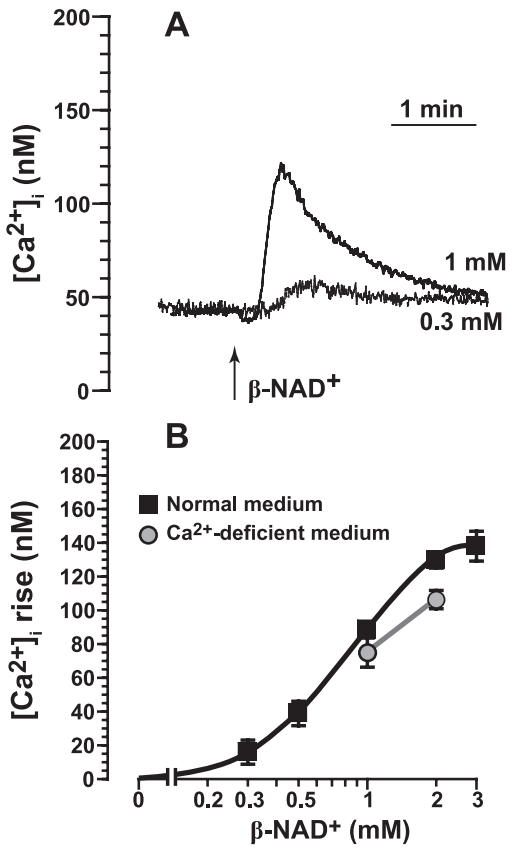

Fig. 4. β-NAD^+–induced $[Ca^{2+}]_i$ rise in intact human neutrophils. A: Typical pattern of $[Ca^{2+}]_i$ rise induced by β-NAD^+ at 0.3 and 1 mM. B: Concentration-response curve of β-NAD^+–induced peak $[Ca^{2+}]_i$ rise in normal medium and Ca^{2+}-deficient medium. Values each represent the mean ± S.E.M. of peak $[Ca^{2+}]_i$ rise induced by β-NAD^+ (n = 5 – 26).

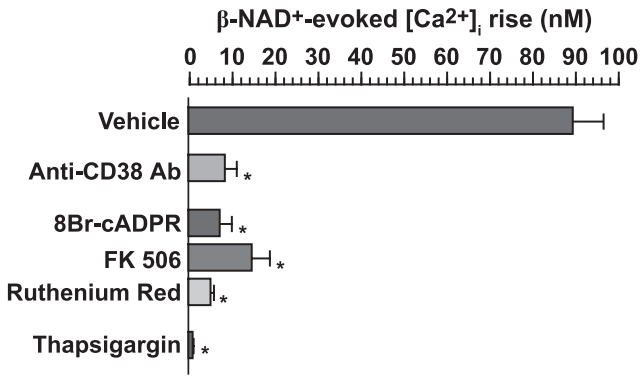

Fig. 5. Effect of various treatments on β-NAD^+–induced $[Ca^{2+}]_i$ rise in Ca^{2+}-deficient medium in intact human neutrophils. Neutophils were suspended in Ca^{2+}-deficient medium; and then anti-CD38Ab (3 μg/ml), 8Br-cADPR (2 mM), FK506 (50 μM), ruthenium red (20 μM), or thapsigargin (1 μM) was added to the cells at 20, 20, 5, 20, or 20 min before the addition of β-NAD^+ (2 mM), respectively. Values each represent the mean ± S.E.M. of peak $[Ca^{2+}]_i$ rise induced by β-NAD^+ (n = 5 – 10). *P<0.01 compared with the control.

inhibitor of vesicle Ca^{2+}-ATPase and thus a depletor of Ca^{2+} in intracellular Ca^{2+} stores, blocked the β-NAD^+–induced $[Ca^{2+}]_i$ rise (Fig. 5). These data suggest that cADPR is synthesized from β-NAD^+ outside the cells, transported into cells, and then induced Ca^{2+} release from intracellular Ca^{2+} stores by the FKBPs/RyR pathway. cADPR added to the medium caused a slight $[Ca^{2+}]_i$ rise (Fig. 6A) and markedly potentiated the $[Ca^{2+}]_i$ rise induced by a low concentration of fMLP, and this potentiation by cADPR became smaller with an increasing concentration of fMLP (Fig. 6B).

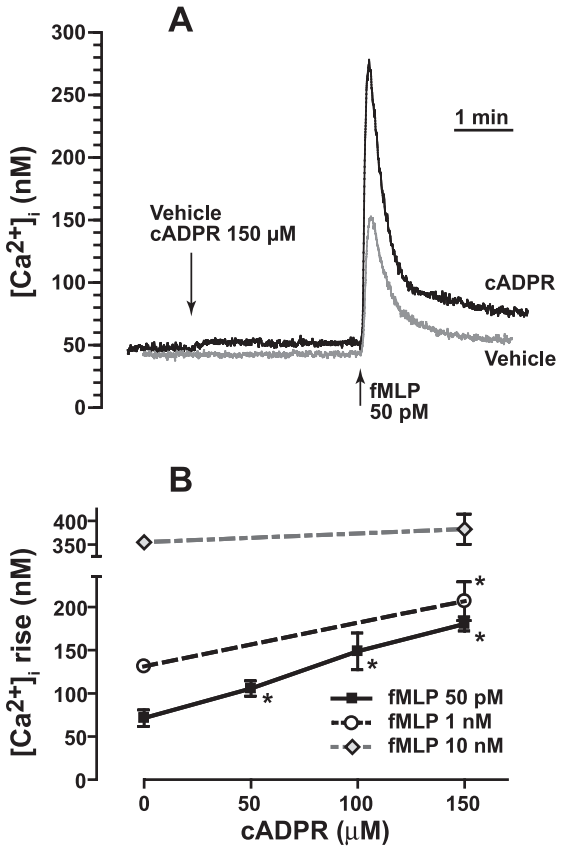

Fig. 6. Effect of cADPR on fMLP-induced $[Ca^{2+}]_i$ rise in intact human neutrophils. A: Typical pattern of fMLP-induced $[Ca^{2+}]_i$ rise in the presence or absence of cADPR. cADPR (150 μM) was added to the cells at 20 min before the addition of fMLP (50 pM). B: Values each represent the mean ± S.E.M. of peak $[Ca^{2+}]_i$ rise induced by fMLP (50 pM, 1 nM, 10 nM) in the absence or presence of cADPR (n = 4 – 10). **P*<0.01 *vs* control.

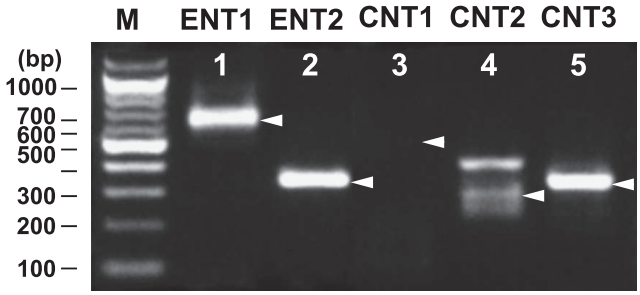

Fig. 7. Expression profile of members of the nucleoside transporter gene family in human neutrophils. Total RNA was prepared and subjected to RT-PCR analysis. cDNA fragments of human nucleoside transporters were amplified from human neutrophil cDNA, as described in Methods. The panel shows a typical example of the results.

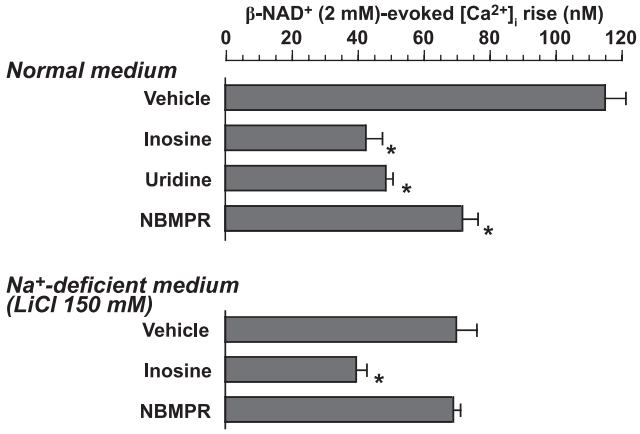

Fig. 8. Effect of inhibitors of nucleoside transporters on β-NAD^+–induced $[Ca^{2+}]$i rise in normal medium and Na^+-deficient medium in intact human neutrophils. Neutrophils were suspended in normal medium or Na^+-deficient medium (NaCl was replaced with equivalent of LiCl); and then pretreated with inosine (10 mM), uridine (10 mM), or NBMPR (20 nM) at 5 min before the addition of β-NAD^+ (2 mM). Values each represent the mean ± S.E.M. of peak $[Ca^{2+}]_i$ rise induced by β-NAD^+ (n = 5 – 10). **P*<0.01 *vs* control.

The next question was how cADPR synthesized by CD38 extracellularly is transported into cells. Recently, cADPR was demonstrated to cross the plasma membrane of a number of CD38-expressing cells through various members of the wide family of nucleoside transporter (NT), equilibrative nucleoside transporter (ENT)2, and concentrative NT (CNT)2 and the not yet molecularly characterized nitrobenzylthioinosine (NBMPR)-inhibitable nucleoside transporting system (20 – 22). In human neutrophils, hENT1 and hENT2, and hCNT2 and hCNT3 are amplified by RT-PCR (Fig. 7). The effects of inhibitors of NTs on the $[Ca^{2+}]_i$ rise induced by β-NAD^+ and fMLP were examined. Inosine and uridine, which can block all of these NTs, inhibited about 60%, and NBMPR significantly inhibited the β-NAD^+–evoked Ca^{2+} rise (Fig. 8). The CNT family forms a Na^+-gradient–dependent synporter. When extracellular Na^+ was deleted, the β-NAD^+-evoked Ca^{2+} rise was significantly reduced. In Na^+-deficient medium, inosine, but not NBMPR, produced significant inhibition (44.1 ± 3.8% inhibition of the control). Inosine, uridine, and NBMPR significantly reduced the $[Ca^{2+}]_i$ rise induced by fMLP at 1 nM (Fig. 9A) and 50 and 100 pM (Fig. 9B). The fMLP (50 pM)–induced $[Ca^{2+}]_i$ rise was potentiated in combination with cADPR, and this $[Ca^{2+}]_i$ rise was reduced to the level of fMLP alone by inosine or uridine (Fig. 9). These inhibitors of NTs antagonized the platelet-activating factor–induced $[Ca^{2+}]_i$ rise, but did not affect the leukotrien B_4–induced $[Ca^{2+}]_i$ rise (data not shown).

Role of cADPR in fMLP-induced migration of neutrophils

fMLP stimulated the migration of neutrophils in a

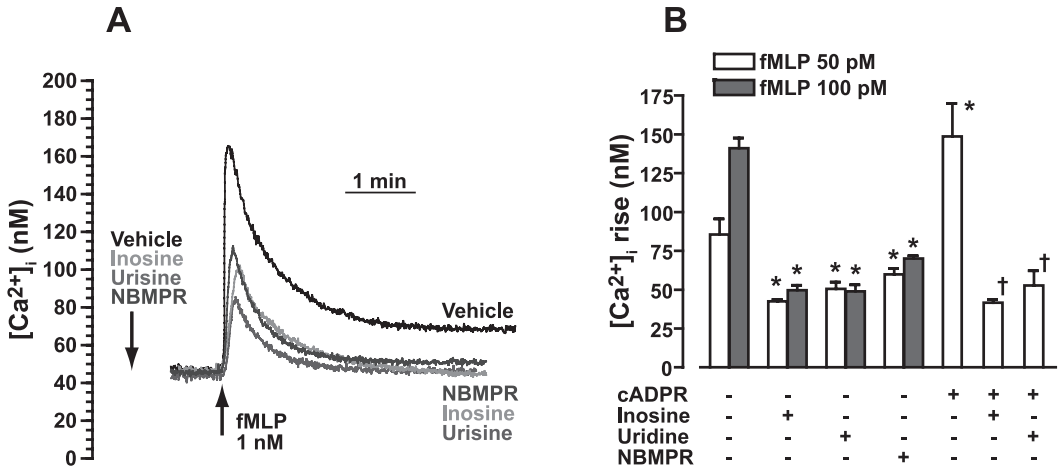

Fig. 9. Effect of inhibitors of nucleoside transporters on fMLP-induced $[Ca^{2+}]_i$ rise in intact human neutrophils. A: Typical pattern of $[Ca^{2+}]_i$ rise induced by fMLP in the absence or presence of inhibitors of nucleoside transporters. Inosine (10 mM), uridine (10 mM), or NBMPR (20 nM) was added to the cells 5 min before the addition of fMLP (1 nM). B: Inosine (10 mM), uridine (10 mM), or NBMPR (20 nM) was added 5 min before the addition of fMLP (50 or 100 pM). Cells were pretreated with cADPR (150 μM) for 20 min before the addition of fMLP (1 nM). Values each represent the mean ± S.E.M. of peak $[Ca^{2+}]_i$ rise induced by fMLP (n = 3-4). *P<0.01 *vs* Control; $^{\dagger}P$<0.01 compared with the control and treatment with cADPR.

concentration-dependent manner from 0.01 to 1 nM (Table 2). At 10 nM of fMLP, the migration decreased, although $[Ca^{2+}]_i$ increased further. fMLP-induced migration was dependent on the presence of Ca^{2+} in the medium. The addition of cADPR to the medium enhanced fMLP-induced migration of neutrophils. Treatments with an antagonist of cADPR, 8Br-cADPR or the blockers of cADPR formation, anti-CD38Ab and NADase, significantly inhibited fMLP-induced migration of neutrophils. Inactivated anti-CD38Ab and NADase had no effect. Inhibitors of nucleoside transporters, inosine, uridine, and NBMPR, significantly inhibited fMLP-induced migration of neutrophils (Table 2).

Discussion

cADPR binds to FKBP 12.6 in the RyR and causes the dissociation of FKBP 12.6 from the RyR to form the FKBP 12.6-cADPR complex, and enhances Ca^{2+} release through the RyR in pancreatic islet cells (7) and in reconstituted RyRs from arterial smooth muscle (8) (see Fig. 10). cADPR stimulates Ca^{2+} release from bovine adrenal chromaffin cells with the requirement of FKBP 12.6 (9). In these cases, the effect of cADPR is lost by FK506 or rapamycin, which are ligands of FKBPs (23 – 27); however, there are cases in which FK506 did not inhibit cADPR-induced Ca^{2+} release in sea urchin eggs and rat pancreatic acinar cells (10, 11). Thus, cADPR-binding proteins are not FKBPs but separate cADPR-sensitive channels or the Ca^{2+} pump may be a target molecule for cADPR in these tissues (28 – 30). The evidence in the present study that cADPR lost its action in the presence of FK506 and rapamycin in digitonin-permeabilized neutrophils suggests that FKBPs are required for cADPR to activate the Ca^{2+} channel on the endoplasmic reticulum in human neutrophils. The Ca^{2+}- and calmodulin-dependent phosphatase, calcineurin, was reported to interact with the RyR via FKBPs (31 – 33). It has been reported that cyclosporin A, which does not dissociate FKBP/calcinurin from RyR, inhibits calcinurin by interacting with cyclophilin, a calcinurin-associated protein. The observation that cyclosporin A did not inhibit, but rather enhanced cADPR-induced Ca^{2+} release, different from the inhibitory effect of FK506 and rapamycin that dissociate FKBPs from RyR, further supports the requirement of FKBPs for the action of cADPR. In intact cells, the fMLP-induced $[Ca^{2+}]_i$ rise was remarkably but partially attenuated by an antagonist of cADPR, 8Br-cADPR, at a concentration to produce maximal inhibition (9, 16). Considering that the fMLP-induced $[Ca^{2+}]_i$ rise was completely blocked by the combination of 8Br-cADPR and an IP_3-receptor antagonist, 2APB, which also produced partial block by itself, it is suggested that the fMLP-induced $[Ca^{2+}]_i$ rise is mainly mediated by both cADPR and IP_3 pathways in human neutrophils.

CD38, the best-characterized mammalian ecto-ADP-ribosyl cyclase, can catalyze the production of cADPR from its substrate β-NAD^+ outside of cells (13), and it is postulated to be an important source of cADPR in many tissues (14, 34) (see Fig. 10). When several cell types

Table 2. Effect of various treatments on fMLP-induced migration in human neutrophils

Chemoattractants		Addition	Transmigrated neutrophils
			Migration ($\times 10^4$ cells)
fMLP	0.01 nM		1.75 ± 0.57
	0.1 nM		8.26 ± 1.98
	0.3 nM		13.76 ± 2.88
	1 nM		25.53 ± 1.68
	3 nM		20.97 ± 1.33
	10 nM		6.21 ± 2.57
fMLP	1 nM	Vehicle	25.33 ± 1.91
		Ca^{2+} (0 mM), EGTA (0.1 mM)	0.61 ± 0.39*
fMLP	1 nM	Vehicle	24.30 ± 0.81
		cADPR (150 μM)	35.31 ± 0.40*
		8Br-cADPR (2 mM)	10.91 ± 0.86*
		Anti-CD38 Ab (3 μg/ml)	10.21 ± 3.10*
		Heat-inactivated anti-CD38 Ab	25.38 ± 0.33
		NADase (0.05 U/ml)	12.71 ± 0.08*
		Heat-inactivated NADase	22.07 ± 0.50
fMLP	1 nM	Vehicle	28.62 ± 0.77
		Inosine 10 mM	14.08 ± 0.36*
		Uridine 10 mM	17.29 ± 0.04*
		NBMPR 20 nM	15.96 ± 1.28*

Purified neutrophils from human peripheral blood were tested for their ability to migrate in response to RPMI1640 medium containing fMLP, in a conventional transwell checkerboard chemokinesis/chemotaxis assay. The number of cells migrating to the lower chamber of the transwell in the absence of any stimulation was not significantly different between the vehicle and various treatments and ranged from 1500 to 2300 cells (data not shown). The number of neutrophils migrating in response to equivalent concentrations of stimuli in both chambers (chemokinesis) and the number of neutrophils migrating in response to a chemotactic gradient (chemotaxis) are shown. Neutrophils were pre-incubated with medium, 0.2 mM EGTA, or 1 mM or 2 mM 8-Br-cADPR, and then they were placed in the upper chamber. Cells that migrated to the bottom chamber in response to the chemotactic gradient were collected and enumerated by fluorescence. Experiments were carried out in triplicate and each experiment was repeated at least 3 times. Data are each expressed as the mean ± S.E.M. (n = 3 – 4). Statistical analysis of significance of the difference of unbiased means was carried by Student's *t*-test. Significantly different from the corresponding control at **P*<0.01. Anti-CD38 Ab was dialyzed for 2 h at 4°C against RPMI 1640 medium before use.

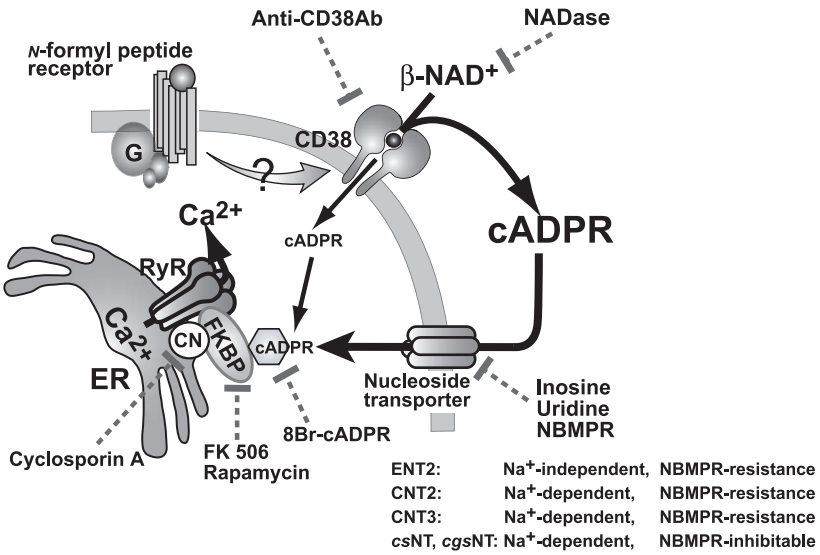

Fig. 10. A schematic model of cADPR-induced Ca^{2+} release from endoplasmic reticulum (ER). CD38, an ectoenzyme possessing ADP-ribosyl cyclase converts extracellular β-NAD^+ to cADPR, which is transported into cells through nucleoside transporters. cADPR requiring FKBPs stimulates Ca^{2+} release from ER through ryanodine receptors (RyR). How the stimulation of fMLP receptors is coupled to the activation of CD38 still remains to be solved. FKBP: FK506 binding protein, CN: calcineurin. Sites of action of various inhibitors are shown with dotted lines.

expressing ADP-ribosyl cylase were incubated in medium added with β-NAD^+, the β-NAD^+ was converted into cADPR (13). β-NAD^+ can be released to the outside of several cell types through an equilibrative transport process across connexin 43 hemichannels (20, 35 – 37). β-NAD^+ is normally present outside the cells in a significant concentration, and its amount increases according with tissue damage (38 – 40). Together with the inhibitory effect of anti-CD38Ab and NADase on fMLP-induced $[Ca^{2+}]_i$ rise, it is suggested that β-NAD^+ present beside CD38 is converted to cADPR and mediates fMLP-induced $[Ca^{2+}]_i$ rise. Actually, β-NAD^+ added to the incubation medium increased $[Ca^{2+}]_i$, and the peak increase was independent of the presence of Ca^{2+} in the medium, and this effect of β-NAD^+ blocked by anti-CD38Ab, which can inhibit ADP-ribosyl cyclase activity. These results suggest that cADPR formed from NAD outside cells stimulated Ca^{2+} release from intracellular Ca^{2+} stores. Considering that the cADPR antagonist 8Br-cADPR, FKBP binding agent FK506, RyR inhibitor ruthenium red, and Ca^{2+}-ATPase inhibitor thapsigargin all inhibited the β-NAD^+–induced $[Ca^{2+}]_i$ rise, cADPR probably stimulates Ca^{2+} release from the RyR channel in the endoplasmic reticulum. The time lag for β-NAD^+ to initiate the $[Ca^{2+}]_i$ rise may be due to the process of conversion of β-NAD^+ to cADPR, and the transport of cADPR to the site of action. The slow effect of β-NAD^+ and small effect of cADPR may be due to lower permeability at non-stimulated neutrophils and the marked potentiation by cADPR of the fMLP-induced $[Ca^{2+}]_i$ rise may be due to activation of the NT system by the stimulation of cells by fMLP. Coupling between the stimulation of fMLP receptors and activation of CD38 is interesting but remains unsolved, as in many other cells.

There have been various proposals on the transport of cADPR; transmembrane CD38 itself is a homodimer acting as a catalytically active transporter of cADPR (41, 42). Another possibility is that cADPR can be transported across the plasma membrane in addition to the process by CD38. Even in cells such as $CD38^-$ 3T3 fibroblast, rat cerebellar granule cells, human hemopoietic progenitors, or Hela cells that lack CD38, cADPR added outside the cells increased $[Ca^{2+}]_i$ and the following cellular events (37, 43 – 46). Recently, it was suggested that cADPR can be transported through the plasma membrane by various NTs: ENT, CNT, and unidentified molecules with sensitivity to NBMPR inhibition *cs*NT (concentrative-sensitive and Na^+-dependent nucleoside transporter) and *csg*NT (concentrative-sensitive, Na^+-dependent and guanosine-preferring nucleoside transporter) (20 – 22) (see Fig. 10). ENT is Na^+-gradient independent and is divided into NBMPR-inhibitable ENT1 and NBMPR-resistant ENT2. The CNT family forms an Na^+-gradient-dependent synporter, including CNT1, CNT2, and CNT3, all of which are resistant to NBMPR inhibition. ENT1 do not transport cADPR (21, 41, 47). In this study, human neutrophil hENT1 and hENT2 and hCNT2 and hCNT3 were amplified by RT-PCR. The β-NAD^+–evoked Ca^{2+} rise was inhibited by about 60% by inosine and uridine, which can block all of these nucleoside transporters, suggesting that a considerable amount of cADPR is transported by NTs. When extracellular Na^+ was deleted, the β-NAD^+-evoked Ca^{2+} rise was reduced by about 50%. Na^+-dependent CNT2-, CNT3-, *cs*NT-, or *csg*NT-mediated transport may be involved in this part of the transport. The remaining part was Na^+-independent, insensitive to NBMPR inhibition, and inhibited by inosine to a similar level as in normal medium, thus suggesting the involvement of ENT2.

In addition to the mobilization of Ca^{2+} from intracellular Ca^{2+} stores, the subsequent influx of extracellular Ca^{2+} plays important roles in developing cellular functions in many types of cells. cADPR has been shown to stimulate the influx of extracellular Ca^{2+}, possibly by activating a store-operated Ca^{2+} channel (SOC) (9, 14, 16, 48). Among the human TRP family, the role of TRPM2 in Ca^{2+} influx became evident in neutrophils. Heiner et al. (49, 50) suggested that ADPR, a metabolite of β-NAD^+ and cADPR, activates TRPM2 channel gating by binding to the Nudix box domain in the C-terminal tail of the channel in neutrophil granulocytes. It is remarkable that NT inhibitors, inosine, uridine, and NBMPR, effectively reduced the sustained $[Ca^{2+}]_i$ rise induced by fMLP in intact neutrophils. Evidence may suggest that cADPR formed in response to stimulation of neutrophils by fMLP participates in the influx of Ca^{2+}.

fMLP stimulated migration of neutrophil in a concentration-dependent manner with peak effect at 1 nM. fMLP-stimulated migration of neutrophils was dependent on the presence of Ca^{2+} in the medium, in agreement with the report that Ca^{2+} influx was required for neutrophil migration (51). However, higher concentration of fMLP (10 nM) decreased neutrophil migration, even though it produced a large increase in $[Ca^{2+}]_i$. This observation is in agreement with the reports that higher concentration of fMLP resulted in diminished polymorphonuclear leukocytes (PMN) migration, a phenomenon known as deactivation (52 – 54). It is also suggested that marked changes in $[Ca^{2+}]$i are not required for fMLP-induced human PMN chemotaxis (54) but transient increases in $[Ca^{2+}]_i$ is required for the migration of adherent neutrophils (55). 8Br-cADPR, anti-CD38Ab, inosine, uridine, NBMPR, and NADase all reduced neutrophil migration to fMLP; therefore,

cADPR-mediated regulation of $[Ca^{2+}]_i$ plays an important role in migration of neutrophils in response to fMLP.

The present study suggested that conversion of β-NAD^+ to cADPR by CD38 and transportation of cADPR by NTs are important cascades for the regulation of $[Ca^{2+}]_i$ in human neutrophils. Together with evidence that the amount of β-NAD^+ outside cells increases with tissue damage (38 – 40). These cascades, especially NTs, may be pharmacological targets to control neutrophil migration.

Acknowledgments

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