Changes in Intracellular $\text{Ca}^{2+}$ Levels Induced by Cytokines and P2 Agonists Differentially Modulate Proliferation or Commitment with Macrophage Differentiation in Murine Hematopoietic Cells*\(^{\ddagger}\)

The role of intracellular $\text{Ca}^{2+}$ ($\text{Ca}^{2+/-}$) on hematopoiesis was investigated in long term bone marrow cultures using cytokines and agonists of P2 receptors. Cytokines interleukin 3 and granulocyte/macrophage colony stimulator factor promoted a modest increase in $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$/]) with activation of phospholipase C$_{\gamma}$, MEK1/2, and $\text{Ca}^{2+}$/calmodulin kinase II. Involvement of protein kinase C was restricted to stimulation with interleukin 3. In addition, these cytokines promoted proliferation (20 times) and an increase in the Gr-1$^+$ population with participation of gap junctions (GJ). Nevertheless ATP, ADP, and UTP promoted a large increase in [Ca$^{2+}$/], a reduction in the primitive Gr-1$^-$Mac-1$^+$ population, and differentiation into macrophages without participation of GJ. It is likely that $\text{Ca}^{2+}$, participates as a regulator of hematopoietic signaling: moderate increases in [Ca$^{2+}$/], would be related to cytokine-dependent proliferation with participation of GJ, whereas high increases in [Ca$^{2+}$/], would be related to macrophage differentiation without maintenance of the primitive population.

Intracellular $\text{Ca}^{2+}$ ($\text{Ca}^{2+/-}$) has important roles in many intracellular signaling pathways that participate in distinct cell functions. Time, intensity, and localization of $\text{Ca}^{2+}$ events control its effects. Several intracellular proteins and ionic channels are sensitive to changes in [Ca$^{2+}$/], translating these changes into cellular physiological effects. The major family of kinases associated with $\text{Ca}^{2+}$, signaling are the classical $\text{Ca}^{2+}/$-dependent protein kinase C (PKC) and calmodulins, which activate the calmodulin-dependent kinases (CaMKs).

Intracellular $\text{Ca}^{2+}$ stores such as those in endoplasmic and nuclear reticula and mitochondria have an important role in $\text{Ca}^{2+}$, signaling. Many types of agonists promote $\text{Ca}^{2+}$, release from cellular stores, e.g. agonists that bind to $G_{\alpha_{i_{1}}}$/coupled receptors, which activate phospholipase C$_{\beta}$ (PLC$_{\beta}$), or agonists that operate receptors with tyrosine phosphorylation, which can be recognized by PLC$_{\gamma}$. Both PLC$_{\beta}$ and PLC$_{\gamma}$ catalyze the hydrolysis of phosphatidylinositol 4,5-biphosphate to produce inositol 1,4,5-triphosphate (InsP$_3$) and diacylglycerol (1, 2). Diacylglycerol and InsP$_3$ act as second messengers; diacylglycerol activates PKC on the cellular membrane, whereas InsP$_3$ is spread in the cytoplasm releasing $\text{Ca}^{2+}$, from the endoplasmic and nuclear reticula.

Cytokines are the most important regulators in hematopoiesis. They activate distinct cytokine receptors with intrinsic kinase activity such as receptor of interleukin 6 (IL-6) and stem cell factor (SCF), which bind to c-Kit receptor; moreover cytokines also activate receptors without intrinsic kinase activity that are Janus kinase-dependent receptors. Janus kinases catalyze tyrosine phosphorylation on a great variety of cytokine receptors such as receptor of IL-3, granulocyte/macrophage colony stimulator factor (GM-CSF), granulocyte colony stimulator factor (G-CSF), macrophage colony stimulator factor (M-CSF), erythropoietin (EPO), etc. Some studies have described that some cytokines such as IL-1, IL-2, and SCF are able to promote $\text{Ca}^{2+}$, increase in chondrocytes, neutrophils, and CD34$^+$ cells (3–5); however, direct participation of $\text{Ca}^{2+}$, in hematopoiesis has not been described.

In recent years, the involvement of P2 receptors in hematopoiesis has been investigated. These receptors play a role in megakaryocytic formation (6), hematopoietic stem cell (HSC) migration (7), and CD34$^+$ cell proliferation (8). P2 receptors are divided into ionic channel P2X receptors and G-protein-coupled P2Y receptors; they are activated by ATP and its analogs, and in most cases they increase [Ca$^{2+}$/], to promote the effects of P2 receptors.

Thus, cytokine and P2 receptor activation may promote $\text{Ca}^{2+}$, release through PLC$_{\gamma}$ or PLC$_{\beta}$ activation, respectively.

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7, Table S1, and Videos S1–S5.

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\(2\) The abbreviations used are: $\text{Ca}^{2+}$, intracellular $\text{Ca}^{2+}$; [Ca$^{2+}$/], $\text{Ca}^{2+}$/ concentration; GM-CSF, granulocyte/macrophage colony stimulator factor; IL, interleukin; PLC, phospholipase C; CaMK, $\text{Ca}^{2+}$/calmodulin kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKC, protein kinase C; GJ, gap junctions; InsP$_3$, inositol 1,4,5-trisphosphate; SCF, stem cell factor; EPO, erythropoietin; HSC, hematopoietic stem cell; IMDM, Iscove’s modified Dulbecco’s medium; FBS, fetal bovine serum; P$_i$, phospho-; LTBMC, long term bone marrow cultures; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N”-tetraacetic acid; AM, acetoxyethyl ester; DMSO, dimethyl sulfoxide; MAPK, mitogen-activated protein kinase; Cx, connexin.
or by Ca\(^{2+}\), influx through P2X receptors. Therefore, Ca\(^{2+}\), is likely to participate in hematopoiesis. In this study, we have shown participation of Ca\(^{2+}\), in proliferation and differentiation of hematopoietic cells in long term bone marrow cultures (LTBMC), which support myelopoiesis. A modest increase in Ca\(^{2+}\) concentration by IL-3 and GM-CSF may act mainly on proliferation with participation of Gi, whereas large [Ca\(^{2+}\)]i increases by ATP and analogs promote weak proliferation and induce differentiation of hematopoietic cells into macrophages without participation of Gi.

**EXPERIMENTAL PROCEDURES**

**Extraction of Bone Marrow and LTBMC**—To establish the stroma layer, femur bones were excised from mice (C57Bl/6), and the medullary cavities were aseptically flushed with Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen). The cells so obtained were seeded and incubated (37 °C in 5% CO\(_2\)) in a humidified incubator. Half of the medium in each flask was replaced weekly with an equal amount of fresh medium. IMDM was supplemented with 5% fetal bovine serum (FBS; Culitlab), 20% horse serum (StemCell Technologies), and 10\(^{-6}\) M hydrocortisone (Sigma). At the end of the 8th week, after stroma formation, the remaining hematopoietic cells were removed. New bone marrows from other mice were collected in supplemented IMDM (10 ml) and cultured (2 h) in tissue culture flasks (75 cm\(^2\)). Non-adherent cells were collected by removing the medium, and 10\(^6\) cells/well (12-well plates) were added to pre-cultured stroma.

After 1-week co-culture, the cells were further cultured in 0.5% FBS, IMDM for 24 h. Subsequently LTBMC were stimulated with IL-3 (Sigma) or GM-CSF (Sigma) in 12-well plates in 0.5% FBS, IMDM (1 ml) to evaluate the proliferation and cell populations. Initiator was added 1 h before stimulation with cytokine. The number of cells present in the supernatant was evaluated every 24 h by using a Neubauer chamber. Cell counts were normalized by the number of cells present before stimulation. The experiments were approved by the Ethics Committee of the Federal University of São Paulo (1464/03).

**Calcium Measurements in LTBMC**—Bone marrow cells were seeded on cover glass slides (25 mm) in 6-well plates. For [Ca\(^{2+}\)], measurements, the cells were incubated (40 min at room temperature) with fluo-4/AM (10 \(\mu\)M) and pluronic acid (0.01%) and washed with Tyrode’s solution. Images were captured in two Z planes with a microscope (Axiovert 100 M, Zeiss, Heidelberg, Germany) equipped with a laser scanner (LSM 510 META, Zeiss) and using an objective (Plan-NeoFluor, 63\(\times\), 1.4 numerical aperture) under oil immersion. The fluo-4 probe was excited with argon laser (\(\lambda_{\text{ex}} = 488\) nm), and light emission was detected by using a bypass filter (\(\lambda_{\text{em}} = 500–550\) nm). The pinhole device was not used for [Ca\(^{2+}\)], measurements. Images were collected at \(\sim 4.5\) s intervals for about 2 min. Fluorescence intensity was normalized with reference to the basal fluorescence by using Examiner 3.2 (Zeiss) and Spectralyzer softwares (Spectralyzer, Philadelphia, PA). Max refers to the value of maximal intensity per pixel after stimulation by the agonists, and Basal refers to the maximal value per pixel obtained before stimulation. The mean intensity by Basal and Max images (8 bits = 256 levels) in supplemental Fig. S1 was obtained using the Image J software.

**Confocal Microscopy**—Bone marrow cells were seeded on cover glass slides (13 mm) under conditions described above. These cells were cultured previously in 0.5% FBS (24 h) and stimulated with GM-CSF or IL-3 (5 min). Subsequently the cells were fixed with formaldehyde (2%; 30 min), washed with glycine (0.1M), permeabilized with saponin (0.01%; 15 min), and washed with phosphate-buffered saline. The cells were incubated for 2 h with rabbit anti-connexin 43 (Cx43) (4 \(\mu\)g/ml; Chemicon) or anti-P-panPKC (4 \(\mu\)g/ml; Cell Signaling Technology Inc.) or with goat anti-P-PLC\(\gamma\) (4 \(\mu\)g/ml; Santa Cruz Biotechnology, Inc.), anti-P-CaMKII (4 \(\mu\)g/ml, Santa Cruz Biotechnology, Inc.), or anti-P-MEK (4 \(\mu\)g/ml, Santa Cruz Biotechnology, Inc.); all of them were diluted in phosphate-buffered saline with 1% albumin. Rabbit or goat anti-IgG-Alexa Fluor 488 conjugate (Invitrogen/Molecular Probes) were used for 40 min as secondary antibodies. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, 20 \(\mu\)g/ml; Sigma) for 20 min. Light microscopy analyses were performed with a confocal laser scanning microscope (LSM 510 META, Zeiss).

**Flow Cytometry Analysis**—To determine the hematopoietic populations present in LTBMC, whole cells where collected after 3 days of stimulation with IL-3, GM-CSF, ATP (Sigma), ADP (Sigma), and UTP (Calbiochem). The cells (10\(^6\)) were fixed and labeled with anti-Gr-1-fluorescein isothiocyanate (1 \(\mu\)g/ml), anti-Mac-1-phycerythrin (1 \(\mu\)g/ml), and anti-c-Kit-allophycocyanin (1.5 \(\mu\)g/ml). Data analyses were performed in a FACSCalibur (BD Biosciences) flow cytometer by using the CellQuest software (BD Biosciences). The antibodies were purchased from BD Biosciences.

**Statistical Analysis**—The fluo-4 fluorescence intensity was normalized with reference to basal intensity and were shown to be representative pseudocolored images according to a fluorescence intensity scale ranging from 0 (black) to 255 (white). Data were expressed as the means ± S.E. Statistical comparisons were performed by using Student’s t test or analysis of variance. \(p\) values <0.05 were considered statistically significant.

**RESULTS**

**Hematopoietic Cytokines Promote Increase in [Ca\(^{2+}\)], of Hematopoietic Cells**—LTBMC reproduce myelopoiesis, the formation of granulocytic, monocyte/macrophage, and erythroid cells, although erythrocyte formation depends on external erythropoietin (9). In this kind of culture, a visible formation of cobblestones is observed where hematopoietic progenitor cells can be found in close relationship with stromal cells (9, 10). LTBMC are composed of stromal cells and hematopoietic cells that grow in the stroma. LTBMC were incubated in IMDM supplemented with 0.5% FBS for 24 h before any stimulus to synchronize the cellular cycle; hematopoietic cells were sensitive to lower concentrations of FBS (data not shown).

To determine participation of Ca\(^{2+}\), in hematopoiesis, Ca\(^{2+}\), concentration was monitored in cobblestone areas of LTBMC after addition of agonists. In the first step it was determined which cytokines promoted an increase in
[Ca\textsuperscript{2+}]\textsubscript{i} in stromal areas. Thus, measurements of [Ca\textsuperscript{2+}]\textsubscript{i} were obtained in two planes: from the inferior Z plane (down) where more stromal and some hematopoietic progenitor cells were found and on the superior Z plane (up) where more hematopoietic cells were observed (Fig. 1). Because hematopoietic cells are smaller cells with circular shape and stromal cells are bigger with variable shape, these morphological features were used in their identification.

EC\textsubscript{50} values for cytokines range from 0.05 to 3 ng/ml as informed by Sigma-Aldrich. These values were confirmed by cellular proliferation assays with IL-3 and GM-CSF, and maximum proliferation efficacy for these agonists occurred at about 10–50 ng/ml (supplemental Fig. S2A).

All cytokines tested (SCF, IL-3 (supplemental Video S1), IL-6, GM-CSF, EPO, and IL-7) promoted an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 1) in hematopoietic and stromal cells in the two Z planes.
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![Graphs and images showing cellular proliferation](image)

**FIGURE 2.** $\text{Ca}^{2+}$ signaling inhibitors decrease hematopoietic proliferation in LTBMC. Cells were incubated for 24 h in IMDM, 0.5% FBS before stimulation with IL-3 or GM-CSF. The percent values represented in the histograms were obtained 3 days after stimulation with or without signaling inhibitors. Inhibitors were added 1 h before stimulation with cytokines. A and B, proliferation induced by IL-3 (A) and GM-CSF (B) 3 days after stimulation. C and D, analysis of Gr-1$^+$ Mac-1$^+$, Gr-1$^+$ Mac-1$^-$, and Gr-1$^-$ Mac-1$^-$ populations for stimulation with IL-3 (C) or GM-CSF (D) was performed by flow cytometry. E and F, any signaling inhibitor used did not alter the fraction of immature Gr-1$^-$ Mac-1$^-$ c-Kit$^+$ cells. Data are expressed as mean ± S.E. (n = 8), * and #, p < 0.05, analysis of variance test. *, statistical analysis was performed against samples stimulated with IL-3 (Fig. 2A and B). Proliferation was followed for 3 days (supplemental Fig. S2B); in addition, cell cycle analysis also revealed a proliferation state in the primitive population Gr-1$^-$ Mac-1$^-$ c-Kit$^+$ (supplemental Fig. S2C). All stimuli by agonists were performed in IMDM, 0.5% FBS.

Lymphocytes, erythrocytes, and remaining cells of bone marrow co-culture can be found in the Gr-1$^-$ Mac-1$^-$ population, and the primitive population c-Kit$^+$ corresponds to 1% of the total cells of the culture. The c-Kit$^+$ population present in LTBMC after stimulus with cytokines was still able to form either colony-forming unit granulocyte-macrophage or colony-forming unit granulocyte-erythroblast-macrophage-megakaryocyte colonies (supplemental Fig. S3).

Participation of $\text{Ca}^{2+}$ in proliferation was evaluated by using $\text{Ca}^{2+}$-signaling inhibitors. LTBMC were incubated with the signaling inhibitors, and the $\text{Ca}^{2+}$ chelator BAPTA-AM for 1 h before stimulation with IL-3 and GM-CSF. Because all inhibitors were diluted in dimethyl sulfoxide (DMSO), stimuli by IL-3 and GM-CSF in DMSO were used as controls in statistical analysis. Supernatant cells in LTBMC stimulated by IL-3 and GM-CSF were counted for 3 days in the presence or absence of signaling inhibitors. InsP$_3$ receptor $(2$APB$)$, PLC (U73122), CaMKII (KN-62), and MEK (PD98059) inhibitors and BAPTA significantly decreased IL-3- and GM-CSF-dependent cell proliferation (Fig. 2A). PKC inhibitors (GF109203 and chelerythrine) partly blocked IL-3-dependent cell proliferation (Fig. 2A) but did not promote a decrease in GM-CSF-dependent cell proliferation (Fig. 2B). The concentration of inhibitors is shown in supplemental Fig. S2B. The signaling inhibitor was also able to decrease the percent fraction of cells in phase S/G2/M (supplemental Fig. S4, A and B). These results confirm the role of PKC in the effect of IL-3 described previously in hematopoietic lineages (11, 12). Because the fraction of apoptotic cells in LTBMC was not altered by the presence of inhibitors, their specific effect in intracellular signaling is inferred (supplemental Fig. S4, C and D).
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Quantification of cell populations in LTBMC was assessed by flow cytometry after the 3rd day of stimulation with cytokines. As expected, LTBMC stimulated by IL-3 and GM-CSF produced myeloid cells. IL-3 and GM-CSF produced an increase in the Gr-1Mac-1 population as compared with its control (0.5% FBS). Proliferation and flow cytometry analyses were performed by using the same samples. When LTBMC were stimulated by IL-3, a significant increase in the Gr-1Mac-1 population occurred in the presence of 2APB and KN-62 (Fig. 2C). When cells were stimulated by GM-CSF in the presence of the CaMKII inhibitor KN-62 a significant difference was observed with an increase in the Gr-1Mac-1 population (Fig. 2D). PDB98059, an MEK inhibitor, promoted an increase in the Gr-1Mac-1 population when cells were stimulated with GM-CSF. The statistical analysis is described in detail in supplemental Table S1. The inhibitors used in this study can inhibit cell proliferation and act at distinct moments of myeloid differentiation. The fraction of progenitor Gr-1Mac-1 c-Kit+ cells in the presence of cell signaling inhibitors was not altered (Fig. 2, E and F).

IL-3 and GM-CSF Activate Intracellular Ca ^2+ -dependent Kinases—

Because Ca supra2+ signaling inhibitors were able to inhibit IL-3- and GM-CSF-dependent cell proliferation, antibodies to active forms related to Ca ^2+ signaling such as PLCγ, PKC, and CaMKII were tested. Anti-P-MEK antibody, a protein that acts on cytokine signaling by activating MAPKs, was used as a positive control. The cells were stimulated by IL-3 and GM-CSF in Tyrode’s solution (37°C for 5 min). Under this condition, an increase in the expression of P-MEK and P-PLCγ was observed (Fig. 3, A and B). Participation of PKC in the IL-3 response was shown previously by using PKC inhibitors (Fig. 2A), and Fig. 3C confirms participation of PKC in IL-3-dependent proliferation. Activation of CaMKII may be associated mainly with GM-CSF (Fig. 3D); however, CaMKII does not act exclusively in GM-CSF signaling because IL-3 partially activated P-CaMKII (Fig. 3D), and IL-3-dependent proliferation was also decreased by KN-62 (Fig. 2A). Therefore, cytokines IL-3 and GM-CSF promote high cell proliferation with participation of Ca ^2+ and activation of kinases without a decrease in the primitive population. As a negative control, a secondary antibody, rabbit or goat anti-IgG-Alexa Fluor 488 conjugate, was used (supplemental Fig. S5).

Activation of P2 Receptors Produces Both a Higher Increase in [Ca ^2+]_i and Macrophage Differentiation of Hematopoietic Cells in LTBMC—[Ca ^2+]_i increase induced by the P2 receptor agonists ATP, ADP, and UTP was transient and higher than the cytokine-induced [Ca ^2+]_i increase (Fig. 4A and supplemental Fig. S1). Oscillatory increases were observed in stromal and hematopoietic cells (supplemental Video S2).

To know the ability of P2 receptors to promote proliferation in hematopoietic cells, LTBMC were stimulated with ATP and analogs. Maximal cell proliferation in LTBMC was obtained with 1 mM ATP and its analogs (supplemental Fig. S6A). P2 agonists induced a significant proliferation on the 1st day (Fig. 4B); however, such proliferation was transient, and its efficacy was lower than that of the cytokine-dependent proliferation (Fig. 2, A and B). Of note, daily stimulation with 1 mM ATP did not cause a higher proliferative effect compared with that induced by a single stimulation (data not shown). However, the possibility that the transient effect of P2 agonists on hematopoietic cell proliferation and differentiation is because of their faster degradation deserves further investigation.

Interestingly evaluation of populations of LTBMC showed that stimulation with ATP, ADP, and UTP induced an increase in the Gr-1Mac-1 population (Fig. 4C) and a decrease in the
Gr-1−Mac-1−c-Kit+ immature population (Fig. 4D). Stimulation of LTBM by ATP and analogs produced mainly macrophages as revealed by Giemsa/May-Grünwald stain (Table 1). Reduction in the Gr-1−Mac-1−c-Kit+ population by ATP and its analogs can account for the transient proliferation observed. In addition, BAPTA blocked the P2 agonist-dependent effects (Fig. 4, C and D). Another compound used to promote Ca2+ increase was the Ca2+ ionophore A23187, which was able to induce an increase in the Gr-1+Mac-1+ population (supplemental Fig. S6B).

**Participation of P2Y Receptors in Maturation of Hematopoietic Cells**—In hematopoietic cells, P2Y receptors are activated mainly by ADP and UTP. The presence of P2Y receptors seems to be a characteristic on hematopoietic cells (7, 13–15). However, the presence of P2X receptors has also been described in macrophages, platelets, and granulocytic cells (14, 16, 17).
IL-3, GM-CSF, UTP, and ADP promoted a decrease in the number of apoptotic cells (Fig. 5A). Only ATP, the agonist that promotes less proliferation, was unable to promote a significant decrease in apoptosis.

Among P2Y receptors, ADP-activated P2Y1 receptor is expressed in the human CD34+ progenitor cell population (8, 18) and murine HSCs (Lin−c-Kit+Sca1+) and is expressed in lesser amounts in mature hematopoietic cells (data not shown). Thus, it is likely that this receptor is involved in hematopoiesis. For these reasons, participation of P2Y1 receptor in the ADP-dependent response was evaluated by using its specific inhibitor, MRS2179, which was able to block both the increase of Gr-1+Mac-1+cells (Fig. 5B) and the decrease of immature Gr-1−Mac-1−c-Kit+ cells (Fig. 5C). MRS2365 (10−5 M), a specific P2Y1 agonist, also induced [Ca2+]i increase (supplemental Fig. S7D). The primitive Gr-1+Mac-1−c-Kit+ population expressed the P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, and P2Y14 receptors (Fig. 5D) similarly to the short term HSC (c-Kit+Sca-1+Linlow) population (data not shown). Participation of P2X receptors in proliferation and differentiation of hematopoietic cells was not tested; however, α,β-methylene ATP and benzoyl ATP promoted a higher [Ca2+]i increase in stromal and hematopoietic cells (supplemental Fig. S7). ADP and UTP induced similar effects, although they bind to different receptors.

**TABLE 1**
Giemsa/May-Grünwald stain of hematopoietic cells was obtained 3 days after stimulation of LTBMC.

| Macrophages | Monocyteid | Neutrophils | Immature forms | Blasts |
|-------------|------------|-------------|----------------|--------|
| IL-3        | 48 ± 8     | 12 ± 2      | 14 ± 3         | 25 ± 3 |
| GM-CSF      | 48 ± 4     | 32 ± 6      | 10 ± 3         | 7 ± 1  |
| ATP         | 92 ± 1     | 7 ± 1       | 0 ± 0          | 1 ± 0.5|
| ADP         | 86 ± 3     | 4 ± 2       | 1 ± 1          | 6 ± 2  |
| UTP         | 64 ± 4     | 7 ± 4       | 11 ± 5         | 16 ± 3 |

**FIGURE 5.** Participation of P2Y receptors in differentiation of cells in LTBMC. A, presence of the agonists ADP, UTP, IL-3, and GM-CSF promoted a decrease in cell death; ATP did not cause a significant decrease in cell death, and proliferation promoted by ATP was lower than that promoted by other agonists. B and C, LTBMC were incubated for 1 h with MRS2179, a specific inhibitor of P2Y1 receptor; this antagonist blocked the ADP-induced differentiation: B shows an increase in the Gr-1+Mac-1−population, and C shows a decrease in the primitive Gr-1+Mac-1−c-Kit+population. Data are expressed as mean ± S.E. (n = 6). * and #, p < 0.05. *, statistical analysis was performed against a control (0.5% FBS). #, statistical analysis was performed against response to ADP. A, analysis of variance test; B and C, Student’s t test. D, expression of P2Y receptors was analyzed by flow cytometry in the Gr-1+Mac-1−c-Kit+ population; this primitive population expresses P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, and P2Y14 receptors. Filled histogram, negative control (antibody and inhibitor peptide); open histogram, labeled sample. Data are representative records of at least four independent experiments.
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FIGURE 6. Ca\textsuperscript{2+} promotes intercellular communication between stromal and hematopoietic cells. Cells were loaded with the Ca\textsuperscript{2+} indicator fluo-4, and fluorescence intensity measurements were performed with a confocal microscope. Scale bar, 20 \(\mu\)m. Images are shown with a pseudocolored representation according to a fluorescence intensity scale (0 = black; 255 = white). In this figure, some records obtained by stimulation with cytokines are shown. A, macrophage colony stimulator factor (M-CSF) (50 ng/ml) induced Ca\textsuperscript{2+} waves between stromal cells. B, an oscillatory increase in [Ca\textsuperscript{2+}]\textsubscript{i} is shown in hematopoietic cells (circle) when stimulated with IL-3 (50 ng/ml). After the third increase in [Ca\textsuperscript{2+}], mobilization of a contiguous cell (arrow) was recorded. When cells were in contact, an increase in [Ca\textsuperscript{2+}]\textsubscript{i} was evidenced (circle). C, an increase in [Ca\textsuperscript{2+}]\textsubscript{i} was induced by IL-3 (50 ng/ml) in (i) a stromal cell (adherent) and (ii) a hematopoietic cell (superior); after stimulation an apparent contact area between cells can be observed, imaged by using a line scan. Scale bar, 5 \(\mu\)m. ii corresponds to the sequence shown in i.

Cytokines are the main controllers in the hematopoietic system; these agonists bind to specific receptors (cytokine receptors) that are classified in many families. The intracellular pathways Ras-Raf-MEK-MAPK and Janus kinases/signal transducers and activators of transcription are the two main pathways triggered by cytokines (20, 21). However, recent studies have shown participation of other intracellular pathways that control proliferation, differentiation, and the cell death process in hematopoietic stem cells. For example, participation of the Wnt and Notch pathways in HSC maintenance and differentiation was reported recently (22, 23). In this report, we describe the role of Ca\textsuperscript{2+} pathway; Ca\textsuperscript{2+} is an important second messenger associated with many intracellular processes in murine myelopoiesis.

We demonstrate that low increases in [Ca\textsuperscript{2+}]\textsubscript{i} are related to high IL-3- and GM-CSF-dependent proliferation because the cytokine effects were sensitive to the PLC (U73122) and Inos\textsubscript{3} receptor (2APB) inhibitors, which are active components in the Ca\textsuperscript{2+} release process (Fig. 2, A and B). In addition, activation of PLC\textgamma, which recognizes phosphorylated tyrosine in cytokine receptors, was demonstrated by confocal microscopy (Fig. 3B). Release of Ca\textsuperscript{2+} induced activation of Ca\textsuperscript{2+}-dependent proteins (PKC and CaMKII) that translate their signals into physiological responses (Fig. 3, C and D).

Participation of PKC in hematopoietic proliferation was partial and restricted to responses to IL-3 (Fig. 2A). Participation of PKC was described previously in the differentiation process of hematopoietic precursors. High concentrations of phorbol 12-myristate 13-acetate induced myelomonocyte differentiation, whereas low concentrations induced eosinophil differentiation (24). Whetton et al. (11) also showed that IL-3 and phorbol 12-myristate 13-acetate are able to promote proliferation, an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and PKC activation in factor-dependent cell established at the Paterson
sary that the intensity of $\text{Ca}^{2+}$ release be directly proportional to the observed effect. For example, in many processes, $\text{Ca}^{2+}$ evokes a biphasic response like that in InsP$_3$ and ryanodine receptors. An initial increase in $[\text{Ca}^{2+}]$, induces a positive feedback and opens $\text{Ca}^{2+}$ channels. Inversely high $\text{Ca}^{2+}$ concentrations induce a negative feedback and block $\text{Ca}^{2+}$ channels (26). $\text{Ca}^{2+}$ sparks released between the cell plasma membrane and the endoplasmic reticulum can produce relaxation in vascular smooth muscle by opening of $\text{Ca}^{2+}$-dependent $K^+$ channels, whereas a global $[\text{Ca}^{2+}]$, increase in the same cell can induce contraction (27). This versatility of $\text{Ca}^{2+}$ signaling allows these ions to act on hematopoiesis in distinct ways depending on stimulus duration, amount of $\text{Ca}^{2+}$ released, and type of protein activated. In the hematopoietic system, cytokines and P2 receptor agonists altered the proportion of Gr-1, Mac-1, and c-Kit populations by different $[\text{Ca}^{2+}]$, increases. IL-3 and GM-CSF induced a low increase in $[\text{Ca}^{2+}]$, high proliferation, and an increase in the Gr-1$^-$/Mac-1$^+$ population without a change in the percent value for the primitive Gr-1$^-$/Mac-1$^-$ c-Kit$^+$ cells and with participation of GJ. However, ATP, ADP, and UTP induced a large $[\text{Ca}^{2+}]$, increase and low proliferation and differentiation of hematopoietic cells (macrophage differentiation and a decrease of the primitive Gr-1$^-$/Mac-1$^-$ c-Kit$^+$ populations). Thus, low $[\text{Ca}^{2+}]$, release may induce proliferation with normal differentiation, whereas high $[\text{Ca}^{2+}]$, release can induce preferential rapid cell differentiation of the primitive population. In

Institute with mixed differentiation potential (FDCP-Mix), an IL-3-dependent stem cell line.

CaMKII, another $\text{Ca}^{2+}$-dependent kinase expressed in most cells, apparently acts on IL-3 and GM-CSF responses because KN-62 partly inhibited IL-3- and GM-CSF-dependent proliferation and induced an increase in the Gr-1$^+$ Mac-1$^+$ population. Other CaMKIs such as CaMKIV, related to quiescence of murine HSCs, also can control hematopoiesis by cyclic AMP response element-binding protein activation (25).

$\text{Ca}^{2+}$ ion is a very versatile messenger, and it is not neces-

human CD34$^+$, monocyte, and HL-60 cells, a large $\text{Ca}^{2+}$, increase by $\text{Ca}^{2+}$ ionophores induces differentiation in dendro-

cytes (28–30).

Participation of GJ is another distinctive characteristic of cytokines and P2 agonist response. A high $[\text{Ca}^{2+}]$, increase by ATP and its analogs was independent of GJ, whereas the IL-3- and GM-CSF-dependent $[\text{Ca}^{2+}]$, increase was partly inhibited by carbenoxolone (Fig. 7C). The function of GJ in hematopoiesis is not clear, but it is known that Cx43 is highly expressed in bone marrow of neonatal animals and decreases after birth (31).
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However, expression of Cx43 can be increased in regeneration processes (32). Montecino-Rodriguez et al. (33) showed that Cx43\textsuperscript{-/-} heterozygote animals have problems with myeloid and lymphoid regeneration. GJ also act on the maintenance of primitive human CD34\textsuperscript{+} cells co-cultivated with L87/4 stromal fibroblasts (34). In addition, the stroma regulates quiescence of primitive cells through GJ by keeping more CD34\textsuperscript{+} cells quiescent in the stromal cell line S17, whereas higher proliferation occurs in the stroma of leukemia patients, who have fewer GJ (35). Proliferation inhibition by carbenoxolone shows the important role of GJ in proliferation (Fig. 7D).

Participation of GJ in Ca\textsuperscript{2+}, release by cytokines explains how stromal cells are also responsive to specific cytokines such as SCF, IL-7, and EPO. Consequently primitive cells may conduct Ca\textsuperscript{2+}, signals to stromal cells that trigger others effects, like cytokine release, modification of extracellular matrix, and cellular mobilization. These effects have not yet been investigated.

In the supplemental Video S3, a Ca\textsuperscript{2+}, wave is clearly observed in the stromal cell showing the functional presence of intercellular communication. Ca\textsuperscript{2+}, waves without stimulation were seldom observed; however, movements between hematopoietic and stromal cells are often observed and would occur by pseudopodia, which have been described previously in murine hematopoietic primitive cells (36).

The action of purinergic receptors is not clear. Herein we propose that these receptors promote differentiation in the primitive population. In another study, action of ATP was shown to be synergic with cytokines, increasing the number of progenitor cells (8). The source of ATP in the hematopoietic system is another intriguing fact. The release of ATP in the bone marrow microenvironment may occur with other neurotransmitters such as adrenergic transmitters that regulate the attraction of stem cells to their niches (37) or by either release of endothelial cells or release of cytoplasm in bone fracture (38 - 40).

These results allowed us both to show an important role for Ca\textsuperscript{2+}, in hematopoiesis and to evidence how Ca\textsuperscript{2+}, signaling contributes to this process. Temporal changes in Ca\textsuperscript{2+}, signals and intercellular communication should be further investigated to better understand the role of Ca\textsuperscript{2+}, in the hematopoietic system. The low cytoplasm volume and the small size of hematopoietic cells raise difficulties in better evaluating subcellular aspects associated to Ca\textsuperscript{2+}, signaling.

In this study, redundancy in Ca\textsuperscript{2+}, signaling was evidenced for all agonists used. Although distinct effects were induced, Ca\textsuperscript{2+}, was utilized as the coordinator of their functions. Participation of different subtypes of Ras, MAPK, PLC\textgreek{cy}, PLC\textgreek{B}, PKC, and CaMK proteins have to be investigated. A cross-talk between Ca\textsuperscript{2+}, signaling and Ras-Raf-MEK-MAPK pathways are under investigation in murine and human HSCs.

It may be stated that Ca\textsuperscript{2+}, participates in hematopoiesis depending on signal intensity, type of kinase activated, and participation of GJ. Herein we report results of an ongoing study aimed at the understanding of Ca\textsuperscript{2+}, signal translators and other factors that control the development of murine hematopoiesis.

REFERENCES

1. Wahl, M. I., Daniel, T. O., and Carpenter, G. (1988) Science 241, 968 – 970
2. Berridge, M. J., and Irvine, R. F. (1984) Nature 312, 315 – 321
3. Tonon, R., and D’Andrea, P. (2002) Biochimie 90, 153 – 160
4. Collison, K., Saleh, S., Parhar, R., Meyer, B., Kwaasi, A., Al-Sedairy, S., and Al-Mohanna, F. (1998) J. Immunol. 161, 3737 – 3745
5. Dutt, P., Wang, J. F., and Groopman, J. E. (1998) J. Immunol. 161, 3652 – 3658
6. Lemaire, I., Falzoni, S., Leduc, N., Zhang, B., Pellegratti, P., Adinolfi, E., Chiozzi, P., and Di Virgilio, F. (2006) J. Immunol. 177, 7257 – 7265
7. Rossi, L., Manfredini, R., Bertolini, F., Ferrari, D., Fogli, M., Zini, R., Salati, S., Salvestrini, V., Gulinelli, S., Adinolfi, E., Ferrari, S., Di Virgilio, F., Baccarani, M., and Lemoli, R. M. (2007) Blood 109, 533 – 542
8. Lemoli, R. M., Ferrari, D., Fogli, M., Rossi, L., Pizzirani, C., Forchach, S., Chiozzi, P., Vasselli, D., Bertolini, F., Foutz, T., Aluigi, M., Baccarani, M., and Di Virgilio, F. (2004) Blood 104, 1662 – 1670
9. Dexter, T. M., Allen, T. D., and Lajtha, L. G. (1977) J. Cell. Physiol. 91, 335 – 344
10. Quesenberry, P. J., Crittenden, R. B., Lowry, P., Kittle, E. W., Rao, S., Peters, S., Ramshaw, H., and Stewart, F. M. (1994) Blood Cells 20, 97 – 106
11. Whetton, A. D., Vallance, S. J., Monk, P. N., Cróg, E. J., Dexter, T. M., and Heyworth, C. M. (1988) Biochem. J. 256, 585 – 592
12. Carroll, M. P., and May, W. S. (1994) J. Biol. Chem. 269, 1249 – 1256
13. Adrian, K., Bernhard, M. K., Breitinger, H., and Oglive, A. (2000) Biochem. Biophys. Acta 1492, 127 – 138
14. Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torboli, M., Bolognesi, G., and Barcording, O. R. (2001) Blood 97, 580 – 600
15. Paredes-Gamero, E. J., Craveiro, R. B., Pesquero, J. B., Faenza, J. P., Oshiro, M. E., and Ferreira, A. T. (2006) Eur. J. Pharmacol. 534, 30 – 38
16. Clifford, E. E., Parker, K., Humphreys, B. D., Kertesy, S. B., and Dubyak, G. R. (1998) Blood 91, 3172 – 3181
17. Paredes-Gamero, E. J., Dreyfuss, J. L., Nader, H. B., Oshiro, M. E. M., and Ferreira, A. T. (2007) Exp. Gerontol. 42, 320 – 326
18. Wang, L., Jacobsen, S. E., Bengtsson, A., and Erlinge, D. (2004) BMC Immunol. 5, 16
19. Cancelas, J. A., Koevoet, W. L., de Koning, A. E., Mayen, A. E., Rombouts, E. J., and Plommecher, R. E. (2000) Blood 96, 498 – 505
20. Bradley, H. L., Hawley, T. S., and Bunting, K. D. (2002) Blood 102, 3983 – 3989
21. Wandzioch, E., Edling, C. E., Palmer, R. H., Carlsson, L., and Hallberg, B. (2004) Blood 104, 51 – 57
22. Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003) Nature 423, 409 – 414
23. Duncan, A. W., Rattis, F. M., DiMascio, L. N., Condgon, D. K., Pizianos, G., Zhao, C., Yoon, K., Crot, J. M., Willert, K., Gaiano, N., and Reya, T. (2005) Nat. Immunol. 6, 314 – 322
24. Rossi, F., McNagny, M., Smith, G., Frampton, J., and Graf, T. (1996) EMBO J. 15, 1894 – 1901
25. Kitos, C. M., Sankar, U., Illario, M., Colomer-Font, J. M., Duncan, A. W., Ribar, T. J., Reya, T., and Means, A. R. (2005) J. Biol. Chem. 280, 33101 – 33108
26. Thower, E. C., Hagar, R. E., and Ehrlich, B. E. (2001) Trends Pharmacol. Sci. 22, 580 – 586
27. Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bone, A. D., Knot, H. I., and Lederger, W. J. (1995) Science 270, 633 – 637
28. Czerniecki, B. J., Carter, C., Rivoltini, L., Koski, G. K., Kim, H. I., Wang, D. E., Roos, J. G., Hjaija, Y. M., Xu, S., Rosenberg, S. A., and Cohen, P. A. (1997) J. Immunol. 159, 3823 – 3837
29. Engels, H. F., Koski, G. K., Bedosian, I., Xu, S., Luger, S., Nowell, P. C., Cohen, P. A., and Czerniecki, B. J. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 10332 – 10337
30. Koski, G. K., Schwartz, G. N., Wang, D. E., Czerniecki, B. J., Carter, C.,
31. Rosendaal, M., Green, C. R., Rahman, A., and Morgan, D. (1994) J. Cell Sci. 107, 29–37
32. Krenács, T., and Rosendaal, M. (1998) Am. J. Pathol. 152, 993–1004
33. Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2000) Blood 96, 917–924
34. Dürig, J., Rosenthal, C., Halfmeyer, K., Wiemann, M., Novotny, J., Bingmann, D., Duhrsen, U., and Schirrmacher, K. (2000) Br. J. Haematol. 111, 416–425
35. Paraguassá-Braga, F. H., Borojevic, R., Bouzas, L. F., Barcinski, M. A., and Bonomo, A. (2003) Cell Death Differ. 10, 1101–1108
36. Frimberger, A. E., McAuliffe, C. I., Werme, K. A., Tuft, R. A., Fogarty, K. E., Benoit, B. O., Dooner, M. S., and Quesenberry, P. J. (2001) Br. J. Haematol. 112, 644–654
37. Katayama, Y., Battista, M., Kao, W. M., Hidalgo, A., Peired, A. J., Thomas, S. A., and Frenette, P. S. (2006) Cell 124, 407–421
38. Bodin, P., and Burnstock, G. (2001) Neurochem. Res. 26, 959–969
39. Hoebertz, A., Arnett, T. R., and Burnstock, G. (2003) Trends Pharmacol. Sci. 24, 290–297
40. Thuringer, D. (2004) Ann. N. Y. Acad. Sci. 1030, 14–27