THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 24, Issue of June 15, pp. 21642–21648, 2001
Printed in U.S.A.
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Paracrine Roles of NAD^+ and Cyclic ADP-ribose in Increasing Intracellular Calcium and Enhancing Cell Proliferation of 3T3 Fibroblasts*

Received for publication, November 21, 2001, and in revised form, March 6, 2001
Published, JBC Papers in Press, March 27, 2001, DOI 10.1074/jbc.M010536200

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CD38 is a bifunctional ectoenzyme synthesizing from NAD^+ (ADP-ribosyl cyclase) and degrading (hydrolase) cyclic ADP-ribose (cADPR), a powerful universal calcium mobilizer from intracellular stores. Recently, hexameric connexin 43 (Cx43) hemichannels have been shown to release cytosolic NAD^+ from isolated murine fibroblasts (Bruzzone, S., Guida, L., Zocchi, E., Franco, L. and De Flora, A. (2001) *FASEB J.* 15, 10–12), making this dinucleotide available to the ectocellular active site of CD38. Here we investigated transwell co-cultures of $CD38^+$ (transfected) and $CD38^-$ 3T3 cells in order to establish the role of extracellular NAD^+ and cADPR on $[Ca^{2+}]_i$ levels and on proliferation of the $CD38^-$ target cells. $CD38^+$, but not $CD38^-$, feeder cells induced a $[Ca^{2+}]_i$ increase in the $CD38^-$ target cells which was comparable to that observed with extracellular cADPR alone and inhibitable by NAD^+-glycohydrolase or by the cADPR antagonist 8-NH_2-cADPR. Addition of recombinant ADP-ribosyl cyclase to the medium of $CD38^-$ feeders induced sustained $[Ca^{2+}]_i$ increases in $CD38^-$ target cells. Co-culture on $CD38^+$ feeders enhanced the proliferation of $CD38^-$ target cells over control values and significantly shortened the S phase of cell cycle. These results demonstrate a paracrine process based on Cx43-mediated release of NAD^+, its CD38-catalyzed conversion to extracellular cADPR, and influx of this nucleotide into responsive cells to increase $[Ca^{2+}]_i$ and stimulate cell proliferation.

CD38, a type II transmembrane glycoprotein of 46 kDa, formerly known as a leukocyte activation antigen (1, 2), has attracted increasing attention since it proved to be a bifunctional ectoenzyme involved in the metabolism of two signal molecules, *i.e.* cyclic ADP-ribose (cADPR)[1] and $NAADP^+$ (3, 4). CD38 is able either to convert NAD^+ to cADPR (ADP-ribosyl cyclase) and then to hydrolyze cADPR (cADPR hydrolase), or to catalyze a base exchange reaction leading to $NAADP^+$ biosynthesis from $NADP^+$ and nicotinic acid (3, 4). Cyclase and base exchange activities are common to other members of the CD38 family, the best known of which is a soluble protein purified and characterized from the marine mollusk *Aplysia californica* (5, 6).

Since both cADPR and $NAADP^+$ are potent calcium mobilizers from distinct intracellular stores (3, 4), CD38 is held to play an essential role in the control of calcium homeostasis in many responsive cells. Specifically, cADPR has been demonstrated to regulate a number of calcium-related cellular events including proliferation, contraction, and secretion (3). Therefore, cADPR can reach its receptor-operated intracellular stores (7), although its site of CD38-catalyzed generation is in fact ectocellular (8, 9). This topological paradox holds both for CD38 in the plasma membrane and for the subcellular fraction of CD38 whose active site is hidden inside either exocytotic or endocytotic vesicles during intracellular trafficking. Indeed, both enhanced exocytosis and ligand-induced endocytosis of CD38-containing membrane vesicles proved to elude such compartmentation and to be causally associated to cADPR-dependent $[Ca^{2+}]_i$ increases (10, 11). Elucidation of the topological paradox of the CD38/cADPR system came from some recent findings as follows. (i) The plasma membrane of several cell types harbors a passive transport system for pyridine dinucleotides, which is responsible for NAD^+ fluxes through the membrane (11), thus providing NAD^+ substrate to the otherwise inaccessible active site of CD38. This dinucleotide transporter has been identified with connexin 43 hemichannels (12). (ii) Transmembrane CD38 is an active transporter of catalytically produced cADPR across its oligomeric structure (13). (iii) A third, CD38-unrelated mechanism of permeation of extracellular cADPR across cell membranes has been postulated in selected cell types (14, 15).

The presence of multiple transport systems for NAD^+ and cADPR in the plasma membrane raises the possibility of a paracrine exchange of these molecules between neighboring cells via Cx43, CD38, and eventually cADPR influx. The possibility of NAD^+/cADPR-related paracrine mechanisms and their potential role in regulating intracellular calcium were experimentally addressed in the present study by means of

* This work was supported in part by grants from Associazione Italiana per la Ricerca sul Cancro, MURST-PRIN 2000, MURST-CNR (5% Project on Biotechnology to A. D. F.), and from the University of Genova and CNR (Target Project on Biotechnology to E. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a fellowship from Fondo Sociale Europeo-MURST.

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[1] The abbreviations used are: cADPR, cyclic ADP-ribose; Cx43, connexin 43; $NAADP^+$, nicotinic acid adenine dinucleotide phosphate; cGDPR, cyclic GDP-ribose; NAD^+ase, NAD^+-glycohydrolase; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; RIA, radioimmunoassay; BrdUrd, bromodeoxyuridine; IFN-γ, interferon-γ.

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co-cultures of CD38 sense- and antisense-transduced 3T3 fibroblasts. $CD38^-$ 3T3 cells were found to respond to the paracrine production of cADPR by co-cultured $CD38^+$ 3T3 cells with a calcium-related increase of proliferation. This previously unrecognized interplay between extracellular NAD^+ and cADPR may represent a means for regulating intracellular calcium homeostasis and relevant cell responses in selected tissue microenvironments featuring $CD38^+$ stromal cells and $CD38^-$ parenchymal cells, *e.g.* bone marrow (14) and smooth muscle (15).

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]NAD^+ (200 Ci/mmol) and [^{3}H]NAD^+ (40 Ci/mmol) were obtained by ICN (Milan, Italy) and PerkinElmer Life Sciences, respectively. cADPR, [^{32}P]cADPR, and [^{3}H]cADPR were prepared enzymatically from NAD^+, [^{32}P]NAD^+, and [^{3}H]NAD^+, respectively, with recombinant ADP-ribosyl cyclase from *A. californica* (courtesy of Prof. H. C. Lee) and HPLC-purified (14). Cx43 antisense (5′-CTCCAGTCACCCATGTCTG-3′) oligodeoxynucleotide, complementary to the AUG translation start codon region of murine Cx43 mRNA and the corresponding sense (5′- CAGACATGGGTGACTGGAG-3′), were purchased from Life Technologies, Inc. Fura 2-AM was obtained from Calbiochem. The anti-cADPR polyclonal antibody (16) and recombinant CD38 (17) were kindly provided by Prof. H. C. Lee. All other chemicals were obtained from Sigma.

Cell Lines—NIH 3T3 cells obtained from ATCC (Manassas, VA) were cultured as described (10). Transfection with sense ($CD38^+$) or antisense ($CD38^-$) CD38 cDNA was performed as described (10). Transfected cells were routinely maintained under geneticin (1 mg/ml) selection.

Assay of Ectoenzyme Activities—NAD^+ glycohydrolase (NAD^+ase), GDP-ribosyl cyclase, and cADPR hydrolase activities were assayed on 1 mM NAD^+, 1 mM nicolinamide guanine dinucleotide, and 0.5 mM cADPR, respectively, by incubating intact $CD38^{+/-}$ 3T3 cells (10^6) in 400 μl of PBS containing 10 mM glucose (PBS/glucose) at 37 °C. At different times, 60-μl aliquots of the incubation mixtures were centrifuged for 30 s at 5,000 × *g,* and the corresponding supernatants were deproteinized with trichloroacetic acid (10% final concentration) as described (14). HPLC analyses of nucleotides in the samples were performed as described (15). Protein content was determined according to Bradford (18).

$CD38^+$-transfected 3T3 cells, but not the $CD38^-$ ones, expressed at their outer surface the three enzymatic activities of CD38 as follows: NAD^+ase (29 ± 3 nmol of ADPR/min/mg), GDP-ribosyl cyclase (2.8 ± 0.2 nmol of cGDPR/min/mg), and cADPR hydrolase (0.61 ± 0.04 nmol of ADPR/min/mg). Nicolinamide guanine dinucleotide was used as substrate for the cyclase activity because its enzymatic product cGDPR is not hydrolyzable and accumulates during the assay (19).

cADPR Influx in Intact $CD38^-$ 3T3 Cells and cADPR Association to Their Membranes—Cell membranes were prepared by submitting $CD38^-$ 3T3 cells (50 × 10^6) to lysis in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3 M sucrose, in the presence of protease inhibitors (10 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml trypsin inhibitor) at 0 °C. Following sonication in ice for 1 min at 3 watts (Heat-System Ultrasonics, W-380, New York), cell lysates were subjected to two subsequent centrifugations at 4 °C as follows: at 3,000 × *g* for 10 min and the corresponding supernatants at 100,000 × *g* for 15 min. Membrane pellets were then washed twice in 2 ml of PBS buffer.

Total membranes (5 mg/ml) and intact 3T3 $CD38^-$ cells (4 × 10^6/ml) were incubated in 1.0 ml of PBS/glucose in the presence of 200 μM cADPR at 37 °C. At different times 100-μl aliquots were withdrawn and centrifuged at 100,000 × *g* for 15 min (total membranes) or at 5,000 × *g* for 30 s (intact cells). Pellets were washed in 2 ml of PBS/glucose, in order to dilute cADPR in the supernatants to HPLC-undetectable concentrations, and then were resuspended in 300 μl of water and sonicated for 1 min at 3 watts in ice. Aliquots of 270 μl were trichloroacetic acid-deproteinized (14), and cADPR content was analyzed by HPLC (see below). Protein content was determined on 30-μl aliquots according to Bradford (18).

HPLC analyses were performed on a Hewlett-Packard 1090 instrument equipped with an HP1040 A diode array spectrophotometric detector set at 260 nm, using a 5-μm, 100-Å, 150 × 3.9-mm Delta pack C18 reverse phase column (Waters, Milford, MA). Solvent A was water, and solvent B was 70% 0.1 M KH_2PO_4 containing 5 mM PIC A reagent (Millipore, Milan, Italy), pH 5, and 30% methanol; the solvent program was a gradient starting at 100% solvent A for 5 min, linearly increasing to 10% solvent B in 25 min, and then increasing to 100% solvent B in 30 min at a flow rate of 0.5 ml/min. Identification and quantitation of the individual peaks were obtained both by co-elution with known standard compounds and by comparison of UV absorption spectra with those of computer-stored standards. cADPR eluted at 35 min, completely separated from other nucleotides and nucleosides. Sensitivity of this HPLC analysis of cADPR was ≥10 pmol per sample.

Co-culture Conditions—CD38-antisense-transfected 3T3 cells (0.25 × 10^6) were seeded in transwell plates (24 mm diameter) on pre-established feeder layers of $CD38^+$ or $CD38^-$ 3T3 cells (1.0 × 10^6), in 1.5 ml of fresh complete medium. Co-cultures were exposed to different cADPR antagonists (20) as follows: 1 μM 8-Br-cADPR for 24 h or 8-NH_2-cADPR for 24, 48, and 72 h; or to the gap junction inhibitor oleamide (50 μM) for 24 h (21); or incubated with Cx43 sense or antisense oligodeoxynucleotide (12) as described below. Purified NAD^+ase (Sigma) was added to 3T3 $CD38^{+/-}$ co-cultures at a final concentration of 1.3 milliunits/ml. Recombinant ADP-ribosyl cyclase from *A. californica* was added to 3T3 $CD38^{-/-}$ co-cultures at a final activity of 3 nmol of cADPR/min/ml. Parallel long term incubation of ADP-ribosyl cyclase with 100 μM NAD^+ revealed the formation of products other than cADPR, among which are ADPR, ADP, and AMP.

Cx43 antisense and sense oligodeoxynucleotides were administered, each at 20 μM, in phosphatidylcholine liposomes (12) to $CD38^{+/-}$ feeders as well as to $CD38^-$ target cells pre-adsorbed on a 20-mm diameter coverslip, separately. After 16 h of culture at 37 °C, cells were refed with complete Dulbecco's modified Eagle's medium, and $CD38^-$-treated cells were transferred onto transwell plates over $CD38^{+/-}$-treated feeders. $[Ca^{2+}]_i$ of $CD38^-$ 3T3 target cells was determined after 24 h of co-culture.

Fluorimetric Determination of $[Ca^{2+}]_i$—$CD38^-$ 3T3 target cells (2.5–5 × 10^4) adherent on 20-mm diameter coverslips treated with or without Cx43 oligodeoxynucleotides were incubated in the presence of Fura2-AM (10 μM) for 45 min at 37 °C. Untreated cells on the coverslips were incubated without or with 50 μM oleamide or 50 μM 8-Br-cADPR or 100 μM 8-NH_2-cADPR in zero calcium standard solution (10) in a 200-μl recording chamber mounted on the stage of an inverted microscope (Zeiss IM35, Stuttgart, Germany). After 20 min incubation at 25 °C, 100 μM cADPR was added into the chamber, and intracellular calcium concentration in Fura2-AM-treated cells was continuously recorded for 30 min, as described (11). No differences were observed between CD38 antisense-transfected and native $CD38^-$ 3T3 cells as concerns the $[Ca^{2+}]_i$ changes.

For determination of $[Ca^{2+}]_i$ in $CD38^-$ cells co-cultured with $CD38^{+/-}$ feeders, target $CD38^-$ cells were harvested at different times from the transwells, washed twice in 1 ml of PBS for 30 s at 5,000 × *g,* and resuspended in 1 ml of fresh complete medium. Calcium measures were performed in a 2-ml cuvette under continuous stirring in zero calcium solution, as described (14). Statistical analysis of different $[Ca^{2+}]$ values was performed using one-way analysis of variance and two-sided Dunnett's *t* test. *p* values were considered statistically significant when <0.05.

Determination of Intracellular cADPR in Co-culture Conditions—After 48 h co-culture on 75-mm diameter plates, CD38 target cells were washed with 10 ml of PBS, detached with trypsin, and washed twice with 1 ml of ice-cold PBS at 5,000 × *g* for 30 s. Pellets were resuspended in 250 μl of cold water and frozen at −20 °C and then thawed and sonicated in ice 1 min at 3 watts. A 50-μl aliquot was withdrawn for assay of protein (18), whereas the rest of the sample was deproteinized with 10% trichloroacetic acid (14). The cADPR content of the cell extracts was analyzed by two subsequent HPLCs after addition of trace amounts of radiolabeled [H^3]cADPR (2 × 1,000 cpm) as internal standard (14). Identification of the cADPR peak in the cell extracts was confirmed by co-elution with the radioactive standard, by comparison of the absorbance spectrum and elution time with standard cADPR, and by the disappearance of the corresponding peak in the matched CD38-hydrolyzed samples (14). The concentration of intracellular cADPR was calculated from the area of the HPLC peak, taking into account the percentage of nucleotide recovery obtained with the radioactive standard.

Determination of Extracellular cADPR—At various times of co-culture of $CD38^-$ over $CD38^{+/-}$ 3T3 feeder cells in complete medium (without phenol red), the medium was collected and clarified by three repeated centrifugations at 300 × *g* for 5 min. The cell-free medium was trichloroacetic acid-deproteinized (14) and submitted to enzyme digestion to hydrolyze nucleotides potentially interfering with the cADPR assay (16). cADPR content in the samples was determined by a sensitive and specific radioimmunoassay (RIA) (16), rather than by HPLC as for intracellular cADPR levels (see above), because high salt concentra-

tions in these samples proved to interfere with the latter type of analyses.

Determination of Extracellular NAD⁺—Fresh co-culture media at different times and in different experimental conditions were collected and cells were removed by 3 subsequent centrifugations at 5,000 × *g* for 30 s. NAD^+ content was measured on 200 μl aliquots by a sensitive enzymatic cycling procedure (22), as described (11). To calculate the percentage of cell lysis, hexokinase activity was assayed in aliquots of the same media (11).

Cell Proliferation Assay—CD38⁻ 3T3 target cells were co-cultured in triplicate over $CD38^{+/-}$ feeders as described above; after 24, 48, and 72 h, target cells were harvested from the transwell with trypsin and washed twice in PBS at 5,000 × *g* for 30 s, and the dry cell pellet was frozen at −20 °C. The DNA content of the cell pellets was estimated with the CyQuant proliferation assay kit (Molecular Probes, OR). DNA fluorescence of the samples was measured on an LS-50B fluorometer (PerkinElmer Life Sciences). Results were expressed as percentages of proliferation compared with control cells ($CD38^-$ 3T3 cells co-cultured with $CD38^-$ 3T3 cells). The Mann-Whitney rank sum test was used to determine the significance of the difference between two cell populations.

Cell Cycle Analysis—Following co-culture of $CD38^-$ cells (0.5×10^6) with $CD38^{+/-}$ 3T3 feeder cells (2×10^6) on 75-mm diameter transwell plates for 72 h, target cells were washed in 10 ml of PBS and incubated for 30 min with 30 μM 5′-bromodeoxyuridine (BrdUrd). Cells were then washed with PBS and either immediately fixed (zero time) or further co-cultured on $CD38^+$ or $CD38^-$ 3T3 feeders for 4 h. BrdUrd-labeled cells were detached with trypsin, washed twice in ice-cold PBS containing 2 mM EDTA, and prepared for flow cytometry-mediated analysis of BrdUrd and DNA contents as described previously (10). Briefly, from flow cytometry-mediated and derived data of the $CD38^-$ target cells cultured on either $CD38^-$ or $CD38^+$ feeder cells, the length of the S phase (T_S) was calculated on the basis of the fact that BrdUrd-labeled cells were allowed to progress through the cell cycle in a BrdUrd-free environment during the so-called "post-labeling" period (4 h). Specifically, T_S values were calculated by comparing the mean DNA content of the cohort of BrdUrd-labeled cells which have moved through the cell cycle during post-labeling time, with that of G_1 and G_2/M phase cells, using the relative movement (RM) method (23). RM values were obtained using Equation 1,

$$RM = (FS - FG_1)/(FG_2 - FG_1) \quad \text{(Eq. 1)}$$

where *F* is the mean red fluorescence of the corresponding phase of the cell cycle. T_S (the length of the S phase) values were calculated from Equation 2,

$$T_S = [RM_{T0}/(RM_{T4h} - RM_{T0})] \times 4 \quad \text{(Eq. 2)}$$

where RM_{T0} is relative movement at the time of pulse labeling, RM_{T4h} = relative movement 4 h after pulse labeling, and 4 is the observation time of 4 h. The Student's *t* test was used to determine the statistical significance of the difference between the two cell populations in the four experiments performed.

RESULTS

Susceptibility of CD38⁻ 3T3 Fibroblasts to Extracellular cADPR—Digitonin-permeabilized murine 3T3 fibroblasts have been shown previously to respond to cADPR with an immediate, 8-NH_2-cADPR-inhibitable elevation of $[Ca^{2+}]_i$ levels (10). Influx of external cADPR has been postulated in cells as murine B-lymphocytes (24), rat cerebellar granule neurons (25), and smooth myocytes from bovine trachea (15), where sustained $[Ca^{2+}]_i$ increases were observed following exposure to extracellular cADPR. Moreover, cADPR influx was measured directly in human hemopoietic progenitors, in which the alternative possibility of surface-bound cyclic nucleotide was ruled out by time dependence of cADPR association to extensively washed cells (14).

Therefore, we investigated the possible permeation of extracellular cADPR across the plasma membrane of native $CD38^-$ 3T3 fibroblasts. Both intact cells and isolated membrane preparations were examined for their content of HPLC-detectable cADPR at different times of incubation with 200 μM cADPR

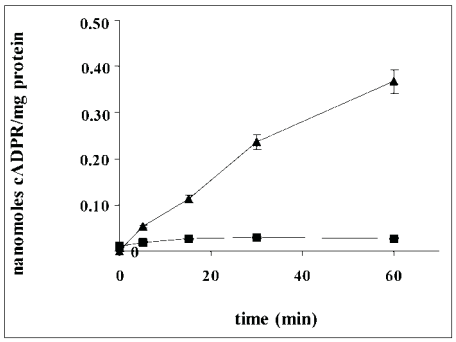

FIG. 1. **Time-dependent influx of extracellularly added cADPR into intact CD38⁻ fibroblasts.** Intact $CD38^-$ 3T3 cells (▲) or their membranes (■), prepared as described under "Experimental Procedures", were incubated in the presence of 200 μM extracellular cADPR at 37 °C. At the times indicated, aliquots were withdrawn, and the cADPR content of each sample was analyzed by HPLC as described under "Experimental Procedures."

(Fig. 1). While membrane-associated cADPR kept stable over time at barely detectable levels, there was a progressive increase of the fraction of cell-associated cADPR which resisted washing of the incubated fibroblasts. This time dependence of association to equally washed cells and failure to record association to isolated cell membranes strongly favor a process of internalization of external cADPR over a simple surface binding.

The data illustrated in Fig. 1 and the intrinsic occurrence of cADPR receptors inside the 3T3 cells (10) prompted us to investigate whether these intact cells respond to externally added cADPR with mobilization of $[Ca^{2+}]_i$ levels. As shown in Fig. 2, addition of the cyclic nucleotide to $CD38^-$ native cells resulted in a progressive increase of their $[Ca^{2+}]_i$ levels. The lowest effective concentration of cADPR was 0.5 μM, similarly to results obtained with bovine tracheal smooth myocytes (15). The kinetics and extent of $[Ca^{2+}]_i$ elevations were identical to those recorded in the same 3T3 fibroblasts exposed to oleamide, a known inhibitor of solute exchange across gap junctions (21) and of specific NAD^+ transport through Cx43 hemichannels in isolated 3T3 cells (12). Also, the progressive $[Ca^{2+}]_i$ increase was completely unaffected in $CD38^-$ cells pre-treated either with a specific anti-Cx43 deoxynucleotide or with the corresponding sense deoxynucleotide. Conversely, $[Ca^{2+}]_i$ levels in $CD38^-$ cells pre-incubated with either of two cADPR analogs and antagonists (100 μM 8-NH_2-cADPR or 50 μM 8-Br-cADPR) failed to increase following addition of extracellular cADPR.

These data demonstrate responsiveness of 3T3 fibroblasts to extracellular cADPR, similarly to earlier results obtained on the same permeabilized cells (10), yet with distinctive patterns of time dependence, *i.e.* a fast peak of $[Ca^{2+}]_i$ elevation in the permeabilized cells (10) and a sustained response in the native 3T3 fibroblasts (Fig. 2). The latter pattern suggests influx of cADPR (and of both cADPR antagonists as well) across the plasma membrane. Cx43 hemichannels are not involved in the $[Ca^{2+}]_i$ increases elicited by extracellular cADPR, in agreement with complete lack of cADPR transport into proteoliposomes reconstituted with homogeneous Cx43 (12).

Effect of the Co-culture Over $CD38^{+/-}$ Feeders on $[Ca^{2+}]_i$ of CD38⁻ 3T3 Cells—These results and the availability of both $CD38^-$ and $CD38^+$ 3T3 fibroblasts prompted us to develop a model for co-culture where $CD38^+$ fibroblasts were used as feeders for $CD38^-$ cells. Control co-cultures were grown on a feeder represented by the same $CD38^-$ cells. The properties we investigated in the $CD38^-$ 3T3 cells were the $[Ca^{2+}]_i$ levels and also the rate of cell proliferation, which had been proved to be enhanced under conditions of *de novo* expression of CD38 cells

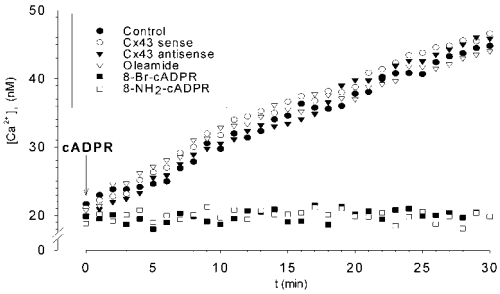

FIG. 2. **Responsiveness of CD38⁻ 3T3 fibroblasts to extracellular cADPR.** CD38⁻ 3T3 cells, either untreated (control) or treated with various Cx43 modulators and cADPR antagonists (see "Experimental Procedures"), were exposed to extracellular 100 μM cADPR. $[Ca^{2+}]_i$ changes were continuously measured as described (11). *Traces* are the mean of 5 different experiments; no S.D. values are shown for the sake of clarity.

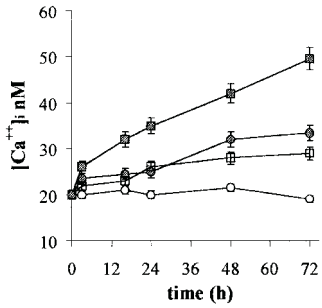

FIG. 3. **$[Ca^{2+}]_i$ changes in CD38⁻ 3T3 cells co-cultured with CD38⁺/⁻ feeders.** Co-cultures were performed as described under "Experimental Procedures." At the times indicated CD38⁻ target cells were harvested, and $[Ca^{2+}]_i$ was determined (10), and extracellular NAD⁺ and cADPR levels were measured as reported under "Experimental Procedures." CD38⁻ feeders, ○; CD38⁺ feeders, ■; CD38⁻ feeders plus recombinant ADP-ribosyl cyclase, ●; CD38⁺ feeders plus NAD⁺-glycohydrolase, □. Values are means ± S.D. of 10 different experiments.

resulting in cADPR-mediated increases of the $[Ca^{2+}]_i$ (10). Co-culturing with CD38⁺ 3T3 feeder cells determined a progressive increase of the $[Ca^{2+}]_i$ of CD38⁻ fibroblasts from a basal value of 20.3 ± 2 to 49.6 ± 4 nM within 72 h (Fig. 3). No increase whatsoever was detectable in the same cells grown in the same conditions, yet over CD38⁻ 3T3 feeders. Addition of NAD⁺ase to the culture medium substantially decreased ($p < 0.05$) the sustained enhancement of $[Ca^{2+}]$ of cells co-cultured with the CD38-transfected fibroblasts (Fig. 3), indicating a causal role of extracellular NAD⁺ on the $[Ca^{2+}]_i$ changes. Moreover, supplementation of soluble ADP-ribosyl cyclase to the medium conditioned by the presence of CD38⁻ feeder cells elicited a significant ($p < 0.05$) and sustained increase of $[Ca^{2+}]_i$ in the CD38⁻ 3T3 fibroblasts over the remarkably stable levels observed in the same cells without any addition (Fig. 3). This increase witnesses NAD⁺ release from the CD38⁻ feeder cells.

In an attempt to investigate whether a quantitative relationship exists between the functional effects on the $[Ca^{2+}]_i$ and the extracellular concentration of NAD⁺ and cADPR, both nucleotides were measured in the media of the various cultures shown in Fig. 3 at different times. Results are reported in Table I. Levels of extracellular NAD⁺ proved to be remarkably stable throughout the co-culture conditions up to 72 h of incubation. Moreover, they were not significantly modified in the CD38⁺/CD38⁻ co-cultures nor in the CD38⁻ cultures supplemented with ADP-ribosyl cyclase as compared with their corresponding CD38⁻ control cultures. The only difference was observed in the CD38⁺ cultures supplemented with NAD⁺ase, where extracellular NAD⁺ was slightly (18%) lower than in CD38⁻ cultures alone (Table I).

On the contrary, concentrations of extracellular cADPR, measured by means of a specific RIA (16), were progressively increasing in the CD38⁺/CD38⁻ co-cultures, until reaching 6.0 ± 0.8 nM at 72 h (Table I). They were remarkably lower in the medium from the same co-cultures supplemented with NAD⁺ase, in agreement with a comparatively reduced $[Ca^{2+}]_i$ increase (Fig. 3). Addition of ADP-ribosyl cyclase to the CD38⁻ feeders resulted in increasing concentrations of extracellular cADPR, although lower than those measured in CD38⁺/CD38⁻ co-cultures. Possible reasons for this apparent discrepancy are as follows: (i) the ADP-ribosyl cyclase is partially inactivated at 37 °C (19); (ii) the K_m value of ADP-ribosyl cyclase for NAD⁺ is higher (39 μM) than that of CD38 (14 μM) (19); (iii) long term incubation of ADP-ribosyl cyclase on NAD⁺ resulted in generation of nucleotides other than cADPR (see "Experimental Procedures").

The results shown in Fig. 3 and in Table I demonstrate a good quantitative correlation between $[Ca^{2+}]$ increases of 3T3 cells and the concentrations of extracellular cADPR in the corresponding culture media. As mentioned, no such correlation was observed with the levels of extracellular NAD⁺, which were almost unaffected even under experimental conditions that result in the enzymatic conversion of the dinucleotide (by either CD38, ADP-ribosyl cyclase, or NAD⁺ase). The levels of extracellular NAD⁺ reported in Table I seem in fact to reflect steady-state concentrations arising from a two-step process of release and enzymatic conversion (see "Discussion"). In any case, the results shown in Fig. 3 suggest the involvement of extracellular NAD⁺ and cADPR in the co-culture medium as responsible for calcium mobilization in the CD38⁻ target cells.

In order to correlate better extracellular cADPR to intracellular calcium release, the specific cADPR antagonists 8-NH_2-cADPR and 8-Br-cADPR were separately added to the co-culture medium. As shown in Fig. 4, supplementation of extracellular 8-NH_2-cADPR or of known membrane permeant (26) 8-Br-cADPR (both at 1 μM) for 24 h completely inhibited the $[Ca^{2+}]_i$ increase in the CD38⁻ target cells grown over the CD38⁺ feeder cells. These cADPR antagonists had no effect on $[Ca^{2+}]_i$ levels of target cells co-incubated over CD38⁻ feeder cells (not shown). Extracellularly added 8-NH_2-cADPR has been recently found to inhibit the $[Ca^{2+}]_i$ increase elicited by cADPR in human hemopoietic progenitors (14) and in tracheal smooth myocytes (15).

Role of Cx43 Hemichannels in the $[Ca^{2+}]_i$ Changes Observed in Mixed CD38⁺/CD38⁻ Co-cultures—The increases of $[Ca^{2+}]_i$ induced in the target CD38⁻ 3T3 fibroblasts by co-culturing with CD38⁺ feeder cells demonstrate a paracrine role of extracellular NAD⁺ and cADPR in the mechanism underlying these changes. Specifically, NAD⁺ release from cells followed by CD38-catalyzed generation of extracellular cADPR seem to be the required steps. Since NAD⁺ release has now been shown to take place in isolated 3T3 cells across hexameric hemichannels of Cx43 (12), we attempted to disrupt the paracrine effects of co-culture by inhibiting the NAD⁺-exporting activity of Cx43 hemichannels. Oleamide proved to block the $[Ca^{2+}]_i$ increase in our co-culture setting almost completely (Fig. 4). Moreover, the specific anti-Cx43 oligodeoxynucleotide inhibited the $[Ca^{2+}]_i$ increase in the target CD38⁻ cells, whereas the corresponding sense deoxynucleotide was totally uneffective (Fig. 4). These results give further support to the idea of Cx43-mediated export of cellular NAD⁺ and of subsequent generation of extracellular cADPR at the outer surface of the CD38⁺ feeder cells followed by influx of cADPR into the target CD38⁻ cells across a Cx43-unrelated transport system (Fig. 2).

Role of cADPR in the Changes Observed in Mixed CD38⁺/⁻ Co-cultures—In an effort to directly demonstrate this paracrine

TABLE I

Extracellular NAD⁺ and cADPR levels in the media from CD38⁺/CD38⁻ co-cultures and from control cultures

Determinations of extracellular NAD^+ and cADPR concentrations were performed as described under "Experimental Procedures." Mean results ±S.D. of duplicate assays from four different experiments are shown. ND, not detectable. The percentage of cell lysis in the media was ≤0.5% as determined by release of hexokinase activity (see "Experimental Procedures"). Accordingly, the NAD^+ and cADPR concentrations measured in the media were not determined by cell lysis during the co-cultures.

Type of culture	NAD^+ 72 h[a]	cADPR		
		24 h	48 h	72 h
	nM		*nM*	
$CD38^+/CD38^-$	146 (±15)	3.1 (±0.4)	5.0 (±0.6)	6.0 (±0.8)
$CD38^-/CD38^-$	138 (±12)	ND	ND	ND
$CD38^+/CD38^-$ + NAD^+-ase	120 (±13)	0.9 (±0.2)	1.2 (±0.3)	1.8 (±0.3)
$CD38^-/CD38^-$ + cyclase	136 (±16)	1.1 (±0.2)	2.8 (±0.4)	3.5 (±0.4)

[a] Concentrations of extracellular NAD^+ at 24 and 48 h of culture were closely similar to those measured after 72 h. Therefore, they are not shown for the sake of clarity.

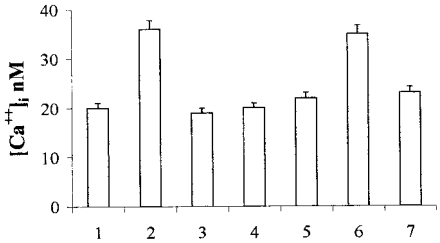

FIG. 4. **Effect of cADPR antagonists and Cx43 inhibitors on the $[Ca^{2+}]_i$ increase in $CD38^-$ 3T3 cells co-cultured over $CD38^+$ feeders.** $CD38^-$ target cells were co-cultured for 24 h on $CD38^-$ feeder cells (*column 1*) or $CD38^+$ cells in the absence (*column 2*) or presence of 1 μM 8-NH_2-cADPR (*column 3*), or 1 μM 8-Br-cADPR (*column 4*), or 50 μM oleamide (*column 5*). $CD38^-$ target cells pre-treated with Cx43 antisense (*column 6*) or sense (*column 7*) oligodeoxynucleotide were co-cultured 24 h on $CD38^+$ feeder cells pre-treated with the same oligodeoxynucleotide. $[Ca^{2+}]_i$ measurements were carried out as described (10). Values are means ± S.D. of five different experiments.

mechanism, we measured intracellular cADPR in the target $CD38^-$ cells during the co-culture experiments. $CD38^-$ cells were incubated on 75-mm diameter transwell plates over pre-established $CD38^{-/+}$ feeder layers for 48 h in the same conditions used for cell cycle analysis (see below). Cell extracts were then analyzed by HPLC. The intracellular cADPR concentration was undetectable in the $CD38^-$ target cells co-cultured over homologous $CD38^-$ layers (controls), whereas it was estimated to be 2.1 ± 0.1 picomoles/mg in the $CD38^-$ grown on $CD38^+$ feeders. This value is in the range of reported intracellular concentrations of cADPR in constitutively $CD38^+$ human lymphoid and myeloid cell lines (27, 28).

Effect of the Co-culture Over $CD38^{+/-}$ Feeders on the Proliferation of $CD38^-$ 3T3 Cells—De novo expression of CD38 has been demonstrated to enhance the rate of proliferation of some cell types, including 3T3, via increases of $[Ca^{2+}]_i$ elicited by intracellular cADPR (10). In order to investigate whether the calcium mobilization in $CD38^-$ cells which is induced by cADPR generated and provided by $CD38^+$ cell feeders could interfere with cell growth, we assayed proliferation of $CD38^-$ 3T3 target cells co-cultured with $CD38^{+/-}$ 3T3 feeder cells. A significant ($p < 0.05$) increase in cell proliferation was observed in $CD38^-$ fibroblasts co-cultured over $CD38^+$ cells for 72 h as compared with the same $CD38^-$ cells grown on homologous $CD38^-$ feeders (Fig. 5*A*). This increase was inhibited by addition of NAD^+ase to the medium, with a maximum effect being recorded after 24 h of culture ($p < 0.05$). The reduced extent of inhibition afforded by NAD^+ase at 48 and 72 h, despite the appearance of detectable levels of extracellular cADPR (Table I), might reflect some compensatory mechanisms promoting cell growth and either located downstream of $[Ca^{2+}]_i$ levels or independent of them.

Involvement of both extracellular NAD^+ and cADPR in the

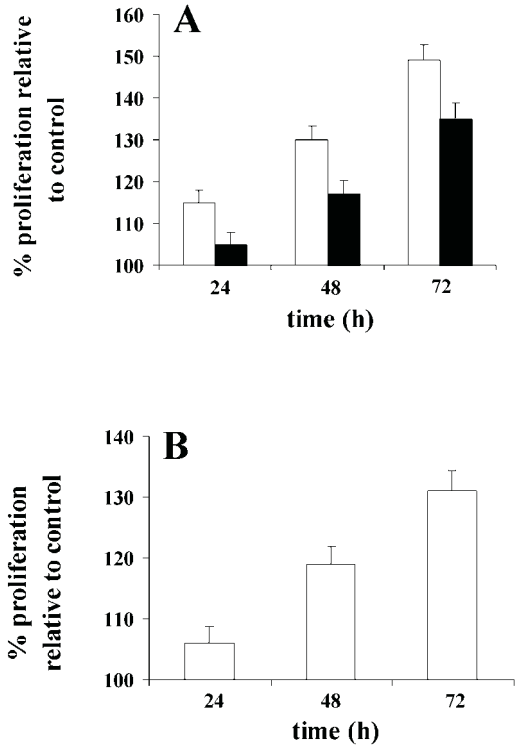

FIG. 5. **Modulation of cell growth of $CD38^-$ 3T3 cells on $CD38^{+/-}$ feeders.** *A,* $CD38^-$ target cells were co-cultured on $CD38^+$ feeders in the absence (*white columns*) or presence of NAD^+ase (*black columns*). *B,* $CD38^-$ target cells were co-cultured on $CD38^-$ feeders in the absence (control) or presence of recombinant ADP-ribosyl cyclase. Results of 10 different experiments are expressed as percentages of proliferation compared with control cells.

growth-enhancing effect was demonstrated by the significantly ($p < 0.05$) higher rate of proliferation that was observed upon co-culturing $CD38^{-/-}$ cells yet in the presence of recombinant ADP-ribosyl cyclase added to the medium (Fig. 5*B*). As observed with NAD^+ase supplementation (Fig. 5*A*), also in these experiments there was a weak quantitative correlation between time-dependent increases of $[Ca^{2+}]_i$ (Fig. 3) and corresponding stimulation of cell growth. Both apparent discrepancies of timing and of extent of effects seem to reflect additional, probably $[Ca^{2+}]_i$-unrelated mechanisms involved in the control of cell proliferation, especially on a long time scale. However, the growth-promoting role of extracellular cADPR was demonstrated by complete abolition of the increases of proliferation afforded by $CD38^+$ feeders on $CD38^-$ target cells that was observed at all times investigated upon adding 8-NH_2-cADPR (1 μM) to the co-culture media (not shown).

Therefore, the NAD^+/cADPR-mediated paracrine cross-talk leading to increases of $[Ca^{2+}]_i$ in $CD38^-$ target cells proved to

TABLE II
DNA synthesis time (T_S) of CD38⁻ 3T3 cells co-cultured over CD38⁺/⁻ feeders

Exponentially growing CD38⁻ target cells were labeled for 30 min with 30 μM BrdUrd. At the times indicated cells were fixed, processed with anti-BrdUrd monoclonal antibody and propidium iodide (DNA content). Measurements of T_S were performed from the relative movement (RM) as described under "Experimental Procedures."

Experiment no.	3T3 feeder cells	RM_{T0}	RM_{T4h}	T_S
				h
1	CD38⁻	0.49	0.56	28
	CD38⁺	0.47	0.61	13
2	CD38⁻	0.49	0.59	20
	CD38⁺	0.39	0.61	7
3	CD38⁻	0.51	0.59	26
	CD38⁺	0.45	0.56	16
4	CD38⁻	0.48	0.58	19
	CD38⁺	0.28	0.56	4

have an important functional outcome, *i.e.* enhanced cell proliferation.

Cell Cycle Analysis—These experiments were performed in co-culture conditions different from those followed for cell proliferation assays (see "Experimental Procedures"). Specifically, a larger culture surface (plates of 75 mm diameter) and an incubation for 72 h were chosen in order to achieve a number of CD38⁻ target cells sufficient for fluorimetric detection of BrdUrd. These conditions resulted in a $[cADPR]_e$ concentration of 18 ± 2.1 nM (not shown).

In Table II the results of four different BrdUrd pulse labeling experiments are reported. In CD38⁻ target cells co-cultured over CD38⁻ feeder cells the RM showed a low variability among the four experiments, and the corresponding T_S values ranged from 19 to 28 h with a mean value of 23 ± 4.4 h. In CD38⁻ target cells co-cultured with CD38⁺ feeder cells the RM showed a wide variability, indicating a less uniform progression through the cell cycle, and the corresponding T_S values ranged from 4 to 16 h with a mean value of 10 ± 5.4 h. Statistic significance of values obtained with the two cell populations ($p < 0.01$) confirmed a shorter S phase for the CD38⁻ target population over CD38⁺ feeders as compared with the control one over CD38⁻ cells.

DISCUSSION

The present investigation was focused on a simple, yet informative, model of paracrine communication impacting on cell growth. Mixed co-culture of two populations of 3T3 fibroblasts differing from each other for CD38 expression on their plasma membrane and use of transwell systems avoiding contact between the two cell populations allowed us to address the occurrence of a NAD⁺/cADPR-related paracrine cross-talk. In addition, use of selected reagents designed to modulate the corresponding effects in the target CD38⁻ cells (NAD⁺ase, ADP-ribosyl cyclase, $8NH_2$-cADPR, and 8-Br-cADPR) enabled us to dissect the individual steps of this intercellular communication and to identify specific roles of NAD⁺ and of cADPR therein.

Fig. 6 depicts the conclusions of this study. Cx43 hemichannels mediate the release of NAD⁺ from feeder cells, thus making it available to the ectocellular active site of CD38 in their plasma membrane (12). Subsequent cADPR generation is followed by its channeling across oligomeric CD38 to reach the cytosol of the feeder cell, thus completing an autocrine loop (13), and also by appearance of cADPR in the extracellular medium. The third step is permeation of cADPR across the plasma membrane of target CD38⁻ cells (Fig. 1), as previously suggested to occur in several cell types responding to extracellular cADPR with calcium mobilization and in some cases with

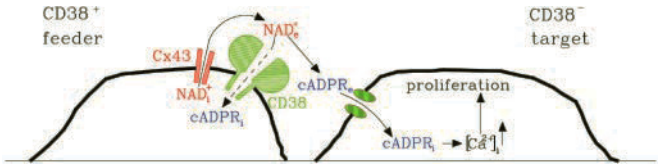

FIG. 6. **The NAD⁺/cADPR/$[Ca^{2+}]_i$ relationship and its role in regulation of proliferation of CD38⁻ target 3T3 fibroblasts co-cultured with CD38⁺ feeders.** For details see text.

remarkable changes in cell functions (14, 15, 24, 25).

The paracrine model summarized in Fig. 6 envisages new roles for NAD⁺ as a cell-to-cell communication signal mimicking a hormone, whereas extra/intracellular cADPR represents its second messenger, and intracellular calcium behaves as a third messenger regulating selected cell functions (3, 9, 29). Recently, a comparable yet intracellularly localized loop has been described in rat heart mitochondria, where opening of the permeability transition pore is followed by release of intramichondrial NAD⁺. This dinucleotide can accordingly behave as substrate for the NAD⁺-glycohydrolase located outside the matrix space (30) which has been shown to express ADP-ribosyl cyclase activity (31). Therefore, release of NAD⁺ through mitochondrial permeability transition pore is expected to produce cytosolic cADPR with consequent calcium release from the sarcoplasmic reticulum (30).

Although the paracrine process involving NAD⁺ and cADPR is demonstrated by the present results, further studies are required to elucidate completely the quantitative aspects of this novel mechanism of cell-to-cell communication. A challenging point is represented by extracellular levels of NAD⁺ and cADPR. Both represent steady-state concentrations resulting from a three-step process, *i.e.* efflux of NAD⁺ from feeder cells, its ectocellular conversion to cADPR, and eventually permeation of the cyclic nucleotide across the plasma membrane of target cells. Therefore, stability of $[NAD^+]_e$ in the co-culture experiments where it is measurably converted to cADPR could be explained by an enhanced, Cx43-mediated release from feeder cells resulting from a continuous and steep gradient of NAD⁺ concentrations across their plasma membrane. This enhanced efflux seems to mimic closely the experimental situation previously observed upon submitting cultured CD38⁻ fibroblasts to extensive and repeated washings (11). Another, probably related issue is that the ectocellular ADP-ribosyl cyclase activity of CD38 in the feeder cells is apparently working at largely non-saturating concentrations of NAD⁺ (Table I), since its reported K_m is 14 μM NAD⁺ (19). Accordingly, generation of cADPR in the extracellular space could play a limiting role in this complex process. Finally, the next step, *i.e.* clearance of extracellular cADPR by the CD38⁻ target cells, requires elucidation of the transport system responsible for cADPR influx (Fig. 1) whose molecular properties are as yet unknown.

With respect to this, an interesting feature is the remarkably high efficiency in the co-culture system of extracellular cADPR concentrations as low as 4.0–6.0 nM (Table I) in triggering $[Ca^{2+}]_i$ increases that are comparable in extent to those elicited by cADPR added at concentrations several orders of magnitude higher. Indeed, pulse addition of extracellular cADPR below 0.5 μM was totally uneffective on $[Ca^{2+}]_i$ levels of CD38⁻ cells (not shown). A closely comparable situation has been recently reported for the potent hemopoietic inhibition mediated by interferon-γ (IFN-γ) constitutively expressed in the stromal microenvironment of human bone marrow cultures (32). In this case, similar decreases of early hemopoietic progenitors were observed with 20 units/ml of endogenous IFN-γ as with exogenous concentration of 200 units/ml added every day or with

1,000 units/ml added weekly (32). Reasons for this concentration disparity of IFN-γ (32) and of cADPR as well (this study) are as yet undefined and might depend on the physical presentation of both signal molecules to their target cells. Specifically, cADPR concentrations in the co-culture media could be in fact non-homogeneous and locally higher in proximity to the CD38$^-$ cells. An alternative explanation might be provided by additional compounds being involved in sensitizing this signaling process; simultaneous release from feeder CD38$^+$ cells of other signal metabolites enhancing cADPR efficiency, *e.g.* dimeric ADP-ribose (33), could make the difference with exogenously supplemented cADPR.

The enhanced cell proliferation induced by CD38$^+$ feeders in CD38$^-$ target cells was prevented by the presence of 8-NH_2-cADPR in the medium. This demonstrates a direct and causal relationship between cADPR generated extracellularly by the CD38$^+$ feeder, $[Ca^{2+}]_i$ increase, and enhanced proliferation of CD38$^-$ cells. As far as the latter process is concerned, the extent of increase of cell proliferation recorded in the BrdUrd experiments following 72 h of co-culture of CD38$^-$ 3T3 cells over CD38$^+$ feeders, accounting for ~100% (Table II), does not match with the 50% increase observed after the same time of co-culture in the experiments measuring total DNA content (Fig. 5*A*). This discrepancy can be reasonably due to the different co-culture conditions used in the two types of experiments and especially to the larger culture surface (75-mm diameter plates) required to obtain a number of CD38$^-$ target cells sufficient for fluorimetric detection of BrdUrd. Specifically, the higher number of CD38$^+$ feeder cells obtained at the end of the 72-h co-culture generated comparatively higher $[cADPR]_e$, as witnessed by a concentration of 18 ± 2.1 nM *versus* 6.0 ± 0.8 nM (Table I). Finally, the time-dependent increase of the proliferation rate measured at 24, 48, and 72 h in CD38$^-$ target cells co-cultured over CD38$^+$ feeders (Fig. 5*A*), which likely reflects a progressive shortening of the S phase of the cell cycle until reaching a T_S value of 10 ± 5.4 h at 72 h (Table II), parallels the increase of $[cADPR]_e$ in the media (Table I).

The growth-enhancing effect featured by CD38$^+$ cell feeders on CD38$^-$ target fibroblasts by virtue of a paracrine NAD$^+$/cADPR mechanism may have important functional consequences that should extend beyond our model system of co-culture. For instance, in bovine tracheal strips, we were able to show that co-incubation of mucosa CD38$^+$ fragments with smooth myocytes induces the NAD$^+$/cADPR-mediated increase of $[Ca^{2+}]_i$ in these cells (15). Moreover, a cADPR-dependent expansion of human hemopoietic progenitors grown on CD38$^+$ stroma cells has been recently observed in our laboratory (34). The mechanism underlying the calcium-related stimulation of cell growth proved to be a significant shortening of the S phase of the cell cycle (Table II). A comparable change had been observed in an earlier study exploring the biochemical consequences of *de novo* expression of CD38 obtained upon transfecting constitutively CD38$^-$ cells, *i.e.* murine 3T3 fibroblasts and human HeLa cells (10). Therefore, $[Ca^{2+}]_i$ increases that follow either enhanced intracellular traffic of NAD$^+$ and cADPR (9) or an extracellular exchange of both signal metabolites can trigger an increased cell proliferation via a significant shortening of the S phase of cell cycle.

REFERENCES

1. Jackson, D. G., and Bell, J. I. (1990) *J. Immunol.* **144,** 2811–2815
2. Mehta, K., Shahid, U., and Malavasi, F. (1996) *FASEB J.* **10,** 1408–1417
3. Lee, H. C. (1997) *Physiol. Rev.* **77,** 1133–1164
4. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F., and Lee, H. C. (1995) *J. Biol. Chem.* **270,** 30327–30334
5. Prasad, G. S., McRee, D. E., Stura, E. A., Levitt, D. G., Lee, H. C., and Stout, C. D. (1996) *Nat. Struct. Biol.* **3,** 957–964
6. Munshi, C., Thiel, D. J., Mathews, I. I., Aahrus, R., Walseth, T. F., and Lee, H. C. (1999) *J. Biol. Chem.* **274,** 30770–30777
7. Walseth, T. F., Aahrus, R., Kerr, J. A., and Lee, H. C. (1993) *J. Biol. Chem.* **268,** 26686–26691
8. De Flora, A., Guida, L., Franco, L., and Zocchi, E. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1149–1166
9. De Flora, A., Franco, L., Guida, L., Bruzzone, S., Usai, C., and Zocchi, E. (2000) *Chem. Immunol.* **75,** 79–98
10. Zocchi, E., Daga, A., Usai, C., Franco, L., Guida, L., Bruzzone, S., Costa, A., Marchetti, C., and De Flora, A. (1998) *J. Biol. Chem.* **273,** 8017–8024
11. Zocchi, E., Usai, C., Guida, L., Franco, L., Bruzzone, C., Passalacqua, M., and De Flora, A. (1999) *FASEB J.* **13,** 273–283
12. Bruzzone, S., Guida, L., Zocchi, E., Franco, L., and De Flora, A. (2001) *FASEB J.* **15,** 10–12
13. Franco, L., Guida, L., Bruzzone, S., Zocchi, E., Usai, C., and De Flora, A. (1998) *FASEB J.* **13,** 1507–1520
14. Podestà, M., Zocchi, E., Pitto, A., Usai, C., Franco, L., Bruzzone, S., Guida, L., Bacigalupo, A., Scadden, D. T., Walseth, T. F., De Flora, A., and Daga, A. (2000) *FASEB J.* **14,** 680–690
15. Franco, L., Bruzzone, S., Song, P., Guida, L., Zocchi, E., Walseth, T. F., Crimi, E., Usai, C., De Flora, A., and Brusasco, V. (2001) *Am. J. Physiol.* **280,** L98–L106
16. Graeff, R. M., Walseth, T. F., and Lee, H. C.(1997) *Methods Enzymol.* **280,** 230–241
17. Fryxell, K., O'Donoghue, K., Graeff, R. M., Lee, H. C., and Branton, W. D. (1995) *Protein Expression Purif.* **6,** 329–336
18. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–252
19. Graeff, R. M., Walseth, T. F., Fryxell, K., Branton, W. D., and Lee, H. C. (1994) *J. Biol. Chem.* **269,** 30260–30267
20. Walseth, T. F., and Lee, H. C. (1993) *Biochim. Biophys. Acta* **1178,** 235–242
21. Guan, X., Cravatt, B. F., Ehring, G. R., Hall, J. E., Boger, D. L., Lerner, R. A., and Gilula, N. (1997) *J. Cell Biol.* **139,** 1785–1792
22. Zerez, C. R., Lee, S. J., and Tanaka, K. R. (1987) *Anal. Biochem.* **164,** 367–373
23. Begg, A. C., McNally, N. J., Shrieve, D. C., and Kaercher, H. (1985) *Cytometry* **6,** 620–626
24. Howard, M., Grimaldi, J. M., Bazan, J. F., Lund, F., Santos-Argumedo, L., Parkhouse, R. M., Walseth, T. F., and Lee, H. C. (1993) *Science* **26,** 1056–1059
25. De Flora, A., Guida, L., Franco, L., Zocchi, E., Pestarino, M., Usai, C., Marchetti, C., Fedele, E., Fontana, G., and Raiteri, M. (1996) *Biochem. J.* **320,** 665–672
26. Sethi, J. K., Empson, R. M., Bailey, V. C., Potter, B. V., and Galione A. (1997) *J. Biol. Chem.* **272,** 16358–16363
27. Da Silva, C., Potter, B. V., Mayr, G. W., and Guse, A. (1998) *J. Chromatogr. B Biomed. Appl.* **707,** 43–50
28. Takabashi, K., Kukimoto, I., Tokita, K., Inageda, K., Inoue, S., Kontani, K., Hoshino, S., Nishina, H., Kanaho, Y., and Katada, T. (1995) *FEBS Lett.* **371,** 204–208
29. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
30. Di Lisa, F., Menabò, R., Canton, M., Barile, M., and Bernardi, P. (2001) *J. Biol. Chem.* **276,** 2571–2575
31. Ziegler, M., Jorcke, D., and Schweiger, M. (1997) *Biochem. J.* **326,** 401–405
32. Selleri, C., Maciejewski, J. P., Sato, T., and Young, N. S. (1996) *Blood* **87,** 4149–4157
33. De Flora, A., Guida, L., Franco, L., Zocchi, E., Bruzzone, S., Benatti, U., Damonte, G., and Lee, H. C. (1997) *J. Biol. Chem.* **272,** 12945–12951
34. 34. Zocchi, E., Podestà, M., Pitto, A., Usai, C., Bruzzone, S., Franco, L., Guida, L., Bacigalupo, A., and De Flora, A. (2001) *FASEB J.*, in press