Equilibrative and Concentrative Nucleoside Transporters Mediate Influx of Extracellular Cyclic ADP-Ribose into 3T3 Murine Fibroblasts*

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In mammals cyclic ADP-ribose (cADPR), a universal calcium mobilizer from intracellular stores, is generated from NAD\(^+\) at the outer cell surface by the multi-functional ectoCD38 and by related ADP-ribosyl cyclases. Recently, influx of extracellular cADPR has been observed in 3T3 murine fibroblasts, where it elicits Ca\(^{2+}\)-mediated enhancement of proliferation. Here we addressed the nature and the properties of cADPR influx into CD38-3T3 cells, which showed pleiotropic mechanisms of both equilibrative and concentrative transport. Based on selective inhibitors or experimental conditions (e.g. abrogation of Na\(^+\)-dependent active symport processes and transient transfection experiments) and on reverse transcriptase-polymerase chain reaction analysis of transcripts in 3T3 fibroblasts and comparatively in HeLa cells, we identified cADPR-transporting activities with specific nucleoside transporters (NT), both equilibrative (ENT2) and concentrative (CNT2 and a nitrobenzylthioinosine (NBMPR)-inhibitable NT). A reciprocal inhibition relationship was observed between inosine and cADPR fluxes across these NT species. Concentrative (but not equilibrative) transport of nanomolar extracellular cADPR took place in CD38-3T3 cells co-cultured for 48 h in transwells on feeders of CD38-transfected, cADPR-generating 3T3 fibroblasts. These results suggest possible, hitherto unrecognized, correlations between ectocellular metabolism of nucleotides/nucleosides and cADPR-mediated regulation of intracellular calcium homeostasis.

Cyclic ADP-ribose (cADPR)\(^1\) is a potent and universal calcium mobilizer from intracellular stores (1–5). During phylogenetics, the physiological role of cADPR has evolved in parallel with the increasing complexity of the biological systems where it is present: from cell cycle regulator in Euglena gracilis (6), to signal molecule in response to environmental stress in higher plants (7) and in Porifera (8), or during oocyte fertilization in marine invertebrates (9). In mammals, cADPR is involved in a number of Ca\(^{2+}\)-dependent cell- and tissue-specific functions, including proliferation, contraction, and secretion (1–3, 10–12).

In several mammalian cells and tissue microenvironments, cADPR and its metabolic precursor NAD\(^-\) have recently been demonstrated to undergo a variable though potentially intense transmembrane trafficking (13). By virtue of these subcellular and intercellular movements, cADPR can access its ryanodine-sensitive intracellular Ca\(^{2+}\) stores/channels despite generation of this cyclic nucleotide by ADP-ribosyl cyclases at sites that are topologically opposite to the stores (13, 14). The most represented member of these cyclases in mammalian cells is CD38 (15), a type II glycoprotein involved in several transduction pathways (16) and a multifunctional ectoenzyme that synthesizes cADPR from NAD\(^-\) and also hydrolyzes cADPR to ADP-ribose (17, 18). An additional functional activity of CD38 is synthesis of the potent calcium mobilizer NAAADP\(^+\) from NADP\(^-\) and nicotinic acid (18, 19).

Availability of intracellular NAD\(^-\) to the ectocellular active site of CD38 is made possible by its controlled release from cells across an equilibrative transport system, represented by hexameric hemichannels of the gap junctional protein connexin 43 (Cx43) (20). Subsequent cADPR generation at the outer cell surface can be steadily followed by its influx into CD38-3T3 cells because of the peculiar property of transmembrane oligomeric CD38 to couple cADPR synthesis to its active translocation across the cell membrane to reach the Ca\(^{2+}\) stores in the intracellular environment (21). Such autocrine cross-talk between NAD\(^-\)-releasing Cx43 hemichannels and CD38 has been demonstrated to finely up-regulate the [Ca\(^{2+}\)]\(_i\) levels in 3T3 murine fibroblasts (22).

However, the peculiar localization and the properties of the catalytic site of CD38 suggest that a significant fraction of ectocellularly produced cADPR might escape transport across CD38 and thus be present in the extracellular environment, especially in close proximity of CD38-3T3 cells. This assumption was strongly supported by the finding of 40–60 nM concentrations of NAD\(^-\) in human blood plasma\(^2\) and by the recent demonstration that nanomolar concentrations of cADPR are...
cADPR Transport Across Nucleoside Transporters

Consistently detectable in the medium of heterologous transwell co-cultures of CD38+/CD38+ 3T3 fibroblasts (23). Both data raised the question whether extracellular cADPR can permeate across the plasma membrane of cADPR-responsive cells through mechanisms (either diffusion or transport) independent of those featured by CD38 as a catalytically active transporter of cADPR itself (21). Recently, such CD38-unrelated transport system was reported to be active in naïve, constitutively CD38+ 3T3 fibroblasts. This process proved to account for the increased [Ca2+]i levels and consequently for the enhanced proliferation of CD38+ cells when these are grown on cADPR-generating CD38+ cell feeders (23). A role for the CD38-independent cADPR transport system can be retrospectively extended to explain the Ca2+-related functional responses elicited by extracellular cADPR in a number of cell types: murine B lymphocytes (24), rat cerebellar granule cells (25), smooth myocytes in intact trabecula fragments (26), and human hemopoietic progenitors (27, 28).

These facts prompted us to start a study aiming to elucidate the properties and the nature of the transport system(s) whereby several cells can respond to extracellularly generated cADPR. The results reported here identify specific equilibrative and concentrative nucleoside transporters (NT) responsible for cADPR translocation: these NT are widely though variably expressed in mammalian cells (29–33), accounting for the widespread efficacy of extracellular cADPR observed in several cell types. Accordingly, a functional link is established between nucleosides and cADPR, which may bear novel and unexpected consequences on the control of Ca2+-related cell processes and on possible therapeutic strategies to control them.

EXPERIMENTAL PROCEDURES

Materials—[14C]Inosine was purchased from Moravek (Brea, CA). All other chemicals were obtained from Sigma.

Cell Cultures—NIH 3T3 fibroblasts, HeLa cells, and COS-7 cells, obtained from ATCC (Manassas, VA), were cultured as described (34).

Assays of GDP-ribosyl Cyclase Activity—The ectoenzymatic activity was estimated on intact CD38+/3T3 cells with NGD+- as substrate, as described (23).

cADPR Influx into Intact 3T3 Fibroblasts, HeLa Cells, and COS-7 Cells—Cells were harvested during exponential growth (50% confluence, a critical requirement to limit variability of results) by trypsin-EDTA treatment and resuspended (106 cells/ml, unless otherwise indicated) in 100 μl of Na+-buffer (135 mM NaCl, 6.3 mM KH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, 0.5 mM MgCl2, 0.9 mM CaCl2, 10 mM glucose, pH 7.4) or Li+-buffer (same as Na+-buffer), but with 155 mM LiCl instead of NaCl) in the presence of different cADPR concentrations at 22 °C. At different times after cADPR addition (from 0 to 30 min), 50-μl aliquots were withdrawn and centrifuged at 5,000 × g for 15 s. Pellets were resuspended in 1.5 ml of ice-cold appropriate buffer (Na+- or Li+-buffer) containing 10 μM uridine (except in experiments reported in Figs. 1 and 2) and submitted to two consecutive centrifugations as above in order to remove the supernatant completely. Pellets were resuspended in 200 or 500 μl of water for HPLC or enzymatic cycling assays, respectively, and the samples were sonicated for 30 s at 3 watts in ice. For HPLC analyses, aliquots of 180 μl were chromatographed on an anion exchange HPLC (see below). For enzymatic cycling assays, 480-μl aliquots were deproteinized with 0.6 M perchloric acid (final concentration). cADPR was detected by the enzymatic cycling assay recently described by Graeff and Lee (35), whose sensitivity is 10 fmol of cADPR. Protein content was determined in 20 μl aliquots according to Bradford (36). HPLC analyses were performed according to Ref. 37, slightly modified, on a Hewlett Packard 1090 instrument equipped with an HP1040 diode array spectrophotometric detector set at 260 nm, using a PL 1000-SAX column (8 μm, 1,000 Å, 50 × 5 mm). Solvent A was 20 mM NH4HCO3, pH 7.6, and solvent B was 500 mM NH4HCO3, pH 7.6; the solvent gradient program was a gradient starting at 100% solvent A linearly increasing to 10% solvent B in 10 min, then linearly increasing to 100% solvent B in 20 min and maintained constant for an additional 5 min. The flow rate was 0.5 ml/min. Identification and quantitation of the cADPR peak was obtained both by co-elution and comparison of UV absorption spectra with that of the standard compound. cADPR elution time was always completely separated from other nucleosides and nucleotides.

Sensitivity of this HPLC analysis was >10 pmol of cADPR per sample. cADPR Influx into Erythrocytes—Human blood samples were obtained after informed consent from normal volunteers, and murine blood was obtained from the axillary vein of C57 mice under ether anesthesia. Heparin was used as anticoagulant; blood samples were immediately centrifuged at 5,000 × g for 30 s, for erythrocytes were resuspended in isotonic Na+-buffer and washed three times. The experiments of cADPR influx were performed over a 30-min incubation as described for 3T3 and HeLa on 200-μl samples containing erythrocytes (at a 50% hematocrit) in Na+-buffer supplemented with 30 μM extra-cellular cADPR. Hemoglobin content in each sample was determined by a pulse-chromatographic cADPR assay in duplicate at 540 nm. Intraerythrocytic cADPR levels were estimated by HPLC on trichloroacetic acid extracts (see above).

Plasma was recovered from human blood, and 1-ml aliquots were deproteinized with 1.2 M perchloric acid (final concentration). cADPR levels in the plasma were determined by the enzymatic cycling assay (23).
sequenced on both strands of cDNA to verify the presence of correct insert. The pcDNA3.1 plasmid and the pcDNA3.1/mCNT2 construct were transfected into COS-7 cells by LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h cells were collected, and the expression of mCNT2 was monitored by RT-PCR, performed as described above (see “Reverse Transcriptase-Polymerase Chain Reaction Analyses”). The primers used for amplification of β-actin cDNA as control (GenBank™ accession number X00351) were: sense, 5′-AATGAGCTGCGTGTGGCTCC-3′ and antisense, 5′-CAATGGTGAATGGCAGGAAA-3′ (nucleotide positions 773–792). The PCR conditions for β-actin were: 30 s, 94 °C; 1 min, 72 °C, for 30 cycles.

Co-cultures of CD38+ with CD38− Cells—3T3 or HeLa cells, transfected with the antisense cDNA for human CD38 (34) (CD38 HeLa), were seeded at a 25% confluence in transwell plates (12-cm diameter) on pre-established confluent feeder layers of CD38− 3T3 cells (transfected with the sense cDNA for human CD38) in 90 ml of fresh complete medium and incubated for 48 h (23). Co-cultures were grown at 37 °C as such (controls) or exposed to 10 mM concentrations of NBMPR (and 300 μM DIDS (A)), or 100 μM dipyridamole (B). Cells were then incubated with 300 μM cADPR. At the times indicated, aliquots were withdrawn and cADPR content of each trichloroacetic acid cell extract was analyzed by HPLC, as described under "Experimental Procedures." Traces are means ± S.D. of five different experiments.

**RESULTS**

**Characterization of Influx of Extracellular cADPR into CD38− 3T3 Fibroblasts**—When CD38− 3T3 fibroblasts are exposed for up to 60 min to extracellular cADPR there is a progressive association of the cyclic nucleotide to cells (23). This is not the case upon incubating cADPR with isolated membranes from the same cells, a fact that, coupled to the sustained increase of [Ca2+]i, levels it elicits in the intact 3T3 fibroblasts, supports influx of cADPR into cells rather than cell surface adsorption. We first examined some general properties of the CD38-unrelated uptake of extracellular cADPR across the plasma membrane of intact CD38− 3T3 fibroblasts, in order to optimize the assay conditions. cADPR influx occurred to a maximal extent in a pH range between 7.4 and 8.1, with a 40% decrease of transport being measured at pH 7.1. No appreciable variations of cADPR influx were observed over a broad range of temperatures: therefore, the process was investigated at 22 °C.

Next, we addressed whether the uptake of extracellular cADPR was affected by known inhibitors of selected transport systems (Fig. 1). Influx of cADPR was neither affected by 4,4-di-iso-thiocyano-2,2-stilbene disulfonic acid (DIDS, an inhibitor of the anion exchanger, Ref. 42) nor by oleamide and β-glycerophosphoric acid (both inhibiting connexin channels and hemicannels, Refs. 43 and 44). On the contrary, the vasodilator dipyridamole (a well known inhibitor of equilibrative nucleoside transporters, Ref. 45) reduced the extent of cADPR uptake to 50% of control values at all time points. A similar extent of inhibition was observed with 100 μM dipyridamole (and 300 μM extracellular cADPR, Fig. 1) or with 20 μM dipyridamole (and 20 μM extracellular cADPR, not shown). In the latter experimental condition the cell-associated cADPR was measured by means of a new, highly sensitive cADPR assay based upon enzymatic cycling (35).

**cADPR Influx Is Mediated by Nucleoside Transporters**—Both equilibrative and concentrative nucleoside transport processes have been demonstrated, identified and characterized in mammalian cells (29–33, 46). The equilibrative nucleoside transporting proteins are broadly selective, widely expressed in different cells and include ENT1 (ex, i.e. equilibrative and sensitive to inhibition by nanomolar concentrations of nitrobenzoxylithoiosine, NBMPR) and ENT2 (ei, equilibrative, NBMPR-insensitive). The family of concentrative NTs com-

![Fig. 1. Time-dependent uptake of extracellular cADPR by 3T3 fibroblasts.](https://www.jbc.org/content/1995/2/7099/F1.large.jpg)

*4.4-di-isothiocyanato-2,2-stilbene disulfonic acid (DIDS, an inhibitor of the anion exchanger, Ref. 42) nor by oleamide and β-glycerophosphoric acid (both inhibiting connexin channels and hemicannels, Refs. 43 and 44). On the contrary, the vasodilator dipyridamole (a well known inhibitor of equilibrative nucleoside transporters, Ref. 45) reduced the extent of cADPR uptake to 50% of control values at all time points. A similar extent of inhibition was observed with 100 μM dipyridamole (and 300 μM extracellular cADPR, Fig. 1) or with 20 μM dipyridamole (and 20 μM extracellular cADPR, not shown). In the latter experimental condition the cell-associated cADPR was measured by means of a new, highly sensitive cADPR assay based upon enzymatic cycling (35).*

**Table 1**

| cDNA     | GenBank™ accession number | Primer positions (5' to 3') | PCR product, size bp | Primer sequence (5' to 3') |
|----------|---------------------------|----------------------------|----------------------|---------------------------|
| mENT1    | AF151213                  | 318–327                    | 421                  | AGCTGTCCCCAGAATGTTGCC    |
| mENT2    | AF183397                  | 738–719                    | 303                  | CCAAGCAGGCCCTGGCCACT     |
| mCNT2    | AF079853                  | 449–467                    | 404                  | TGGGACGCTGCTGGCCAT       |
| mCNT3    | AF305211                  | 751–731                    | 176                  | CACAGGGTGTTAGAAGTATCC    |
| hENT1    | NM_004955                 | 272–253                    | 666                  | AGCTGTCCCAAGAATGTTCC     |
| hENT2    | AF029358                  | 974–953                    | 342                  | ATTCCTCTTGGCTGCTTTGG     |
| hCNT1    | U62966                    | 751–731                    | 434                  | GATTCGCTTCTCAGTCCACAGAG |
| hCNT2    | NM_004212                 | 2130–2105                  | 533                  | TACTCAACCTCTTCAGTCCACAG |
| hCNT3    | AF305210                  | 1712–1731                  | 302                  | GTGAACAGTGGTGGCTAACCAGG |

*Figure 1: Time-dependent uptake of extracellular cADPR by 3T3 fibroblasts. Intact 3T3 fibroblasts (105 cells/ml) were preincubated for 15 min at 22 °C in Na+-buffer without addition (●) or in the presence of 50 μM DIDS (●), or 50 μM oleamide and 50 μM 18-β-glycyrrhetinic acid (●A), or 100 μM dipyridamole (●B). Cells were then incubated with 300 μM cADPR. At the times indicated, aliquots were withdrawn and cADPR content of each trichloroacetic acid cell extract was analyzed by HPLC, as described under "Experimental Procedures." Traces are means ± S.D. of five different experiments.*
Fig. 2. Effect of nucleosides on cADPR influx into 3T3 fibroblasts. 3T3 fibroblasts were incubated for 5 min at 22 °C in the presence of 10 μM cADPR in Na\(^+\)/K\(^-\) buffer in the absence (control) or in the presence of 10 μM of various nucleosides (uridine, inosine, thymidine, or guanosine). Washings of the cell pellets were performed without uridine. Uptake of cADPR in the controls was 8.5 ± 1.1 pmol/mg/5 min. cADPR was measured in the perchloric acid-deproteinized cell extracts by the enzymatic cycling assay (35). Values are means ± S.D. of six different experiments.

Fig. 3. Effect of selective inhibitors of equilibrative and concentrative nucleoside transporters on influx of cADPR into 3T3 fibroblasts. 3T3 cells were preincubated for 15 min at 22 °C in Na\(^+\) buffer in the absence (○) or in the presence of 100 μM dipyridamole (●), or in Li\(^+\)-buffer, in the absence (□) or in the presence of 100 μM dipyridamole (■). Cells were incubated with 300 μM cADPR, and, at the times indicated, aliquots were withdrawn, cell pellets were washed with the appropriate buffer (either Na\(^+\)- or Li\(^+\)-buffer) containing uridine (see “Experimental Procedures”), and the cADPR content was determined in each deproteinized sample by HPLC. Traces are means ± S.D. of six different experiments.

supplemented in Na\(^+\)-free buffers (Fig. 3). Since uptake was not completely abolished in this condition; however, a minimal passive diffusion mechanism of cADPR influx in 3T3 fibroblasts cannot be ruled out.

The same approach, i.e. use of selective conditions to dissect equilibrative from concentrative cADPR-translocating activities, was followed in HeLa cells. These cells have been reported to express only the equilibrative transporters ENT1 and ENT2, while lacking known concentrative NT (50). Indeed, 100 μM dipyridamole, known to inhibit hENT1 and hENT2 (51), completely abrogated translocation of extracellular cADPR into HeLa cells (Fig. 4). Replacement of Na\(^+\) by Li\(^+\) in the buffer had no effect on cADPR influx, as expected for cells expressing only equilibrative transporters.

The differences between 3T3 fibroblasts and HeLa cells in susceptibility of cADPR transport to specific inhibitors of equilibrative and concentrative NT suggested to compare the influx of cADPR at different extracellular concentrations in the two cell types. Whereas in HeLa cells this showed a clearly monophasic behavior, uptake of cADPR by 3T3 fibroblasts occurred consistently according to a biphasic pattern (Fig. 5). Thus, a saturable component was always detected in the nanomolar range of extracellular cADPR concentrations, with an apparent \(K_m\) of 0.25 ± 0.04 μM cADPR. This was followed by a second type of cADPR transport, occurring at extracellular cADPR concentrations above 5 μM and almost superimposable to that recorded in HeLa cells. Half-saturation of the second component of cADPR influx into both cell types was achieved at 2–3 mM concentrations of cADPR (not shown). The sharp differences observed in the two cell types indicated that cADPR translocation occurs across multiple NT in the 3T3 fibroblasts, one of which is kinetically identified as concentrative by saturation at low cADPR concentrations and the other one reflects an equilibrative process, showing comparatively much lower affinity for the cyclic nucleotide. As illustrated in Fig. 5, HeLa cells express only the equilibrative type of cADPR transport, in agreement with the data shown in Fig. 4.

Reverse Transcriptase-PCR Analyses of Nucleoside Transporter Transcripts in 3T3 and HeLa Cells—Next, we explored...
cADPR Transport Across Nucleoside Transporters

mRNA expression of the five molecularly defined mNT (ENT1, ENT2, CNT1, CNT2, and CNT3) in 3T3 fibroblasts and, by comparison, of the corresponding hNT in HeLa cells, previously reported to express only the two equilibrative NT (50). In fact, this was not the case as the HeLa cell line in our hands also expressed CNT3, in addition to ENT1 and ENT2 (Fig. 6). However, cADPR influx into these HeLa cells did not occur via a concentrative mechanism because it was unaffected by replacement of Na⁺ with Li⁺ (Fig. 4). Thus, CNT3 does not seem to be competent for cADPR transport. The 3T3 fibroblasts had a different pattern of expression, i.e. both equilibrative transporters ENT1 and ENT2 and in addition CNT2, but not CNT3. Since CNT1 was absent in the 3T3 fibroblasts, the observed

![Fig. 4. Time course of cADPR uptake by HeLa cells.](image)

Fig. 4. Time course of cADPR uptake by HeLa cells. Intact HeLa cells (10⁶ cells/ml) were preincubated for 15 min at 22 °C in Na⁺-buffer in the absence (○) or in the presence of 100 μM dipyridamole (■), or in Li⁺-buffer without any addition (□). Cells were incubated with 300 μM cADPR, and, at the times indicated, aliquots were withdrawn, as indicated in the legend to Fig. 3, washed with the appropriate buffer (either Na⁺- or Li⁺-buffer), and deproteinized with 10% trichloroacetic acid and analyzed for cADPR content by HPLC. Traces are means ± S.D. of six different experiments.

![Fig. 5. Concentration-dependent uptake of extracellular cADPR by 3T3 fibroblasts and HeLa cells.](image)

Fig. 5. Concentration-dependent uptake of extracellular cADPR by 3T3 fibroblasts and HeLa cells. 3T3 (A) and HeLa (B) cells (3 x 10⁶ cells/ml) were incubated for 5 min at 22 °C with different concentrations of extracellular cADPR as described under “Experimental Procedures.” CADPR levels in each deproteinized cell extract were estimated by the enzymatic cycling assay (35). Traces are the mean of four different experiments; no S.D. values are shown for the sake of clarity.

![Fig. 6. Expression of nucleoside transporters in 3T3 fibroblasts and in HeLa cells.](image)

Fig. 6. Expression of nucleoside transporters in 3T3 fibroblasts and in HeLa cells. Reverse-transcriptase PCR, analyzed by agarose gel electrophoresis and ethidium bromide staining, was carried out as reported under “Experimental Procedures.” RNA from 3T3 (A) and HeLa (B) cells was reverse-transcribed, and cDNA was subjected to PCR using ENT1, ENT2, CNT1, CNT2, and CNT3 specific primers (see also Table I). Na⁺/cADPR symport activity (Fig. 3) cannot be featured by this pyrimidine nucleoside selective transporter. This assumption is also supported by the fact that thymidine (accepted by ENT1, ENT2, and CNT1) did not substantially affect cADPR influx (Fig. 2). Absence of CNT1, failure of CNT3 to mediate concentrative cADPR uptake and the mRNA patterns observed in extracts from 3T3 fibroblasts (Fig. 6) might suggest the involvement of CNT2 in concentrative cADPR transport, but expression of the two other not yet cloned concentrative proteins responsible for csg and cs activities (47–49) could not be investigated (see below). On the contrary, HeLa cells and 3T3 fibroblasts equally expressed the two equilibrative transporters ENT1 and ENT2 and therefore both of them emerged as reasonable candidates for the equilibrative cADPR-transporting activity actually identified in the two cell lines (Figs. 1–4). ENT2, but Not ENT1, Mediates cADPR Influx—In an attempt to dissect the roles of ENT1 and ENT2 in cADPR transport, we exploited their distinctive susceptibility to inhibition by NBMPR, with ENT1 being inhibited in human cells by nanomolar NBMPR and ENT2 requiring much higher concentrations of NBMPR (micromolar) to be down-regulated (51). HeLa cells were investigated to this purpose because they lack any concentrative type of cADPR influx (Figs. 4 and 5), this simplifying our comparative analysis of ENT1 versus ENT2. As shown in Fig. 7A, NBMPR at 10 nM failed to inhibit influx of extracellular cADPR which, on the contrary, was almost completely abrogated by 20 μM NBMPR. These findings implicate ENT2 as the only equilibrative NT responsible for cADPR uptake. Unequivocal confirmation of this view was obtained by additional experiments in which 300 μM extracellular cADPR failed to cross the membrane of human erythrocytes where the only NT present is ENT1 (30): more specifically, we detected only 2 μM intraerythrocytic cADPR (a level, which might be due to passive diffusion) following a 30-min incubation with 300 μM extracellular cADPR, while by comparison a 200 ± 15 μM intracellular cADPR concentration was measured in HeLa cells. Identical results were obtained with murine erythrocytes, which proved to be virtually impermeable to extracellular cADPR (Fig. 7B).

Correlation between Inosine and cADPR Fluxes in 3T3 Fibroblasts—The occurrence of cADPR transport across NT prompted us to investigate whether inosine fluxes through the plasma membrane of 3T3 cells were affected by extracellular cADPR. Influx of 10 μM [14C]inosine was ~80% inhibited either
by 100 nM dipyridamole (blocking ENT1 function) (45) or by replacing Na\(^+\) with Li\(^+\) in the buffer (abolishing Na\(^+\)/nucleoside concentrative symport activities) (Table II). Extracellular cADPR at 5 mM was a poor inhibitor of inosine influx because this nucleoside can freely permeate into 3T3 fibroblasts across ENT1, which proved not to accept cADPR (see above). However, when ENT1 was inhibited by 100 nM dipyridamole, cADPR completely abrogated the residual uptake of inosine (Table II). Therefore, a reciprocal inhibition exists between influxes of inosine (see also Fig. 2) and of cADPR, respectively, across the plasma membrane of cells expressing both equilibrative and concentrative transport systems.

**Properties of Concentrative cADPR-transporting System(s)—**

After identifying ENT2 as the only equilibrative transport system that accepts cADPR, and cADPR uptake was evaluated on perchloric acid-deproteinized samples by the enzymatic cycling assay (35). Values are means ± S.D. of four different experiments.

### Table II

| Addition | \[^{[14]C}\]inosine Inhibition |
|----------|---------------------------|
| None     | 0.226 ± 0.032             |
| Dipyridamole (100 nM) | 0.041 ± 0.005    |
| Li\(^+\)-buffer | 0.051 ± 0.006  |
| cADPR (5 mM) | 0.200 ± 0.035  |
| cADPR (5 mM) + NBMPR | N.D. \(^a\) | 100 |

\(^a\) Not detectable.

Control COS-7 cells expressed a weak level of CNT2 (Fig. 9A, lane 5), both ENT1 and ENT2, but not CNT1 and CNT3 (not shown). RT-PCR analyses on pcDNA3.1/mCNT2-transfected cells showed a fairly abundant expression of mCNT2 (Fig. 9A, lane 2). The control cells transfected with the plasmid backbone featured NBMPR-inhibitable \[^{14}C\]inosine transport, whereas the COS-7 cells transfected with the pcDNA3.1/mCNT2 construct acquired, by comparison, a remarkably higher Na\(^+\)-dependent inosine-transporting activity, thus confirming transient expression of functionally active CNT2. cADPR uptake assays were carried out in the presence or in the absence of Na\(^+\) and in the presence of 10 mM NBMPR in order to abrogate the endogenous concentrative transport activity present in COS-7 cells and related to ENT2. Whereas in these conditions the control cells transfected with pcDNA3.1 showed in Na\(^+\)-buffer a cADPR influx of 49 ± 7 pmol/mg of protein/5 min, the uptake of the cyclic nucleotide in Na\(^+\)-buffer was comparably much higher in the COS-7 cells transfected with pcDNA3.1/mCNT2 (Fig. 9B). This cADPR influx decreased extensively in Li\(^+\)-buffer (from 98 ± 12 to 12 ± 3 pmol of cADPR/mg of protein), in agreement with the high Na\(^+\) dependence of transiently expressed CNT2. The corresponding change of cADPR uptake by the control, plasmid-transfected cells was from 49 ± 7 (Na\(^+\)-buffer) to 15 ± 3 (Li\(^+\)-buffer) pmol of cADPR/mg of protein. Therefore, the pcDNA3.1-transfected...
COS-7 cells apparently show a significant level of an endogenous Na+-dependent cADPR transport (Fig. 9B), quite similar to that observed earlier for nucleoside transport in native COS-1 cells (41), which might be related to basally expressed CNT2.

On the whole, the transient transfection experiments indicated that CNT2 per se features Na+-dependent cADPR-transporting activity, thus potentially cooperating in the 3T3 fibroblasts (although to a so far undetermined extent) with the different concentrative, NBMPR-sensitive system (see above) in mediating influx of extracellular cADPR. CD38−3T3 fibroblasts, but Not HeLa Cells, Accumulate cADPR When Grown in Co-culture over CD38+ Feeders—Influx of extracellular cADPR into CD38−3T3 fibroblasts (23) or CD38low human hemopoietic progenitors (28) was recently demonstrated to occur upon transwell co-culturing either cADPR-responsive cell type over a feeder of CD38-transfected, cADPR-generating 3T3 fibroblasts. This influx causes a Ca2+-mediated increase of cell proliferation in both experimental systems. Therefore, we comparatively measured the intracellular cADPR concentrations either in CD38−3T3 fibroblasts or in CD38−HeLa cells grown for 48 h on CD38−3T3 fibroblasts as feeders (whose ectocellular levels of GDP-ribosyl cyclase activity were 11.8 ± 2.7 nmol of cADPR/min/mg of protein), in the presence and in the absence, respectively, of selective inhibitors of NT. The effect of the replacement of Na+ with Li+, as well as of the addition of guanosine, on concentrative cADPR-transporting activity could not be evaluated, due to the extensive cytotoxicity induced by both treatments over extended time periods. On the contrary, Li+-buffers did not affect at all cell viability during the short-term (up to 30 min) experiments of cADPR transport (Figs. 3 and 4).

As reported in Table III, HeLa cells, expressing ENT1, ENT2, and CNT3, did not accumulate any cADPR over the extended time of transwell co-cultures. On the contrary, 3T3 fibroblasts accumulated cADPR, as previously reported to occur in this experimental setting (23), well above the concentrations of extracellular cADPR in the co-culture medium, which were as low as in the nanomolar range. Based on the cell volume of 3T3 fibroblasts (34), the intracellular cADPR content reached a concentration of 0.53 ± 0.11 μM after a 48-h co-culture with CD38+3T3 cells. The presence of cADPR in CD38−3T3 cells was not due to de novo expression of ADP-ribosyl cyclase activity in these cells, following their co-culture, because no cyclase activity whatsoever could be detected.

TABLE III
| Cell type      | cADPR content | Influxa | Inhibition |
|---------------|---------------|---------|------------|
|               | pmol cADPR/mg | pmol cADPR/mg | %          |
| HeLa          | 0.66 ± 0.18   | 0       |            |
| 3T3           | 1.06 ± 0.23   | 0.74    |            |
| + Uridine (10 μM) | 0.51 ± 0.08   | 0.19    | 74         |
| + Thymidine (10 μM) | 0.58 ± 0.11   | 0.26    | 65         |
| + NBPMP (1 μM)   | 1.15 ± 0.22   | 0.83    | 0          |
| + Dipyridamole (10 μM) | 0.51 ± 0.06 | 0.19    | 74         |

Influx of extracellular cADPR into 3T3 murine fibroblasts occurs both after incubation of intact cells with the cyclic nucleotide and following their co-culture with cADPR-producing, ectocyclase-expressing feeder layers (23).

The results obtained in this study demonstrated that both equilibrative and concentrative nucleoside transporters are responsible for this influx, which had been previously described...
at a phenomenologic level (23). Therefore, rather unexpectedly, some of these transmembrane proteins proved to behave as promiscuous transporters beyond the current concept of broad acceptance of both pyrimidine and purine nucleosides, to include also the cyclic nucleotide cADPR. The quite compact structure of cADPR, as revealed by x-ray crystallography (52), might be responsible for such behavior.

The use of two cell types, 3T3 fibroblasts and HeLa cells, that exhibit different nucleoside transport systems, helped discriminate among the cADPR-transporting NT forms. Equilibrative uptake of cADPR occurs through ENT2, whereas ENT1 is not involved. Na+/cADPR symport activities have been detected in 3T3 fibroblasts, but not in HeLa cells. The almost complete inhibition afforded by uridine, guanosine, and inosine identified these activities with nucleoside transport systems. The inhibition afforded by uridine, guanosine, and inosine identified these activities with nucleoside transport systems. The largest part of Na+-dependent cADPR uptake is susceptible to inhibition by low concentrations of NBMPR (affording 75% inhibition), a property shared with cs and csg (47–49). Whether this NBMPR-sensitive Na+/cADPR symport system is identifiable with either cs or csg is still unknown, however. The residual concentrative transport activity (25%) could be featured by ENT2, which is expressed in 3T3 cells and is not sensitive to NBMPR. This low level might explain the variability of cADPR influx observed in co-cultures where the CD38+ 3T3 fibroblasts were pretreated with ENT2 antisense oligodeoxynucleotides (see “Results”). Indeed, transient transfection experiments on COS-7 cells demonstrated that ENT2 has intrinsic cADPR-transporting activity (Fig. 9). Whether additional concentrative Na+/cADPR symport systems exist, as reported earlier for Na+/nucleoside transport in the related COS-1 cell line (41), requires further investigations, aimed also to establish whether they are identifiable with, or unrelated to, professional nucleoside transporters.

As widely recognized for NT species, also unequivocal identification and characterization of concentrative cADPR transporters seems to be a difficult task because of the following reasons: (i) co-expression of multiple equilibrative and concentrative transporters in the same cell type and their possible distinctive regulation, as that afforded by cytokines on murine macrophages (40); (ii) intrinsic variability of expression of NT; (iii) possible interferences between influx and efflux of permeating solutes in the presence of selective inhibitors (53); a relevant example is the apparently low extent of inhibition of cADPR influx into 3T3 cells by thymidine (Fig. 2), which might in fact be due to blockade of cADPR release from the cells via ENT2 while cADPR is actively pumped into the same cells by CNT forms; and (iv) lack of availability of related transporting proteins, as those corresponding to csg and cs NT (47–49), this hampering their localization in specific cells and tissues.

Despite these limitations, the identification in the plasma membrane of 3T3 fibroblasts of pleiotropic cADPR-transporting NT opens new perspectives for elucidating the mechanisms of regulation of intracellular calcium homeostasis in cADPR responsive cells. Our findings cast serious doubts on the possibility of a similar role being played by cADPR-transporting ENT2 (Table III), although extracellular cADPR might reach, in specific districts and under particular conditions (e.g. extensive cell death) levels high enough to determine its equilibrative uptake by neighboring cells. On the contrary, the NT responsible for concentrative cADPR influx proved in the heterologous co-cultures of CD38−/CD38+ 3T3 fibroblasts to actively accumulate cADPR into the CD38− cells against largely unfavorable concentration gradients. In this experimental system, the eventual, remarkably high gradient between intracellular (almost micromolar) and extracellular (nanomolar) concentrations of cADPR, despite presence of ENT2-mediated equilibrative activity which could dissipate the gradient itself, might be accounted for by progressive sequestration of intracellular cADPR by specific binding proteins, notably by its receptors on the ryanodine stores/channels (54–59). Indeed, the balance between kinetics of cADPR generation by CD38− cells and of its concentrative influx into the cADPR-responsive CD38− cells proved to be functionally adequate to elicit Ca2+-mediated responses in the co-culture conditions (23, 28). From a more general standpoint, the principle of concentrative uptake of extracellular cADPR, possibly at sites distant from those of its generation, is reminiscent of endocrine processes in which hormones, still at very low concentrations, regulate a number of functions in target cells quite far from the endocrine tissues that release them in the bloodstream. Interestingly, the concentrative cADPR concentration in human plasma was estimated to be around 1 nM (not shown).

The concentrative nature of at least two components of cADPR uptake (i.e. CNT2 and the NBMPR-sensitive system identified in 3T3 fibroblasts) bears relevance to “in vitro” events occurring in several tissue microenvironments including intact trachea (26), synapses (60), and bone marrow (28), where Ca2+-related cell functions are elicited by protracted generation of extracellular cADPR (13, 14). The molecular characterization of concentrative cADPR transporter(s) should allow us to elucidate to what extent and through which mechanisms a site-targeted and constant supply of extracellularly generated cADPR can influence Ca2+-related responses. The molecular and functional interactions between NAD+-releasing Cx43 hemichannels and either CD38 or concentrative Na+/cADPR symport systems require challenging “in situ” studies, whose results would be a biochemical paradigm for many physiological and pharmacological processes involved in the regulation of [Ca2+]i levels.

Finally, the present results raise the possibility that several ectoenzymes involved in nucleotide and nucleoside metabolism at the outer surface of different cells have a role, although indirect, in the regulation of [Ca2+]i levels. The site-specific, ectocellular synthesis of a number of nucleosides potentially inhibiting influx of extracellular cADPR into cADPR-responsive cells seems to support this contention. Topological proximity of these ectoenzymes, including CD38, and of the selective transporters of NAD” and cADPR, as well as possible preferential interactions thereof within the plasma membrane, might confer high efficiency to the related regulatory mechanisms. In any case, the cADPR transporters in the plasma membrane, and especially the concentrative ones, seem to be promising targets for new therapeutic strategies designed to control calcium-related dysfunctions in cADPR-responsive cells and tissues.

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Equilibrative and Concentrative Nucleoside Transporters Mediate Influx of Extracellular Cyclic ADP-Ribose into 3T3 Murine Fibroblasts
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