Cyclic AMP Raises Intracellular Ca\(^{2+}\) in Human Megakaryocytes Independent of Protein Kinase A

Els den Dekker, Johan W.M. Heemskerk, Gertie Gorter, Hans van der Vuurst, José Donath, Christine Kroner, Katsuho Mikoshiba, Jan-Willem N. Akkerman

Abstract—The immature megakaryoblastic cell line MEG-01 responds to iloprost with an increase in cytosolic Ca\(^{2+}\) and cAMP. The Ca\(^{2+}\) response is almost absent in CHRF-288-11 cells, but cAMP formation is preserved in this more mature megakaryoblastic cell line. Also, in human hematopoietic stem cells, iloprost induces a Ca\(^{2+}\) response and cAMP formation. The Ca\(^{2+}\) response is downregulated during megakaryocytopoiesis, but cAMP formation remains unchanged. The Ca\(^{2+}\) increase may be caused by cAMP-mediated inhibition of Ca\(^{2+}\) sequestration, because it is (1) independent of Ca\(^{2+}\) entry; (2) mimicked by forskolin, an activator of adenylyl cyclase, and isobutylmethylxanthine, an inhibitor of phosphodiesterases; and (3) preserved in the presence of inhibitors of protein kinase A and inositol-1,4,5-triphosphate receptors. The small GTPase Rap1 has been implicated in the control of Ca\(^{2+}\) sequestration. Indeed, Rap1 activation parallels the iloprost- and forskolin-induced Ca\(^{2+}\) increase and is unaffected by the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid-AM. These findings reveal a novel mechanism for elevating cytosolic Ca\(^{2+}\) by cAMP, possibly via GTP-Rap1. (Arterioscler Thromb Vasc Biol. 2002;22:179-186.)

Key Words: calcium ■ cAMP ■ stem cells ■ megakaryocytes ■ Rap1

Megakaryocytopoiesis is accompanied by downregulation of stem cell properties and upregulation of properties that later determine platelet functions. One of the first characteristics of megakaryocytopoiesis is the appearance of the fibrinogen receptor, integrin \(\alpha_{IIb}\beta_{3}\) (glycoprotein Ib/IIa, or CD41/CD61), together with the disappearance of the stem cell marker CD34. A second early event is the synthesis of von Willebrand factor (vWF), which starts in immature, both CD61\(^{+}\) and CD34\(^{+}\) megakaryocytes. At a later stage, the vWF receptor, glycoprotein Ib\(\alpha\) (CD42b), is expressed, which marks the beginning of polyploidization.\(^1\) The transition from proliferating to differentiating megakaryocytes is accompanied by loss of nuclear-associated acetylcholinesterase activity.\(^2\)

The megakaryoblastic cell lines MEG-01, DAMI, and CHRF-288-11 have properties in common with normal megakaryocytes at different stages of maturation.\(^3\) The immature MEG-01 cells already show an increase in cytosolic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)], on stimulation by thrombin and platelet-activating factor. The more mature DAMI and CHRF-288-11 cells upregulate this property and, in addition, become sensitive to thromboxane A\(_2\). These cells respond to the prostacyclin analogue iloprost with an increase in cAMP, a response that is also upregulated in the more mature cell lines. Hence, the immature megakaryoblast already has the capacity to regulate Ca\(^{2+}\) and cAMP via mechanisms also seen in platelets. An interesting exception is that in MEG-01 cells, iloprost-induced cAMP formation is accompanied by a rise in [Ca\(^{2+}\)]. This is in sharp contrast with platelets, which do not raise [Ca\(^{2+}\)], when treated with prostacyclin and show Ca\(^{2+}\) responses by thrombin and other platelet-activating agents that are completely blocked by an increase in cAMP.\(^4\)

In the present study, we aimed to clarify how prostacyclin raises [Ca\(^{2+}\)], in immature megakaryoblasts. Studies were focused on iloprost-induced signaling pathways to [Ca\(^{2+}\)], and on the small GTPase Rap1, which has been implicated in Ca\(^{2+}\) regulation in platelets in the same way that phospholamban regulates Ca\(^{2+}\) signaling in cardiac and muscle cells. Earlier studies have shown that megakaryoblastic cell lines express the prostacyclin receptor\(^5\) Rap1\(^6\) and the 97-kDa sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase SERCA3b, which functions in Ca\(^{2+}\) sequestration in platelets.\(^6\) The data show that in addition to MEG-01 cells, human stem cells and immature megakaryocytes respond to iloprost with an increase in cAMP and a rise in [Ca\(^{2+}\)], and that cAMP controls cytosolic Ca\(^{2+}\), possibly via Rap1-GTP.
Iloprost Induces a Ca\textsuperscript{2+} Increase in Megakaryoblastic Cell Lines

Stimulation of MEG-01 cells with iloprost induced both an increase in [Ca\textsuperscript{2+}], and a rise in cAMP, confirming earlier observations.\textsuperscript{3} In CHRF-288-11 cells, the rise in [Ca\textsuperscript{2+}], had almost disappeared, but the increase in cAMP was \textasciitilde 2-fold higher (Figures 1A and 1B). These data are consistent with the concept that cell maturation is accompanied by the downregulation of iloprost-induced Ca\textsuperscript{2+} increases and the upregulation of iloprost-induced cAMP formation. Stimulation of MEG-01 cells with thrombin induced a Ca\textsuperscript{2+} response similar to that of iloprost. Thrombin induced a 3-fold higher Ca\textsuperscript{2+} response in CHRF-288-11 than in MEG-01 cells, illustrating that the failure of iloprost to raise [Ca\textsuperscript{2+}], in CHRF-288-11 cells was not caused by abnormalities in Ca\textsuperscript{2+} storage or influx. To assess the contributions of Ca\textsuperscript{2+} mobilization and influx in the total [Ca\textsuperscript{2+}], increase, experiments were repeated in Ca\textsuperscript{2+}-free medium. Ca\textsuperscript{2+} mobilization by iloprost and thrombin was 42\pm 6\% and 31\pm 12\%, respectively, of the peak Ca\textsuperscript{2+} level in MEG-01 cells; for CHRF-288-11 cells, these values were 18\%\pm 3\% and 22\%\pm 2\%, respectively. As expected, stimulation with thrombin failed to raise cAMP levels in both cell lines. Interestingly, in MEG-01 cells, iloprost was able to induce a further Ca\textsuperscript{2+} rise after thrombin (Figure 1C), suggesting that these 2 agonists raise [Ca\textsuperscript{2+}], by a different mechanism.

Methods

For the Methods section, please see http://www.atvb.ahajournals.org.

Results

Iloprost Induces a Ca\textsuperscript{2+} Increase in Megakaryoblastic Cell Lines

Figure 1. Calcium and cAMP increases in megakaryoblastic cell lines. Megakaryoblastic cell lines were loaded with fura-2/AM, and Ca\textsuperscript{2+} increases, in the presence of 1 mmol/L extracellular Ca\textsuperscript{2+} (A, C), and cAMP formation (B), induced by iloprost (1 \textmu mol/L) or thrombin (5 U/mL), were measured in MEG-01 cells (open bars) and CHRF-288-11 cells (hatched bars). Basal levels of [Ca\textsuperscript{2+}] were 131\pm 15 and 112\pm nmol/L for MEG-01 and CHRF-288-11 cells, respectively. Basal levels of cAMP were 12\pm 3 and 8.0\pm 3 pmol/10\textsuperscript{6} cells for MEG-01 and CHRF-288-11 cells, respectively. Data are expressed as mean\pm SD (n=3).

Figure 2. Ca\textsuperscript{2+} responses in stem cells and cultured megakaryocytes. A (upper panel) shows Ca\textsuperscript{2+} responses in the presence of 1 mmol/L extracellular Ca\textsuperscript{2+} induced by iloprost (1 \textmu mol/L) in suspensions of stem cells (1), immature (2), and mature megakaryocytes (3). A representative tracing for 3 observations with similar results is shown. A (lower panel) shows Ca\textsuperscript{2+} responses of single immobilized cells: (1) stem cells (day 1 cells adhering to anti-CD34); (2) immature megakaryocytes (CD42b\textsuperscript{low} day 7 cells adhering to anti-CD61); and (3) mature megakaryocytes (CD42b\textsuperscript{high} day 14 cells adhering to anti-CD61). A representative tracing for at least 7 observations with similar results is shown. B shows similar experiments for cells stimulated by 1 U/mL thrombin.

Iloprost Induces a Ca\textsuperscript{2+} Increase in Megakaryocytes

To investigate whether iloprost induced similar increases in Ca\textsuperscript{2+} and cAMP in megakaryocytes, CD34\textsuperscript{+} stem cells were immunomagnetically purified from umbilical cord blood and cultured in vitro with recombinant human thrombopoietin and cultured in vitro with recombinant human thrombopoietin and recombinant human stem cell factor. After 7 and 14 days, immature and mature megakaryocytes were isolated by a second immunomagnetic sorting based on expression of CD61 and CD42b, respectively. The 3 isolation procedures resulted in almost pure (>95\%) suspensions of CD34\textsuperscript{-}, CD61-, and CD42b-expressing cells. The 3 isolation procedures resulted in almost pure (>95\%) suspensions of CD34\textsuperscript{-}, CD61-, and CD42b-expressing cells. The CD34\textsuperscript{+} cells were predominantly diploid and contained few megakaryocytes. The immature megakaryocytes, represented by the CD61-expressing cells, were diploid (77\%) and tetraploid (23\%) and contained properties of stem cells (44\%) and mature megakaryocytes (25\%), illustrating an intermediate stage of megakaryocyte maturation. The mature megakaryocyte suspension consisted for >95\% of CD61\textsuperscript{+} and CD42b\textsuperscript{+} cells, without a further increase in ploidy. For a detailed characterization of the cell suspensions, please see Table 1 at http://www.atvb.ahajournals.org. As shown in Figure 2A (upper panel), iloprost induced a Ca\textsuperscript{2+} response in stem cells as well as in immature and mature megakaryocytes. This response was the sum of Ca\textsuperscript{2+} mobilization (60\%) and influx (40\%), and the peak value of [Ca\textsuperscript{2+}], was higher in stem cells (36\pm 13 nmol/L) and immature megakaryocytes (33\pm 3 nmol/L) than in mature megakaryocytes (12\pm 6 nmol/L), illustrating a similar downregulation as observed in mature megakaryo-
Illoprost-Induced \( \text{Ca}^{2+} \) Signaling Is Mimicked by cAMP-Elevating Agents

Illoprost is known to bind with high affinity to receptors of the IP class (which bind prostaglandins of the I type) and with a low affinity to receptors of the EP1 class (which bind prostaglandins of the E type). To clarify which receptor takes part in illoprost signaling to \( \text{Ca}^{2+} \), experiments were repeated in nominally \( \text{Ca}^{2+} \)-free buffer (Figure 3A, insert), illustrating that carbaprostacyclin triggered both mobilization and influx. Stimulation of IP receptors is known to activate Gs, the trimeric G protein that activates adenylyl cyclase. To investigate whether this enzyme takes part in illoprost-induced \( \text{Ca}^{2+} \) signaling, immature megakaryocytes were stimulated with the adenylyl cyclase activator forskolin. In 23% (11 of 48) of the cells, this treatment induced a \( \text{Ca}^{2+} \) response (Figure 3B) and \( \text{Ca}^{2+} \) mobilization (Figure 3B, insert) similar to that of illoprost, indicating that illoprost presumably signals to \( \text{Ca}^{2+} \) by way of adenylyl cyclase.

In MEG-01 cells, forskolin (40 to 100 \( \mu \text{mol/L} \)) induced a \( \text{Ca}^{2+} \) increase of 105±20 \( \text{nmol/L} \) (Figure 3C, left panel). The structurally dissimilar compound isobutylmethylxanthine (IBMX, 500 \( \mu \text{mol/L} \)), which inhibits phosphodiesterases and thereby prevents cAMP breakdown, also induced an increase in [Ca\(^{2+}\)], although this response was much lower than the forskolin-induced \( \text{Ca}^{2+} \) increase (Figure 3C, middle panel). A similar, albeit weaker, \( \text{Ca}^{2+} \) increase was also induced by dibutyryl cAMP (data not shown). To further investigate the role of cAMP in \( \text{Ca}^{2+} \) increases, the effect of an inhibitor of cAMP metabolism was investigated. MEG-01 cells were preincubated with IBMX (500 \( \mu \text{mol/L} \), 5 minutes, 37°C) and subsequently treated with illoprost (1 \( \mu \text{mol/L} \)). As shown in Figure 3C (right panel), IBMX treatment potentiated the illoprost-induced rise in \( \text{Ca}^{2+} \) by 15%, strongly supporting the concept that cAMP plays a role in the illoprost-induced \( \text{Ca}^{2+} \) increase.

As expected, stimulation of IP receptors and direct activation of adenylyl cyclase induced formation of cAMP in cultured megakaryocytes (please see Table II at http://www.atvb.ahajournals.org). Thus, the increase in [Ca\(^{2+}\)], by these treatments was accompanied by an increase in cAMP.

The Forskolin-Induced \( \text{Ca}^{2+} \) Increase Does Not Involve IP\(_3\) Receptors or Protein Kinase A

To investigate whether increases in intracellular inositol triphosphate (IP\(_3\)) levels mediated the forskolin-induced \( \text{Ca}^{2+} \) response, MEG-01 cells in nominally \( \text{Ca}^{2+} \)-free buffer were treated with the IP\(_3\) receptor antagonist 2-aminoethoxydiphenylborate (2-
In platelets, 100 μmol/L 2-ABP completely abolished thrombin- and thromboxane A₂–induced Ca²⁺ responses (Figure 4A, left panel). At concentrations of 50 μmol/L (not shown) and 100 μmol/L (Figure 4A), 2-ABP partially inhibited the forskolin-induced Ca²⁺ increases (middle panel) and totally inhibited the thrombin-induced Ca²⁺ increases (right panel) in MEG-01 cells. Similar results were obtained with immature megakaryocytes (data not shown). Thus, in contrast to thrombin, which fully depended on IP₃ receptors for raising [Ca²⁺]ᵢ, forskolin-induced Ca²⁺ increases were partly independent of IP₃ receptors.

To investigate whether the forskolin-induced Ca²⁺ increase also involved protein kinase A (PKA), MEG-01 cells were treated with the PKA inhibitor H89. As shown in Figure 4B (left panel), the forskolin-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) was completely abolished by pretreatment with H89, indicating that H89 is a good inhibitor of PKA in MEG-01 cells. Treatment with H89, however, did not abolish the forskolin-induced Ca²⁺ increase. Instead, a slight but consistent increase in the release of Ca²⁺ from intracellular stores was observed (Figure 4B, middle panel). PKA inhibition thus “unmasks” a slight suppression of Ca²⁺ increases by PKA. The same effect was seen when MEG-01 cells were stimulated with thrombin (Figure 4B, right panel). Immature megakaryocytes reacted the same way as MEG-01 cells did (data not shown). The specific PKA activator Sp-5,6-DCI-cBIMPS induced the same extent of VASP phosphorylation as forskolin (Figure 4C, left panel) but did not raise [Ca²⁺], in MEG-01 cells (Figure 4C, right panel). Even after stimulation of PKA with Sp-5,6-DCI-cBIMPS, forskolin induced a Ca²⁺ increase (Figure 4C, right panel). These findings argue in favor of a direct effect of cAMP on Ca²⁺ homeostasis, without involvement of PKA.

Iloprost and Thrombin Induce Rap1 Activation

The small GTPase Rap1 has been implicated in the regulation of Ca²⁺ sequestration, because (1) it coimmunoprecipitates with the sarco/endoplasmic reticulum Ca²⁺-ATPase isoform 3b (SERCA3b) and (2) binding of GTPγS to Rap1 has been suggested to be related to SERCA3b activity. Because the guanine nucleotide exchange factor (GEF) for Rap1, Epac1/2, is a target for PKA-independent signaling by cAMP, Rap1...
activation of Rap1 was observed on stimulation with thrombin, which was transient and disappeared after 1 minute. Incubation of blots with antibodies that specifically recognize Rap1α or Rap1β showed that iloprost activated both Rap1α and Rap1β to the same extent and with the same kinetics (Figure 5B). In CHRF-288-11 cells, the responses were completely different. In these cells, iloprost induced only little Rap1 activation, but thrombin induced a 15-fold increase in GTP-Rap1, which remained high throughout the incubation period (Figure 5C). Thus, the downregulation of iloprost-induced Ca²⁺ responses and upregulation of thrombin-induced Ca²⁺ responses, observed when immature and mature megakaryoblastic cell lines were compared, were accompanied by similar changes in the formation of GTP-Rap1, suggesting that these responses go hand in hand.

cAMP Induces Rap1 Activation and Ca²⁺ Signaling in MEG-01 but Not in CHRF-288-11 Cells

To investigate whether direct activation of cAMP formation induced similar changes in [Ca²⁺], and GTP-Rap1 as seen with iloprost, MEG-01 and CHRF-288-11 cells were treated with forskolin. In agreement with the effect of iloprost, forskolin induced an increase in [Ca²⁺], and formation of GTP-Rap1 in MEG-01 but not in CHRF-288-11 cells (Figures 6A, 6B, left panel, and 6C). The onset of forskolin-induced Ca²⁺ increases in MEG-01 cells was ∼10 seconds later than the beginning of Rap1 activation, suggesting that Rap1 activation precedes the Ca²⁺ increase. Rap1 was also activated by the phosphodiesterase inhibitor IBMX (Figure 6B, right panel), although activation was much weaker and slower than that induced by forskolin. This difference corresponds with the difference in Ca²⁺ responses induced by these compounds (Figure 3C, left and middle panels).

To clarify the role of PKA in the forskolin-induced Rap1 activation, studies were repeated in the presence of the PKA inhibitor H89. As shown in Figure 6D, the forskolin-induced Rap1 activation was not affected by this inhibitor. To determine whether Rap1 activation is an upstream step of Ca²⁺ increases or a result of changes in [Ca²⁺], Ca²⁺ increases were prevented by treating the cells with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-AM. The Ca²⁺-chelating capacity of BAPTA-AM was sufficient to abolish the forskolin-induced Ca²⁺ increase completely (data not shown). As also shown in Figure 6D, this treatment failed to interfere with the activation of Rap1. Together, these data favor the concept that Rap1 is activated by a rise in cAMP and that this activation is independent of changes in [Ca²⁺].

cAMP-Induced Rap1 Activation Is Not Accompanied by Phosphorylation

To determine whether Rap1 phosphorylation by PKA occurs concurrently with Rap1 activation, MEG-01 cells were treated with forskolin (100 μmol/L), and Rap1 phosphorylation was determined by a gel-shift assay (Figure 6E, upper panel). Because Rap1 has been described to be phosphorylated in iloprost-treated platelets after 15 minutes, control samples of platelets treated with iloprost (5 μmol/L) were analyzed for Rap1 phosphorylation (Figure 6E, lower panel). At the times when forskolin induced Rap1 activation in MEG-01 cells (between 5 and 25 seconds), Rap1 was not

could be the link between cAMP and Ca²⁺. Using a pulldown technique based on the specific binding of active, GTP-bound Rap1 to the Rap-binding domain of RaLGDS, we investigated the effect of iloprost and forskolin on Rap1 activation. Because the quantities of cultured megakaryocytes were below the detection limit of this technique, the studies were performed with MEG-01 and CHRF-288-11 cells only. Results from different experiments were related to internal standards as defined in the legend to Figure 5. In MEG-01 cells, addition of iloprost induced a 10-fold, sustained increase in GTP-Rap1 (Figure 5A). A much weaker 5-fold
Discussion

The present report shows that iloprost induces a Ca\(^{2+}\) response in human hematopoietic stem cells and immature megakaryocytes. Analysis of the signaling pathway by which iloprost raises [Ca\(^{2+}\)], reveals a central role of cAMP. First, iloprost-induced [Ca\(^{2+}\)] increases are accompanied by cAMP production. Second, the effect of iloprost is mimicked by carbaprostacyclin, a more specific agonist of the IP receptor, which is coupled to the adenyl cyclase–activating G protein, G\(_s\). Third, direct activation of adenyl cyclase by forskolin raises cAMP and [Ca\(^{2+}\)]. Fourth, the phosphodiesterase inhibitor IBMX, which prevents cAMP breakdown, induces a Ca\(^{2+}\) response and potentiates the iloprost-induced rise in [Ca\(^{2+}\)]. Together, these data illustrate that in immature megakaryocytes, rises in cAMP and [Ca\(^{2+}\)], go hand in hand.

The observations that dibutyryl cAMP, IBMX, and forskolin raise [Ca\(^{2+}\)], indicate that cAMP is an upstream regulator of Ca\(^{2+}\). Elevated cAMP levels have been shown to induce Ca\(^{2+}\) mobilization in HEK-293 cells, hepatocytes, neuronal cells, and articular chondrocytes.\(^{16-19}\) In these cells, the increase in [Ca\(^{2+}\)], was caused by PKA-mediated phosphorylation of IP\(_3\) receptors. This resulted in sensitization for IP\(_3\), leading to a 4-fold leftward shift of the dose-response curve of IP\(_3\)-mediated Ca\(^{2+}\) release.\(^{17}\)

The present findings in MEG-01 cells and immature megakaryocytes differ from those observations, because neither PKA nor IP\(_3\) receptors seem to be involved in the cAMP-induced [Ca\(^{2+}\)] increase. The PKA inhibitor H89, which inhibits PKA-mediated suppression of IP\(_3\)-induced Ca\(^{2+}\) responses in rat megakaryocytes\(^{20}\) and completely suppresses forskolin-induced VASP-Ser157 phosphorylation in MEG-01 cells, did not suppress the forskolin-induced Ca\(^{2+}\) response. Moreover, direct activation of PKA by the specific activator Sp-5,6-DCI-cBIMPS induced the same extent of Ca\(^{2+}\) response, illustrating IP\(_3\)-dependent as well as IP\(_3\)-independent routes. Cerebellar microsome,\(^{21}\) which express predominantly the type 1 IP\(_3\) receptor, and submandibular gland cells, which possess mainly type 2 and type 3 IP\(_3\) receptors,\(^{21}\) show the same sensitivity to 2-APB for IP\(_3\)-mediated Ca\(^{2+}\) responses (authors’ unpublished results, 2001). Thus, it is unlikely that forskolin raises Ca\(^{2+}\) via an IP\(_3\) receptor subtype that is resistant to 2-APB inhibition. An explanation for the IP\(_3\) receptor–mediated Ca\(^{2+}\) response might be sought in the Ca\(^{2+}\)-induced Ca\(^{2+}\) release triggered by the cAMP-mediated release of Ca\(^{2+}\) from intracellular stores. Although these responses occur independently of PKA, it is clear that phosphorylation of IP\(_3\) receptors by this kinase plays a role in MEG-01 cells like it does in platelets.\(^{22}\)

The findings in this report reveal a novel mechanism by which cAMP directly regulates [Ca\(^{2+}\)], independent of PKA and IP\(_3\) receptors. Interestingly, iloprost further increases phosphorylation. However, at later times (15 and 30 minutes), weak phosphorylation was visible (not shown). In platelets, a clear iloprost-induced phosphorylation of Rap1 was observed after 15 and 30 minutes, which corresponds with results from previous studies.\(^{14,15}\)
[Ca\(^{2+}\)], after thrombin in MEG-01 cells, indicating that these 2 compounds activate different Ca\(^{2+}\)-mobilizing mechanisms. Few PKA-independent effects of cAMP have been reported so far. Recently, De Rooij et al.\(^{12}\) and Kawasaki et al.\(^{13}\) described that the small GTPase Rap1 is activated by cAMP via cAMP-sensitive guanine nucleotide exchange factors (cAMP GEFs). Two types of cAMP GEF for Rap1 have been reported: cAMP–GEF–I, also called exchange protein, directly activated by cAMP (Epac), and cAMP–GEF–II. Interestingly, earlier work by Corvazier et al.\(^{11}\) and Lacabaratz-Porret et al.\(^{10}\) indicated that Rap1 might be involved in Ca\(^{2+}\)-regulation in platelets. First, proteins in crude platelet plasma membrane vesicles, ranging in molecular mass from 22 to 29 kDa, bound GTPγS. One of these proteins was identified as Rap1, and GTPγS binding was accompanied by inhibition of Ca\(^{2+}\)-ATPases in these vesicles. Second, Rap1 coimmunoprecipitated with the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase isoform 3b (SERCA3b), suggesting a physical interaction of these 2 proteins. The association of Rap1 with SERCA3b was lost on PKA-mediated phosphorylation of Rap1 on Ser179. The present results show that Rap1 activation is correlated with the release of Ca\(^{2+}\) from stores in stem cells and immature megakaryocytes. The cAMP-elevating agents iloprost, forskolin, and IBMX induce an increase in the active, GT-P-bound Rap1 together with Ca\(^{2+}\) release from stores in MEG-01 cells. Both Rap1a and Rap1b were activated by iloprost, indicating that cAMP-mediated Rap1 activation does not involve a specific subtype. The Rap1 activation and Ca\(^{2+}\) increase have similar kinetics and are independent of PKA, as shown by the insensitivity to H89. Rap1 was not phosphorylated by forskolin in MEG-01 cells within the time range (5 to 25 seconds) when this compound induced a Ca\(^{2+}\) increase and Rap1 activation. This result corresponds with that obtained in platelets, in which iloprost and prostacyclin induced Rap1 phosphorylation after a lag time of 15 minutes or later. Furthermore, Rap1 activation was not affected by PKA-mediated phosphorylation.\(^{15}\) Because the Ca\(^{2+}\) chelator BAPTA-AM did not suppress the cAMP-induced Rap1 activation, it is unlikely that Rap1 is downstream of Ca\(^{2+}\) increases. These findings are in correspondence with the hypothesis that Rap1 is a molecular “switch” that regulates SERCA activity: the cAMP-induced Rap1 activation is correlated with the inhibition of SERCA and a rise in [Ca\(^{2+}\)], (present data), and the PKA-mediated phosphorylation of Rap1 relieves the inhibition, resulting in activation of Ca\(^{2+}\)-ATPases and a decrease in [Ca\(^{2+}\)].\(^{10,11}\) The latter property is also observed in Ca\(^{2+}\) regulation by phospholamban in cardiac and muscle cells.\(^{23,24}\) Phosphorylation of this 24-kDa protein by PKA triggers stimulation of cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPases, leading to a fall in [Ca\(^{2+}\)].

In CHRF-288-11 cells, the Rap1 activation and release of Ca\(^{2+}\) from stores by forskolin had disappeared, suggesting that one of the steps in cAMP-induced Rap1 activation is downregulated during maturation and is probably absent in platelets. Indeed, previous studies indicated that cAMP does not induce Rap1 activation in platelets.\(^{15}\) Although the expression of Rap1 is upregulated during megakaryocytopenesis,\(^{5,10,25}\) the mechanism of cAMP-mediated Rap1 activation is downregulated. A likely candidate to be downregulated is a cAMP-dependent GEF for Rap1. However, we were unable to demonstrate expression of cAMP–GEF–I and cAMP–GEF–II mRNA in MEG-01 cells, CHRF-288-11 cells, or platelets by reverse transcription–polymerase chain reaction (data not shown). This suggests that either another subtype of cAMP-dependent GEF may exist or that downregulation of a yet-unknown cAMP-inhibitable GTPase-activating protein for Rap1 causes the loss of cAMP-induced Ca\(^{2+}\) rises during megakaryocyte maturation.

In MEG-01 and CHRF-288-11 cells, thrombin induced Rap1 activation, a process that is upregulated during maturation. This points to a major difference between Rap1 activation by forskolin and that by thrombin. In platelets, the first phase of thrombin-induced Rap1 activation critically depends on a rise in Ca\(^{2+}\) and is followed by an activation phase, in which PKC, phosphatidylinositol-3-kinase, and the integrin α\(_i\)β\(_i\) play a role.\(^{15,26}\) The first phase of Rap1 activation appears to be present already in MEG-01 cells, possibly reflecting the activity of Ca\(^{2+}\)-dependent GEFs, such as Ca\(^{2+}\)-and diacylglycerol-dependent GEF. The higher thrombin-induced activation of Rap1 in CHRF-288-11 cells is probably due to the higher Ca\(^{2+}\) response compared with that in MEG-01 cells. The sustained Rap1 activation in CHRF-288-11 cells may arise from a more sustained PKC or phosphatidylinositol-3-kinase activity in these more mature cells.

An interesting question is whether prostacyclin-induced Rap1 activation also occurs in vivo and what its role might be in megakaryocyteopoiisis. Functions of active Rap1 include induction of cell adhesion,\(^{27}\) inhibition of gene expression,\(^{28}\) and inhibition of cell proliferation by suppression of Rasm-mediated activation of Raf and mitogen-activated protein kinase.\(^{29,30}\) It is possible that immature megakaryocytes become exposed to prostacyclin in vivo, because bone marrow stromal cells, which are in close proximity to maturing megakaryocytes, may produce prostacyclin on stimulation by interleukin-1β.\(^{11,32}\) Thus, stromal cells may regulate megakaryocyte gene expression and proliferation via prostacyclin-induced Rap1 activation.

The present report shows that in immature megakaryocytes, cAMP, independent of PKA, induces release of Ca\(^{2+}\) from intracellular stores, a property that is downregulated during megakaryocyte maturation. Rap1 activation might play a role in the cAMP-induced [Ca\(^{2+}\)] increase, but further studies are needed to provide definite proof. Together these data reveal a novel mechanism for the regulation of cytosolic Ca\(^{2+}\) concentration, in stem cells and immature megakaryocytes, in which cAMP plays a central role.

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cAMP raises Ca$^{2+}$ in human megakaryocytes independent of protein kinase A

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Materials and Methods

Materials

The IP-receptor agonist carbaprostacyclin (carbaprost) and prostacyclin were from Cayman Chemical Company (Ann Arbor, MI, USA). The prostacyclin analog iloprost was a gift from Schering AG (Berlin, Germany). Fura-2-acetoxymethylester (Fura-2/AM), dibutyryl cyclic AMP, 3-isobutyl-1-methyl-xanthene (IBMX), ribonuclease A and α-thrombin were from Sigma (StLouis, MO, USA). Forskolin was from Calbiochem (San Diego, CA, USA). Sp-5,6-DCI-cBIMPS was obtained from Biolog (Bremen, Germany). The protein kinase A (PKA) inhibitor H89 was from Alexis (Läufelfingen, Switzerland). Ficoll Paque was from Pharmacia (Uppsala Sweden). rhTPO and rhSCF were from Pepro Tech (Rocky Hill, NJ, USA).

Monoclonal antibodies (MoAbs) against CD42b (6.20) and CD61 (7F12) were kindly provided by Dr. H.K. Nieuwenhuis, Dept. of Haematology (University Medical Center Utrecht, the Netherlands). FITC-labeled MoAbs against human CD61 (F803), CD42b (F802), a negative control X0949 and rabbit-anti-goat-peroxidase (RAGPO) were from DAKO (Glostrup, Denmark). MoAb against total Rap1 was from Transduction Laboratories (Lexington, NY, USA). FITC-labeled monoclonal antibodies against CD34 (HPCA-2) and against CD14 were from Becton Dickinson (Lincoln Park, NJ, USA). Goat polyclonal antibody against VASP and goat polyclonal antibodies specific for either Rap1a or Rap1b were from Santa Cruz (Santa Cruz, CA, USA).

Isolation of human CD34⁺ stem cells

Umbilical cord blood was collected during normal full-term deliveries and used within 48 hours. Written informed consent was obtained from all mothers before labor and delivery. Low density mononuclear blood cells were isolated on a layer of Ficoll Paque (d = 1.077 g/mL) and CD34⁺ stem cells were isolated using the miniMACS CD34 progenitor cell isolation system from Miltenyi Biotec (Bergisch Gladbach, Germany). The percentage CD34⁺ cells after isolation was determined by FACS analysis (FACS Calibur, Beckton Dickinson, Lincoln Park, NJ, USA) after labeling the cells with the FITC-labeled monoclonal antibody HPCA-2. The purity was typically 94-99%.

Cultures of megakaryoblastic cell lines and megakaryocytes

MEG-01 and CHRF-288-11 cells were cultured as described earlier.¹ Freshly isolated CD34⁺ cells were cultured in a 12-well culture plate at 37°C in a humidified atmosphere with 5% CO₂. The initial cell density was approximately 4 x 10⁵ cells/mL medium. The composition of the medium was as described by Zauli et al² but with the omission of nucleosides. rhTPO and rhSCF were added to a final concentration of 20 ng/mL and 50 ng/mL respectively. After three days of culture, 1 mL fresh medium was added supplemented with 20 ng/mL rhTPO and 50 ng/mL rhSCF (final concentrations). After seven and ten days of culture, 1 mL fresh culture medium containing 20 ng/mL rhTPO (final concentration) was added.

Purification of megakaryocytes

Cells cultured for either 1, 7 or 14 days were washed twice (5 minutes, 125g, 21°C) in phosphate buffered saline (PBS) containing 0.5% BSA and 2.5 mmol/L EDTA (buffer A). Day 7 cells were labeled with mouse-anti-human IgG directed against CD61 (7F12) and day 14 cells were labeled with mouse-anti-human IgG directed anti-CD42b (6.20), for 20 minutes at 4°C. After washing with buffer A, cells were labeled (20 minutes, 4°C) with goat-anti-mouse magnetic microbeads and washed again with buffer A. The cell suspension was brought on a ferromagnetic column, type MS⁺ (Miltenyi Biotec), and after three washing steps with 700 µl buffer A each, the retained cells were eluted with buffer A. To determine the composition of purified megakaryocyte suspensions, cells were incubated with FITC-labeled MoAbs against human CD34, CD61 or CD42b. Because the suspensions also contained a few cells with a monocytic appearance, they were also labeled with anti-CD14-FITC, to determine contamination by these cells. A FITC-labeled negative control was used to determine aspecific antibody binding. FACS analysis was performed with 5000 cells.
Cellular ploidy was determined according to Debili et al..\(^3\) Cells were labeled with propidium iodide and either a FITC-labeled negative control or CD61-FITC. On a FACS 5000 cells were analyzed, and megakaryocytes were identified by CD61-expression.

**cAMP determination**

cAMP determinations were performed with an enzyme immuno assay from Amersham Pharmacia Biotech (Buckinghamshire, England). For each measurement, 3 x 10^4 cells were resuspended in 100 µl Hepes-Tyrode (HT) buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L NaH$_2$PO$_4$, 1 mmol/L MgSO$_4$, 10 mmol/L Hepes, 0.1% (w/v) glucose, pH 7.4) and stimulated with different agents as defined in the Results section. Stimulation was terminated by addition of lysis buffer and samples were further treated according to the manufacturer's instructions for the non-acetylation assay.

**Platelet isolation**
The donors claimed not to have taken any medication during the preceding 10 days. After informed consent freshly drawn venous blood from healthy volunteers was collected into 0.1 volume of 130 mM trisodium citrate. Citrated blood was centrifugated (150·g, 15 min, 20°C), the platelet rich plasma was supplemented with prostaglandin I$_2$ (10 ng/ml) and ACD (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 ml distilled water) for acidification to pH 6.5 and centrifugated again (330·g, 15 min, 20°C). Platelets were resuspended in HT buffer and used for calcium measurements.

**Calcium measurements in cell suspensions and in single cells**

Cell suspensions were pelleted (5 minutes, 125 g, 21°C) and loaded with 3 µmol/L Fura-2/AM in HT buffer (1 mmol/L CaCl$_2$, 0.1% (w/v) glucose, 0.1% (w/v) BSA, pH 7.4) for one hour at a concentration of 1 x 10^6 cells/mL at 37°C in the dark. The cells were pelleted, resuspended in HT-buffer without BSA and without CaCl$_2$ to a final concentration of 1.6 x 10^6 cells/mL, and stored at room temperature in the dark. Immediately before analysis of [Ca$^{2+}$], the cells were resuspended to a final concentration of 2 x 10^5/mL in prewarmed (37°C) HT, containing 1 mmol/L CaCl$_2$ but no BSA. For determination of Ca$^{2+}$ mobilization from internal stores, cells were resuspended in nominally Ca$^{2+}$-free HT. Measurements and calibration were performed as described previously.^1^ For single cell determinations, cells cultured for 1 day were adhered to a round glass coverslip (22 mm diameter) coated with a MoAb against human CD34 (QBEND/10) and blocked with BSA. Cells cultured for 7 or 14 days were adhered to an anti-CD61 (7F12) coated coverslip for 15 minutes at room temperature. This procedure did not activate the cells, as indicated by a low resting level of [Ca$^{2+}$], and the absence of membrane blebbing. The coverslips were washed and the adhered cells were incubated with fura-2/AM (0.25 µmol/L) in buffer B (145 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L MgCl$_2$, 1 mmol/L CaCl$_2$, 10 mmol/L Na-HEPES, 0.1% (w/v) glucose, 0.1% (w/v) BSA, pH 7.4) for 45 minutes at room temperature. After loading, the cells were washed with buffer B and the coverslip was positioned in a preheated chamber (37°C) on an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). Changes in fura-2 fluorescence in individual cells were detected using a Quanticell 700 fluorometric video imaging system equipped with excitation and emission filterwheels (Visi Tech, Sunderland, Tyne & Wear, U.K.) as described by Heemskerk et al.^4^ Averaged, ratioed and background subtracted fluorescence images were measured every 2-3 seconds.

Cells were stimulated by replacing buffer B by prewarmed buffer B containing different agonists. At the end of each experiment the cells were first labeled with anti-CD14-FITC and thereafter with anti CD42b-FITC for 5 minutes in PBS/0.5% BSA at 37°C, to distinguish between megakaryocytes, monocytes and other cell types. Geometric regions matching individual cells were analyzed off-line, and CD14-expressing cells were excluded. Three cell populations were analyzed: (1) stem cells (day 1 cells adhering to anti CD34), (2) immature megakaryocytes (CD42b$^{low}$ day 7 cells adhering to anti-CD61) and (3) mature megakaryocytes (CD42b$^{high}$ day 14 cells adhering to anti-CD61). Calibration parameters were derived as described previously.\(^5\)
Determination of PKA activity
To determine whether the forskolin-induced PKA activation was inhibited by H89, and whether Sp-5,6-DCl-cBIMPS stimulated PKA, MEG-01 cells were resuspended in Ca2+-free HT buffer (pH 7.2, 0.1% glucose) and treated with these agents as defined in the Results section. Subsequently, the mobility-shift of VASP, caused by PKA-mediated phosphorylation on Ser157, was determined on Western blot.6

Rap1 activation in megakaryoblastic cell lines
MEG-01 and CHRF-288-11 cells were resuspended in Ca2+-free HT buffer (pH 7.2, 0.1% glucose) and treated with agents as defined in the Results section. Active GTP-bound Rap1 was measured via binding to RalGDS-RBD, as described by Franke et al.7 Precipitates were subjected to SDS-PAGE (15%) and transferred to Immobilon-P polyvinylidene difluoride membranes. For studies in MEG-01, each lane contained precipitated GTP-Rap1 from 0.7 x 10^6 cells; for studies in CHRF-288-11 cells, each lane contained GTP-Rap1 from 2 x 10^6 cells. Each blot contained a concurrently run sample of a total cell lysate (9 x 10^4 MEG-01 cells or 3 x 10^5 CHRF-288-11 cells) as an internal standard. Rap1 was detected with a MoAb which recognizes both Rap1a and Rap1b, or with polyclonal antibodies recognizing specifically Rap1a or Rap1b, as indicated in the Results section. Immune complexes were detected by enhanced chemiluminescence. This method ensures recovery of almost all immunodetectable Rap1 in complex with RalGDS-RBD.7 To determine the percentage of active Rap1 (relative to the total amount of Rap1 (GDP- + GTP-bound), the lanes were scanned using ImageQuant, and the pixel density of each lane was related to that of the reference.

Rap1 phosphorylation
Phosphorylation of Rap1 was determined by the gel-shift assay as previously described.8 In short, MEG-01 cells or platelets were resuspended in Ca2+-free HT buffer (pH 7.2, 0.1% glucose), and treated with forskolin (100 µmol/L) or iloprost (5 µmol/L). Cells were lysed at different time points in Laemmli sample buffer. After SDS-PAGE on a 12.5 % gel and blotting, Rap1 was detected with a MoAb which recognizes both Rap1a and Rap1b.

Statistical analysis
Statistical analysis was performed using SPSS software (SPSS Inc.). A two-tailed paired Student's t-test was used for single comparisons. Multiple comparisons were performed with ANOVA. The criterion for significance was P<0.05. Data are expressed as mean ± SD (for n data).
Table I. Surface marker expression and ploidy of suspensions of stem cells, immature and mature megakaryocytes

<table>
<thead>
<tr>
<th>suspension</th>
<th>surface marker</th>
<th>ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34</td>
<td>CD61</td>
</tr>
<tr>
<td>stem cells</td>
<td>95.0±1.4</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>immature Mk’s</td>
<td>44.2±6.6</td>
<td>95.3±1.2</td>
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<tr>
<td>mature Mk’s</td>
<td>7.2±2.5</td>
<td>97.4±2.0</td>
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</table>

Stem cells were isolated from umbilical cord blood, and after 7 and 14 days, immature and mature megakaryocytes (Mk’s) were immunomagnetically purified as described in Materials and Methods. The fraction of CD34, CD61 and CD42b expressing cells, and the ploidy were determined by FACS analysis. In the purified immature and mature megakaryocyte suspensions a minor contamination of 5-10% monocytes (CD14⁺) was detected. Data are expressed as percentage of total cell number (mean ± SD, n=3).

Table II. cAMP accumulation in stem cells, immature and mature megakaryocytes

<table>
<thead>
<tr>
<th></th>
<th>basal</th>
<th>iloprost</th>
<th>forskolin</th>
<th>carbaprost</th>
</tr>
</thead>
<tbody>
<tr>
<td>stem cells</td>
<td>1.0±0.2</td>
<td>5.6±1.6</td>
<td>5.5±1.3</td>
<td>ND</td>
</tr>
<tr>
<td>immature Mk’s</td>
<td>1.0±0.4</td>
<td>11.1±2.2</td>
<td>6.4±1.7</td>
<td>3.7±1.0</td>
</tr>
<tr>
<td>mature Mk’s</td>
<td>1.2±0.8</td>
<td>6.9±1.7</td>
<td>3.4±0.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cell suspensions were stimulated for 1 min at 37°C with either 1 µmol/L iloprost, 100 µmol/L forskolin or 1 µmol/L carbaprost. Data show the cAMP accumulation in pmol cAMP/10⁶ cells (mean ± SD, n=3). ND = not determined. ANOVA indicated that iloprost, forskolin and carbaprost induced significant cAMP increases compared to the basal levels (P<0.05, n=3-4).
References


