Spatio-temporal propagation of Ca\(^{2+}\) signals by cyclic ADP-ribose in 3T3 cells stimulated via purinergic P2Y receptors

Santina Bruzzone,¹,² Svenja Kunerth,³ Elena Zocchi,¹,² Antonio De Flora,¹,² and Andreas H. Guse³

¹Department of Experimental Medicine, Section of Biochemistry, and ²Center of Excellence for Biomedical Research, University of Genova, 16132 Genova, Italy
³University Hospital Hamburg-Eppendorf, Centre for Experimental Medicine, Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, 20246 Hamburg, Germany

The role of cyclic ADP-ribose in the amplification of subcellular and global Ca\(^{2+}\) signaling upon stimulation of P2Y purinergic receptors was studied in 3T3 fibroblasts. Either (1) 3T3 fibroblasts (CD38\(^{−}\) cells), (2) 3T3 fibroblasts preloaded by incubation with extracellular cyclic ADP-ribose (cADPR), (3) 3T3 fibroblasts microinjected with ryanodine, or (4) 3T3 fibroblasts transfected to express ADP-ribose (cADPR), (3) 3T3 fibroblasts microinjected with ryanodine, or (4) 3T3 fibroblasts transfected to express the ADP-ribosyl cyclase CD38 (CD38\(^{−}\) cells) were used. Both preincubation with cADPR and CD38 expression resulted in comparable intracellular amounts of cyclic ADP-ribose (42.3 ± 5.2 and 50.5 ± 8.0 pmol/mg protein).

P2Y receptor stimulation of CD38\(^{−}\) cells yielded a small increase of intracellular Ca\(^{2+}\) concentration and a much higher Ca\(^{2+}\) signal in CD38-transfected cells, in cADPR-preloaded cells, or in cells microinjected with ryanodine. Confocal Ca\(^{2+}\) imaging revealed that stimulation of ryanodine receptors by cADPR or ryanodine amplified localized pacemaker Ca\(^{2+}\) signals with properties resembling Ca\(^{2+}\) quarks and triggered the propagation of such localized signals from the plasma membrane toward the internal environment, thereby initiating a global Ca\(^{2+}\) wave.

Introduction

Cyclic ADP-ribose (cADPR), a potent Ca\(^{2+}\) mobilizer from ryanodine-sensitive calcium stores and functionally active in a wide variety of cell types, is generated from NAD\(^{+}\) as substrate by a family of multifunctional enzymes designated ADP-ribosyl cyclases (Guse, 2002; Lee, 2001, 2002). Two ecto-ADP-riboyl cyclases have been cloned and characterized: the transmembrane type II glycoprotein CD38 and the GPI-anchored protein CD157 (BST-1). Cytosolic ADP-riboyl cyclase activities have been observed in the marine mollusk *Aplysia californica*, where the first cyclase was cloned and fully characterized (Lee, 2002), but also in sea urchin eggs (Graeff et al., 1998), human T-lymphocytes (Guse et al., 1999), human blood mononuclear cells (Bruzzone et al., 2000), rat pancreatic acinar cells (Sternfeld et al., 2003), and bovine brain (Matsumura and Tanuma, 1998).

S. Bruzzone and S. Kunerth contributed equally to this work.

Address correspondence to Andreas Guse, University Hospital Hamburg-Eppendorf, Centre for Experimental Medicine, Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, Martinistr. 52, 20246 Hamburg, Germany. Tel.: 49-40-42803-2828. Fax: 49-40-42803-9880. email: guse@uke.uni-hamburg.de

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cADPR is but one of various signal metabolites (cADPR, D-myo-inositol 1,4,5-trisphosphate [IP\(_{3}\)], and nicotinic acid adenine dinucleotide phosphate [NAADP]) that can release Ca\(^{2+}\) from specific internal stores, in some cases coproduced in the same cell type (for review see da Silva and Guse, 2000). Many cell types have been reported to harbour IP\(_{3}\)-, cADPR-, and NAADP-sensitive stores (Albrieux et al., 1998; Guse et al., 1999; Berg et al., 2000; Cancela et al., 2000; Santella et al., 2000; Churchill and Galione, 2001; Hoesch et al., 2002; Brailoiu et al., 2003). The complex spatio-temporal patterns of functional interplay among these Ca\(^{2+}\)-mobilizing second messengers and their target receptors represent a central issue in order to elucidate the mechanisms that underlie mobilization of Ca\(^{2+}\) from the different stores, thereby affecting fundamental and diverse cell functions (Meldolesi and Pozzan, 1998; Berridge et al., 2000; Meldolesi, 2002; Carafoli, 2003).

A long recognized paradox of the NAD\(^{+}\)/cADPR system is its compartmentation in several mammalian cell types (for...
review see De Flora et al., 2002). Thus, for instance, the exposure of intact cells to extracellular cADPR has been shown to upgrade the functional response to different agonists (De Flora et al., 1996; Podestà et al., 2000; Franco et al., 2001a; Zocchi et al., 2001). Studies performed on murine 3T3 fibroblasts revealed that internalization of extracellular cADPR occurs through a number of equilibrative and concentrative nucleoside transporters (Guida et al., 2002) and that influx of cADPR into intact 3T3 cells is paralleled by a sustained increase of the basal intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) (Franco et al., 2001b). Indeed, comparable increases of the basal [Ca$^{2+}$], are also observed after “de novo” expression of CD38 in 3T3 cells, as a result of the related generation of intracellular cADPR, which is responsible for the doubling of [Ca$^{2+}$], in CD38$^{–}$ 3T3 compared with antisense-transfected (CD38$^{+}$) or wild-type cells (Zocchi et al., 1998).

Murine 3T3 fibroblasts seem to represent a good experimental model to study the interplay between the Ca$^{2+}$-mobilizing metabolites cADPR and IP$_3$ for a number of reasons: (1) 3T3 cells respond to extracellular micromolar ATP with an IP$_3$-dependent calcium release mediated by P2Y purinergic receptors (Giovannardi et al., 1992); (2) cADPR can be internalized by intact 3T3 fibroblasts across the above-mentioned nucleoside transporters, without the need to permeabilize the cells (Guida et al., 2002); (3) sense- and antisense CD38-transfected cells have a significantly different [Ca$^{2+}$], due to presence or absence, respectively, of intracellular cADPR (Zocchi et al., 1998); and (4) both IP$_3$- and cADPR-sensitive calcium stores are present in this cell type (Giovannardi et al., 1992; Zocchi et al., 1998).

Therefore, we investigated whether the presence or absence of intracellular cADPR can trigger distinctive Ca$^{2+}$ responses to ATP stimulation in 3T3 fibroblasts. The results obtained indicate that cADPR and IP$_3$ act in a functionally and spatially coordinated fashion and specifically that the presence of intracellular cADPR elicits a clearcut amplification of IP$_3$-mediated [Ca$^{2+}$], responses to extracellular ATP.

## Results

### Different [Ca$^{2+}$], responses to ATP in CD38$^{+}$ and CD38$^{–}$ 3T3 fibroblasts

Intact sense (CD38$^{+}$)- and antisense (CD38$^{–}$)-transfected 3T3 fibroblasts were comparatively challenged with 100 µM ATP, a concentration known to stimulate P2Y receptors (Giovannardi et al., 1992; Di Virgilio et al., 2001). The immediate increase of cytosolic [Ca$^{2+}$], was remarkably different in the two cell types, with the CD38$^{–}$ cells exhibiting much higher peak and plateau responses to ATP (Fig. 1 A). When ATP was supplemented in the presence of EGTA, the extent of [Ca$^{2+}$], increase was almost superimposable to that recorded in a Ca$^{2+}$-containing buffer, thus indicating release from intracellular stores as the main underlying mechanism (Fig. 1 B). On the contrary, when CD38$^{+}$ and CD38$^{–}$ cells were stimulated with 3 mM ATP (a concentration that triggers the P2X receptors, see Di Virgilio et al., 2001), the two cell populations showed quite comparable [Ca$^{2+}$], increases. These were abolished by the presence of EGTA in the buffer, therefore demonstrating that calcium influx follows stimulation of the P2X receptors (not depicted). Thus, the [Ca$^{2+}$], increases elicited by calcium influx across ATP-gated ion channels (P2X receptors) are not influenced by CD38 expression in 3T3 fibroblasts.

### Causal role of intracellular cADPR in the different [Ca$^{2+}$], responses to ATP of CD38$^{+}$ and CD38$^{–}$ 3T3 fibroblasts

A distinctive feature between the CD38$^{–}$ and CD38$^{+}$ cells is the presence of intracellular cADPR in the latter cell population, as a consequence of the expression of ADP-ribose cyclase activity (Zocchi et al., 1998). The amount of intracellular cADPR in CD38$^{+}$ 3T3 fibroblasts was assayed by a highly sensitive procedure of enzymatic cycling (Graeff and Lee, 2002) and estimated to be 50.48 ± 8.03 pmol/mg protein ($n = 8$). Conversely, the concentration of cADPR in CD38$^{–}$ cells was hardly detectable (0.25 ± 0.11 pmol/mg protein, $n = 9$). The corresponding levels of ectocellular ADP-ribosyl cyclase activity, taken as a measure of CD38 content, were 91.25 ± 8.83 ($n = 4$) and 0.28 ± 0.06 ($n = 5$) pmol cADPR/min/mg, respectively, in the CD38$^{–}$ and CD38$^{+}$ cells. To investigate whether the different response to 100 µM ATP could be due to the presence of cADPR, intact CD38$^{–}$ cells were preincubated for 10 min with extracellular cADPR (50 µM), which was recently reported to be internalized by these cells through equilibrative and concentrative nucleoside transporters (Guida et al., 2002).

After preincubation with cADPR, the CD38$^{–}$ 3T3 fibroblasts acquired an ATP-evoked Ca$^{2+}$ release that was quanti-
In an attempt to elucidate the mechanisms responsible for the difference in the ATP-stimulated global Ca\(^{2+}\) signals between wild-type 3T3 cells and cells with increased cADPR contents (obtained either by extracellular addition of cADPR or by transfection with CD38), rapid confocal Ca\(^{2+}\) imaging experiments were performed (Kunerth et al., 2003). Besides confirming the previous results, single cell Ca\(^{2+}\) imaging clearly defined that the limited average response induced by extracellular ATP in CD38\(^{-}\) 3T3 cells (Fig. 1) was due to a full response occurring in very few cells (2/23), with the same amplitude observed in CD38\(^{+}\) cells. In contrast, most of the CD38\(^{-}\) cells were responsive increase of the [Ca\(^{2+}\)], observed after stimulation with ATP was markedly inhibited (Fig. 2 B).

It is well documented that 100 \(\mu\)M ATP evokes a calcium release from IP\(_3\)-sensitive stores (Giovannardi et al., 1992; Di Virgilio et al., 2001). Therefore, CD38\(^{-}\) 3T3 cells were first incubated either with 2-APB, a membrane-permeant IP\(_3\) antagonist, or with U73212, a membrane-permeant inhibitor of PLC. As shown in Fig. 3 A, 2-APB (250 \(\mu\)M) completely abrogated the response to 100 \(\mu\)M ATP, whereas the response to 3 mM ATP was not impaired (not depicted). Thus, the use of 2-APB was instrumental for discriminating between the two different mechanisms that underlie the [Ca\(^{2+}\)]\(_{i}\) increases after stimulation of the P2Y receptors (Ca\(^{2+}\) release) and of the P2X receptors (Ca\(^{2+}\) influx), respectively. Likewise, U73122 at 1 \(\mu\)M completely inhibited the Ca\(^{2+}\) release triggered by 100 \(\mu\)M ATP, while the same concentration of the inactive analogue U73343 proved to be ineffective (Fig. 3 B).

Next, we investigated a possible increase of the intracellular cADPR concentration in CD38\(^{+}\) cells as a consequence of their pulse exposure to ATP. To this purpose, cells were incubated for 0, 10, and 30 s in the presence of 100 \(\mu\)M ATP; the intracellular concentrations of cADPR, however, were not significantly modified after this treatment, i.e., 43.21 ± 2.05, 40.11 ± 4.31, and 45.94 ± 2.45 pmol cADPR/mg protein (n = 3) after 0, 10, and 30 s of treatment, respectively.

To check the specificity of the effect of cADPR, CD38\(^{+}\) 3T3 cells were preincubated for 2 h in the presence of 100 \(\mu\)M 8-Br-cADPR, a membrane-permeant cADPR antagonist (Walseth and Lee, 1993). Under these conditions, the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** ATP-induced Ca\(^{2+}\) release in CD38\(^{-}\) 3T3 fibroblasts preincubated with 8-Br-cADPR and in CD38\(^{+}\) 3T3 fibroblasts preincubated with 8-Br-cADPR. (A) CD38\(^{-}\) 3T3 cells were preincubated for 10 min in the presence (filled square) or absence (open square) of 50 \(\mu\)M cADPR, as described in the Materials and methods, before ATP addition (100 \(\mu\)M final concentration). (B) CD38\(^{-}\) 3T3 cells were preincubated for 2 h with (filled rhombus) or without (open rhombus) 100 \(\mu\)M 8-Br-cADPR, as described in the Materials and methods, before ATP addition (100 \(\mu\)M final concentration). [Ca\(^{2+}\)]\(_{i}\) was monitored using a fluorescence plate reader, as described in the Materials and methods. Characteristic tracings are shown (n = 13 for CD38\(^{-}\) in the presence of 50 \(\mu\)M cADPR, n = 11 for CD38\(^{-}\) in the absence of 50 \(\mu\)M cADPR, n = 4 for CD38\(^{-}\) in the presence of 100 \(\mu\)M 8-Br-cADPR, n = 4 for CD38\(^{-}\) in the absence of 100 \(\mu\)M 8-Br-cADPR).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Role of IP\(_3\) in the ATP-induced Ca\(^{2+}\) release in CD38\(^{-}\) 3T3 fibroblasts. (A) CD38\(^{-}\) 3T3 cells were incubated for 5 min in the presence of 250 \(\mu\)M 2-APB. (B) CD38\(^{-}\) 3T3 cells were incubated for 5 min in the presence of 1 \(\mu\)M U73343 (open rhombus) or U73122 (filled rhombus). ATP (100 \(\mu\)M) was then added to cells, and [Ca\(^{2+}\)]\(_{i}\) was measured using a plate reader fluorometer, as described in the Materials and methods. Characteristic tracings are shown (n = 6 for CD38\(^{-}\) in the presence of 250 \(\mu\)M 2-APB, n = 3 for CD38\(^{-}\) in the presence of 1 \(\mu\)M U73343, n = 3 for CD38\(^{-}\) in the presence of 1 \(\mu\)M U73122).
to ATP (17/20). This agrees with the earlier observation that in each individual wild-type 3T3 cell, the ATP-induced Ca\textsuperscript{2+} rise occurs in an all-or-none fashion (Gionannardi et al., 1992). When the CD38\textsuperscript{-} cells were preloaded with cADPR by incubation with 50 μM extracellular concentrations of this cyclic nucleotide, 60% of cells acquired the ability to respond to ATP (16/27). On the contrary, most CD38\textsuperscript{-} cells, after preincubation with 100 μM 8-Br-cADPR, lost their responsiveness to ATP, and only in one third of these cells was the release evoked with the same amplitude (7/21).

Cooperation between cADPR and IP\textsubscript{3} in the [Ca\textsuperscript{2+}]i response to extracellular ATP

Detailed analysis of high-resolution Ca\textsuperscript{2+} images acquired under the four different conditions, (1) CD38\textsuperscript{-} cells, (2) cADPR-loaded CD38\textsuperscript{-} cells, (3) CD38\textsuperscript{-} cells microinjected with ryanodine, and (4) CD38\textsuperscript{-} cells, revealed fundamental differences in the spatio-temporal patterns of Ca\textsuperscript{2+} signaling (Figs. 4 and 5). While CD38\textsuperscript{-} cells showed a low [Ca\textsuperscript{2+}], throughout the cell (Fig. 4 A, left), a slightly increased [Ca\textsuperscript{2+}], was observed in unstimulated CD38\textsuperscript{-} cells preincubated with cADPR (Fig. 4 B, left) or microinjected with ry-
anodine 15 min before (Fig. 4 C, left), or in CD38− cells (Fig. 4 D, left). Upon stimulation by ATP, only very small local Ca2+ signals close to the plasma membrane were observed in the CD38− cells (Fig. 4 A, 117.9 s), whereas CD38− cells either preincubated with cADPR or microinjected with ryanodine, or CD38 transfectants, developed a rapid and global response that travelled across the whole cell as a regenerating wave (Fig. 4, B–D). In CD38− cells preincubated with cADPR or microinjected with ryanodine, and in CD38-transfectants, too, Ca2+ waves started at specific hot-spots at the cell border and travelled toward the perinuclear region where a significant amplification occurred (Fig. 4, B–D). Interestingly, ryanodine receptors (RyRs) were localized in high density in the perinuclear region (not depicted), compatible with their involvement in the amplification process. Detailed analyses of [Ca2+]i distribution in differently localized regions of interest (ROIs) in the cell further illustrated the patterns of wave propagation and amplification observed when cADPR was present in the cells, either after direct loading or in the CD38-expressing cells (Fig. 4 E). Microinjection of an activating concentration of ryanodine 15 min before addition of ATP mimicked the effect of both cADPR preloading or transfection of CD38 (Fig. 4, C and E), suggesting that cADPR indeed acts on the RyR.

For the onset of global Ca2+ waves to occur, local subcellular Ca2+ signals are required as pacemaker signals to ini-

Figure 5. Analysis of subcellular Ca2+ signals in 3T3 fibroblasts. Confocal pseudo-color Ca2+ images of the early pacemaker phase upon ATP stimulation of a CD38− cell (A), a CD38− cell preincubated with 50 μM cADPR (B), a CD38− cell microinjected with ryanodine 15 min before addition of ATP (Ry; pipette concentration 100 μM, final intracellular concentration ~1 μM) (C), and a CD38− cell (D), and magnifications of subcellular regions are displayed (Bars, 2.5 μm). The cells were stimulated at time point 115 s by 100 μM ATP. Right panel, Ca2+ tracings of selected ROIs (as indicated and color coded in the middle panel) are displayed. Characteristic cells were selected from n = 18 CD38− cells (A), n = 19 CD38− cells preincubated with cADPR (B), n = 15 CD38− cells microinjected with ryanodine, and n = 20 CD38− cells.
tiate the global signal (Bootman et al., 1997; Meldolesi, 2002). Analysis of subcellular Ca\textsuperscript{2+} signals before stimulation of P2Y receptors (basal condition) revealed a small increase in magnitude in CD38\textsuperscript{−} cells previously loaded with cADPR or in CD38\textsuperscript{+} cells, while microinjection of ryanodine 15 min before had no stimulatory effect (Table I).

After stimulation with ATP, increasing pacemaker signals, localized in proximity of the plasma membrane and a few micrometers inside the cell, were rarely visible in CD38\textsuperscript{−} cells, while microinjection of ryanodine (Table I). Interestingly, a similar frequency was observed in CD38\textsuperscript{−} cells previously loaded with cADPR and in CD38\textsuperscript{+} cells, which express in their plasma membrane both equilibrative and concentrative transporters previously demonstrated to functionally circumvent to-
Furthermore, 3T3 murine fibroblasts proved to represent a profitable model to investigate functional interactions between IP₃R and RyR, i.e., a cell type featuring "channel cross-talk" (Patel et al., 2001; Morgan and Galfione, 2002). Enhanced activity of either of the two Ca²⁺ release systems in fibroblasts by overexpression of type 1 IP₃R (Davis et al., 1999) or by de novo inducing intracellular cADPR production (Zocchi et al., 1998; this study) resulted in an increased mean [Ca²⁺] in unstimulated cells. Analysis of the subcellular Ca²⁺ distribution by confocal Ca²⁺ imaging revealed the presence of localized Ca²⁺ signals with increased Ca²⁺ concentrations both in L-fibroblasts overexpressing the type 1 IP₃R (Davis et al., 1999) and in the CD38⁺ 3T3 cells either preincubated with cADPR or microinjected with ryanodine (Fig. 5; Table I).

Analysis of the subcellular Ca²⁺ release events in 3T3 fibroblasts revealed amplitudes of 44–98 nM and areas of 0.35–0.42 μm² (corresponding to diameters of ~0.57 and 0.65 μm). These values are considerably smaller as compared to typical sparks (amplitude 71–300 nM, diameter 2–5 μm, for an extended list of references see Discussion section of Kunerth et al., 2003). However, so-called "fundamental" Ca²⁺ signals produced by very few RyRs (possibly one) were described as Ca²⁺ quarks in skeletal and cardiac muscle (Tsugorka et al., 1995; Lipp and Niggli, 1998). These Ca²⁺ quarks were characterized by diameters between 0.3 and 0.85 μm and amplitudes of ~40 nM (Tsugorka et al., 1995; Lipp and Niggli, 1998), values very similar to the ones described here. This indicates that the subcellular Ca²⁺ release events observed under basal conditions and during the very early pacemaker phase in 3T3 cells are comparable to fundamental Ca²⁺ quarks that have been observed so far only in excitable cells. Moreover, upon ATP stimulation, the quark-like early pacemaker signals were further increased in amplitude and in frequency, but not in diameter. This indicates that longer and more frequent opening of the RyR channel, but not recruitment of further channels, is the major mechanism for signal amplification by both cADPR and ryanodine in this early pacemaker phase.

In conclusion, it is of remarkable interest that a very similar phenotype, namely a global Ca²⁺ wave upon P2Y receptor stimulation, could be obtained in fibroblasts either by enhancing the IP₃/Ca²⁺ signaling pathway (Davis et al., 1999) or by providing the cell with the cADPR/Ca²⁺ signaling system (this study). As overexpression of different IP₃R subtypes may increase or decrease dramatically upon certain conditions (Davis et al., 1999), the transition from local to global Ca²⁺ signals related to increased density of IP₃Rs can be of physiological significance.

Also, the cADPR/Ca²⁺ signaling pathway is susceptible to be widely modulated in RyR-expressing cells. Relevant examples include (1) the massive expression of CD38 that is causally related to retinoic acid–induced granulocytic differentiation of HL60 cells (Munshi et al., 2002); (2) the increased concentrations of intracellular cADPR elicited by lipopolysaccharide in human blood mononuclear cells (Bruzzone et al., 2003); and (3) the delivery of extracellular cADPR by CD38⁺ neighboring cells across various nucleoside transporters that allow cells negative for CD38, but positive for RyR, to feature cADPR-dependent Ca²⁺ responses and increased Ca²⁺-mediated processes (for review see De Flora et al., 2002).

In conclusion, cADPR behaves as a paracrine messenger able to switch different cell types from low to high "excitability" (Franco et al., 2001a,b; Verderio et al., 2001; Zocchi et al., 2001). Our present findings indicate that in 3T3 fibroblasts, the underlying mechanism is the amplification of quark-like subcellular Ca²⁺ signals by cADPR, both in the basal phase and in the early pacemaker phase.

Materials and methods

Materials

Fura 2-AM and 2-APB were obtained from Calbiochem. Other chemicals were obtained from Sigma-Aldrich.

Cell lines

NIH 3T3 cells, obtained from American Type Culture Collection, were cultured as previously described (Zocchi et al., 1998). Transfection with sense (CD38⁺) or antisense (CD38⁻) CD38 cDNA was performed as described elsewhere (Zocchi et al., 1998).

Determination of intracellular cADPR content

Resting CD38⁺ and CD38⁻ 3T3 fibroblasts were extracted in 0.5 ml of 0.6 M PCA, and an aliquot of the cell suspension was submitted to protein determination, according to Bradford (1976). In another set of experiments, CD38⁺ cells were seeded in six-well plates and then extracted by the addition of 0.6 M PCA after 0, 10, and 30 s exposure to 100 μM ATP. Protein determination was performed on cells from wells prepared in parallel. After removal of proteins, cADPR was measured on the neutralized extracts by a highly sensitive enzymatic cycling assay (Graeff and Lee, 2002). The intracellular cADPR concentrations were expressed as pmol/mg protein.

Assay of ADP-ribosyl cyclase activity

ADP-ribosyl cyclase activity was assayed as previously described (Bruzzone et al., 2003). In brief, intact CD38⁺ and CD38⁻ cells (10⁶) were resuspended in 400 μl of PBS–glucose (10 mM) with 0.1 mM NAD⁺. At different times (0, 2, 5, 10, and 60 min), 100-μl aliquots were withdrawn, and 220 μl of 0.9 M PCA was added to each aliquot. After deproteination, PCA was removed, and cADPR content was measured in each aliquot according to the cycling enzymatic assay (Graeff and Lee, 2002). Protein determination was performed on an aliquot of the incubation (Bradford, 1976).

cADPR influx into intact CD38⁺ 3T3 fibroblasts

cADPR influx into intact CD38⁺ cells was performed as previously described (Guida et al., 2002). In brief, cells were harvested and resuspended in 100 μl of Na⁺ buffer (135 mM NaCl, 6.3 mM K₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, 0.9 mM CaCl₂, 10 mM glucose, pH 7.4) in the presence of 50 μM cADPR at 22°C for 10 min. The suspension was then centrifuged at 5,000 g for 15 s. Pellets were washed with 1.5 ml of ice-cold appropriate Na⁺ buffer containing 10 mM uridine (to inhibit loss of internalized cADPR across equilibrative nucleoside transporters, see Guida et al., 2002) and submitted to two consecutive centrifugations as described above to remove the supernatants completely. Pellets were resuspended in 300 μl water, and the samples were sonicated for 30 s at 3 W in ice. Aliquots of 280 μl were deproteinized with 0.6 M perchloric acid (final concentration), and cADPR was detected by the enzymatic cycling assay as described in "Determination of intracellular cADPR content" (Graeff and Lee, 2002). Protein content was determined on 20-μl aliquots according to Bradford (1976).

Calcium measurements in cell populations

Both 3T3⁺ and 3T3⁻ cells were seeded in 96-well plates (50 × 10⁴ cells/well). Cells were loaded with 10 μM FURA-2/AM (or with Fluo-3/AM) for 30 min in complete medium. Cells were then washed twice with 200 μl of calcium buffer (135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Hepes, pH 7.4), and 100 μl of the same buffer was added in each well. Calcium-free buffer was prepared without CaCl₂ and with the addition of 2 mM EGTA. Fluorescence was measured every 2.12 s (excitation, 355 nm and 390 nm, alternatively; emission, 520 nm) using a fluorescence plate reader (Fluostar Optima; BMG Labtechnologies GmbH). The ratio of emitted light after excitation at 355 nm/390 nm was calculated and displayed as a function of time. In the experiments with CD38⁺-loaded CD38⁻ cells, the cyclic nucleotide (50 μM) was added to the complete medium during incubation with FURA-2/AM (last 10 min). Cells were

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washed as described above, and 50 μM cADPR was added to the buffer during the calcium measurements. CD38+ cells were preincubated with 8-Br-cADPR for 2 h in complete medium, and FURA-2-AM was added during the last 30 min.

Confocal calcium imaging

The cells were cultured overnight in chamber slides consisting of a plastic chamber and a thin glass cover slip. At the day of measurements, the cells were loaded in these chamber slides with FURA-2/AM (10 μM) for 30 min. After loading, the medium was exchanged against a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM NaH2PO4, 5.5 mM glucose, and 20 mM HEPES (pH 7.4). The chamber slide was mounted on the stage of a fluorescence microscope (Leica DM IRE2).

Ratiometric calcium imaging was done as described in an earlier report (Kunert et al., 2003). In brief, we used an Immunofluorescence imaging system at 100-fold magnification (Leica objective type HCX APO 100x/1.3 OIL U-V.I; thus, further stimulation by ATP was performed 15–20 min later, and 50 mM Hepes, 110 mM KCl, 2 mM MgCl2, 5 mM KH2PO4, 10 mM NaCl, pH 7.2) and filtered (0.2 μm) before use. A volume amounting to 1% of the cell volume was injected, resulting in an intracellular ryanodine concentration of ~1 μM. Upon ryanodine injection, the cells displayed increased [Ca2+], thus, further stimulation by ATP was performed 15–20 min later, when [Ca2+] had returned to basal values.

Microinjection

Microinjections were performed as previously described (Guse et al., 1997). An Eppendorf microinjection system (transjector type 5246, microinulator-1 type 5171) equipped with Femtostats II as pipettes was used. The system was operated in the semiautomatic mode with the following instrumental settings: injection pressure 40 hPa, compensatory pressure 30 hPa, injection time 0.5 s, and velocity of the pipette 600 μm/s. The system was operated in the semiautomatic mode with the following instrumental settings: injection pressure 40 hPa, compensatory pressure 30 hPa, injection time 0.5 s, and velocity of the pipette 600 μm/s, resulting in a pixel size of 0.129 μm/pixel. A volume amounting to ~1% of the cell volume was injected, resulting in an intracellular ryanodine concentration of ~1 μM. Upon ryanodine injection, the cells displayed increased [Ca2+]; thus, further stimulation by ATP was performed 15–20 min later, when [Ca2+] had returned to basal values.

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