Development of Platelet Inhibition by cAMP during Megakaryocytopoiesis* 

Received for publication, November 29, 2001, and in revised form, April 17, 2002 
Published, JBC Papers in Press, May 7, 2002, DOI 10.1074/jbc.M111390200

Els den Dekker†, Gertie Gortert‡, Johan W. M. Heemskerkt§, and Jan-Willem N. Akkerman¶

From the †Laboratory for Thrombosis and Haemostasis, Department of Haematology, University Medical Center Utrecht and the Institute for Biomembranes, Utrecht University, 3508 GA Utrecht, The Netherlands and the §Department of Biochemistry and Human Biology, Maastricht University, 6200 MD Maastricht, The Netherlands

Prostacyclin is a potent inhibitor of agonist-induced Ca\textsuperscript{2+} increases in platelets, but in the megakaryocytic cell line MEG-01 this inhibition is absent. Using human megakaryocytic cell lines representing different stages in megakaryocyte (Mk) maturation as well as stem cells and immature and mature megakaryocytes, we show that the inhibition by prostacyclin develops at a late maturation stage shortly before platelets are formed. This late appearance is not caused by insufficient cAMP formation or absent protein kinase A (PKA) activity in immature cells. Instead, the appearance of Ca\textsuperscript{2+} inhibition by prostacyclin is accompanied by a sharp increase in the expression of the catalytic subunit of PKA (PKA-C) but not by changes in the expression of the PKA regulatory subunits I\(\alpha\), I\(\beta\), and II\(\beta\). Overexpression of PKA-C in the megakaryocytic cell line CHRF-288-11 potentiates the Ca\textsuperscript{2+} inhibition by prostacyclin. Thus, up-regulation of PKA-C appears to be a key step in the development of Ca\textsuperscript{2+} inhibition by prostacyclin in platelets.

A rise in cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) is a key step in platelet activation. Platelet agonists raise [Ca\textsuperscript{2+}], by the direct stimulation of Ca\textsuperscript{2+} channels in the plasma membrane (1) and by stimulating G-protein- and tyrosine kinase-coupled receptors, which initiate signaling pathways that generate inositol 1,4,5-trisphosphate (IP$_3$)\textsuperscript{2} (2, 3). IP$_3$ activates IP$_3$ receptors in the endoplasmic reticulum, releasing Ca\textsuperscript{2+} into the cytosol. The depleted Ca\textsuperscript{2+} stores trigger store-operated Ca\textsuperscript{2+} entry across the plasma membrane (4). Plasma membrane Ca\textsuperscript{2+}-ATPases and sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPases (SERCA\textsubscript{3}) then remove Ca\textsuperscript{2+} ions from the cytosol and restore the basal [Ca\textsuperscript{2+}], concentration of about 70 nM (5).

* This work was supported by The Netherlands Heart Foundation Grant 97-142. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported by The Netherlands Thrombosis Foundation. To whom correspondence should be addressed: Laboratory for Thrombosis and Haemostasis, Department of Haematology, University Medical Center Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: 31-30-250-6512; Fax: 31-30-251-1893; E-mail: J.W.N.Akkerman@lab-aanzu.nl.
§ The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate; Mk, megakaryocyte; PKA, cAMP-dependent protein kinase; PKA-C, PKA catalytic subunit (\(\alpha\), \(\beta\), and \(\gamma\); PKA-R, PKA regulatory subunit (I\(\alpha\), I\(\beta\), I\(\alpha\), and I\(\beta\)); BSA, bovine serum albumin; Fura-2/AM, Fura-2-acetoxyethyl ester; rIP$_3$, recombinant human thrombopoietin; rIL-3, recombinant human interleukin-3; EGFP, enhanced green fluorescent protein; AKAP, PKA anchoring protein.

In the circulation, uncontrolled platelet activation is prevented by the platelet inhibitors prostacyclin (prostaglandin E$_2$) and nitric oxide produced by endothelial cells. Prostacyclin binds to IP receptors that start cAMP formation via the GTP-binding protein G$_i$, and adenyl cyclase. The importance of this pathway is reflected by the occurrence of acute myocardial infarction, coronary artery disease, and angina in patients with impaired platelet cAMP production (6–8).

The inhibitory effects of cAMP are mediated via protein kinase A (PKA), which phosphorylates and thereby inactivates numerous signaling elements in platelet-activating pathways such as the thromboxane A$_2$ (TxA$_2$) receptor type \(\alpha\) (9), phospholipase C$_{\beta}$ (PLC-\(\beta_\text{2}\)) (10), and IP$_3$ receptors (11, 12). Because a small rise in cAMP already leads to a strong activation of PKA, Ca\textsuperscript{2+} signaling in platelets is extremely sensitive to increases in cAMP (13).

Because platelets are synthesized by megakaryocytes (Mks), one would expect a similar suppression of Ca\textsuperscript{2+} responses by prostacyclin in these progenitor cells. There are indications that the regulation of Ca\textsuperscript{2+} homeostasis is different in these cells. Megakaryocytic cell lines thought to mimic immature Mks respond to the stable prostacyclin analog iloprost with an expected rise in cAMP but also with a rise in [Ca\textsuperscript{2+}]. Thus, in these cells Ca\textsuperscript{2+} responses are insensitive to inhibition by cAMP (14, 15). It is possible that the cause of this different [Ca\textsuperscript{2+}]\textsubscript{i} regulation must be sought in the action of PKA.

PKA is activated by cAMP through binding to its regulatory subunits. One PKA holoenzyme complex is composed of two catalytic subunits and two regulatory subunits. Three isoforms of the PKA catalytic subunit (PKA-C) have been characterized and designated the C\(\alpha\), C\(\beta\), and C\(\gamma\) isoforms, respectively. They appear on SDS-PAGE as proteins with molecular masses ranging from 41 to 50 (PKA-C\(\alpha\)), from 42 to 50 (PKA-C\(\beta\)), and from 39 to 40 kDa (PKA-C\(\gamma\)) (16–18). Four isoforms of the PKA regulatory subunit (PKA-R) have been found and named the R\(\alpha\), R\(\beta\), R\(\gamma\), and R\(\delta\) isoforms, respectively. They have apparent molecular masses of 49 (PKA-R\(\alpha\)), 54–55 (PKA-R\(\beta\)), 51 (PKA-R\(\gamma\)), and 53 kDa (PKA-R\(\delta\)) (19). PKA is activated by the binding of two cAMP molecules to each of the regulatory subunits, thereby releasing and activating the catalytic subunits.

The present study was undertaken to clarify the factors that make cAMP an inhibitor of Ca\textsuperscript{2+} responses in platelets. Earlier work has shown that immature megakaryocytic cell lines and Mkcs produce cAMP when stimulated with prostacyclin (15, 20). The present results show that PKA is present and functional in platelets and all stages of megakaryocytopoiesis but does not become an inhibitor of Ca\textsuperscript{2+} increases before Mk maturation is almost completed and platelets are formed.
Inhibition by cAMP during Megakaryopoiesis

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin, bovine serum albumin (BSA), and saponin were from Sigma. The stable prostacyclin analog iloprost was a gift from Schering AG (Berlin, Germany). Prostacyclin (prostaglandin I₂) was from Cayman Chemicals (Ann Arbor, MI). Fura-2-acetoxymethyl ester (Fura-2/AM) and Fluo-3 were from Molecular Probes (Eugene, OR). The fibrinogen-derived peptide GRGDS was from Bachem (Bubendorf, Switzerland). Lysine-modified Dulbecco’s medium was from Invitrogen. The cAMPP-dependent protein kinase inhibitor H89 was obtained from Alexis (Laufelfingen, Switzerland). IP₃ was from ICN Biochemicals (Irvine, CA). The fat-free dry milk, Proftar, was from Nutricia (Zoetermeer, The Netherlands). Nitrocellulose membranes were from Schleicher & Schull. Ficol-Paque was from Amer sham Biosciences. Recombinant human thrombopoietin (rhTPO) and recombinant human stem cell factor (rhSCF) were from PeproTech, Rocky Hill, NJ. The immunomagnetic progenitor cell isolation kit (miniMACS) for the isolation of CD34-positive cells (using QBEND/10 anti-CD34) was from Miltenyi Biotec (Bergisch Gladbach, Germany). Protein concentrations were determined by a BCA (bicinchoninic acid) protein assay from Pierce. All other chemicals were of analytical grade.

Antibodies—Monoclonal anti-CD61 (7F12) and monoclonal anti-CD42b (6.20), which were used for immunomagnetic purification of Mks, were kindly provided by Dr. H. K. Nieuwenhuis, Dept. of Hematology (University Medical Center Utrecht, The Netherlands). Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD61 (F803) and CD42b (F802) and FITC-labeled negative control IgG, which were used to determine the purity of immunomagnetically purified Mks, and peroxidase-conjugated swine antibody against rabbit IgG (SWARPO) and goat-anti-mouse peroxidase and rabbit-anti-goat peroxidase were from Dako. Fcγ receptor IIb (CD61/CD42b, which is a marker for more mature Mks (22). DAMI cells resemble immature Mks because they express CD41/CD61 but hardly any CD42b, which is a marker for more mature Mks (22). DAMI cells were cultured in a 12-well culture plate at 37 °C in a humidified atmosphere with 5% CO₂. The initial cell density was 4 × 10⁵ cells/ml of medium. The composition of the medium was the same as described by Zauli et al. (25) with the omission of nucleosides. rhTPO and rhSCF were added to a final concentration of 20 and 50 ng/ml, respectively. After 3 days of culture, 1 ml of fresh medium supplemented containing 50 ng/ml rhSCF (final concentrations) was added. After 7 and 11 days of culture, 1 ml of fresh culture medium containing 20 ng/ml rhTPO (final concentration) was added.

Purification of Mks—To obtain immature and mature Mks, CD34⁺ cells were cultured with rhTPO and rhSCF for 7 and 14 days, respectively, as described above. Cells were harvested and washed twice (5 × 10⁹ platelets/ml) in phosphate-buffered saline containing 0.5% BSA and 2.5 mM EDTA (buffer A). Subsequently, the cells were labeled for 20 min at 4 °C with mouse-anti-human IgG directed against CD61 (antibody 7F12) for the isolation of immature Mks and against CD42b (antibody 6.20) for the isolation of mature Mks. After washing with buffer A, cells were labeled (20 min, 4 °C) with goat-anti-mouse magnetic microbeads and washed again with buffer A. The cell suspension was resuspended on a ferromagnetic column (miniMACS) and after three washing steps with 700 μl of buffer A each, the retained cells were eluted with buffer A. The purity of the final immature (CD61⁺) and mature (CD42b⁺) Mk suspensions was determined by flow cytometry and was typically between 94 and 98%. These populations have been characterized thoroughly in previous studies (26, 27).

Measurement of Calcium Responses in Suspension Cells—Platelet-rich plasma was supplemented with 3 μM Fura-2/AM and incubated for 45 min at 37 °C in the dark. The platelet-rich plasma was acidified with AC (2.5 g of trisodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of distilled water) to pH 6.5 and centrifuged again (3300 × g, 15 min, 20 °C). Platelets were resuspended in HT buffer (0.1% (w/v) glucose, pH 6.5) to a concentration of 2 × 10⁶/ml and stored at room temperature in the dark. Immediately before the measurement of [Ca²⁺]i, the platelet suspension was diluted 10 times in prewarmed (37 °C) HT buffer (0.1% glucose, pH 7.4), and GRGDS peptide (100 μM, final concentration) was added to prevent platelet aggregation.

Megakaryocytic cell lines, freshly isolated stem cells, and purified immature and mature Mks were pelleted (5 min, 125 g, 20 °C) and resuspended in HT buffer, pH 7.4, supplemented with 0.1% (w/v) BSA and 0.1% (w/v) glucose at a concentration of 5 × 10⁹ cells/ml. Fura-2/AM was added (final concentration) and the platelet suspension was incubated for 1 h at 37 °C in the dark. Cells were spun down, resuspended in HT buffer without BSA to a final concentration of 1.6 × 10⁶ cells/ml, and stored at room temperature in the dark. Immediately before the analysis of [Ca²⁺]i, the cells were resuspended in prewarmed (37 °C) HT buffer to a final concentration of 2 × 10⁶/ml. Measurements were performed at 37 °C with mild stirring (50 rpm) on a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). Fura-2 fluorescence was measured at 340- (F1) and 380-nm (F2) excitation and 510-nm emission wavelength. [Ca²⁺]i was calculated using the formula of Grynkiewicz et al. (28). Calculations were based on a dissociation constant of the Fura-2-Ca²⁺ complex of 224 nM.

Measurement of IP3-induced Ca²⁺ Mobilization—IP3-induced Ca²⁺ mobilization was determined according to a method described previously (29). Mks were preincubated with 1 mM oligomycin. Washed platelets were resuspended in Ca²⁺-free HT buffer (0.2% glucose, pH 6.5) at a concentration of 5 × 10⁹ platelets/ml. MEK-01 and CRH-288-11 cells were washed and resuspended in the same buffer at a concentration of 15 × 10⁶ and 7.5 × 10⁶ cells/ml, respectively. Immediately before the measurements, the 60-μl cell suspension was diluted with 240 μl of a buffer composed of 20 mM HEPES, 100 mM KCl, 1.4 mM MgCl₂, 100 mM sucrose, 1.25 mM KCl, and 1.3 mM CaCl₂. Platelets or platelet suspensions were prewarmed at 37 °C, treated with 1 μM iloprost for 1 min, and subsequently permeabilized with 50 (platelets) or 65 μg/ml (MEK-01 and CRH-288-11 cells) saponin at 37 °C for 2–5 min until a stable base line was obtained. When necessary, EGTA was added to lower the Ca²⁺ concentration to 350–400 nM, which is an optimal concentration for the measurement of IP3-induced Ca²⁺ release (29). Subsequently, IP₃ (50 mM, final concentration) was added, and changes in fluorescence inten-
Inhibition of PKA Activation—For the measurement of PKA activation, cells were resuspended in Ca²⁺-free HT buffer (pH 7.4, 0.1% glucose) to a final concentration of 4 × 10⁶ cells/ml and incubated with iloprost for 10 min at 37 °C with or without preincubation with 8H9 (15 min, 10 μM, 37 °C). The activity of PKA at different time points was deduced from the mobility shift of VASP on a Western blot caused by PKA-mediated phosphorylation on Ser577, as described elsewhere (30).

Western Blotting—Cells were pelleted and lysed in a buffer consisting of 1% (w/v) Nonidet P-40, 0.5% (w/v) octyl glucoside, 5 mM EDTA, 0.1% SDS supplemented with 2 mM sodium orthovanadate, and protease inhibitors (1% inhibitor mixture from Sigma; catalog no. P8340). A sample was taken for determination of the protein content with the BCA assay, and a Laemmli electrophoresis sample buffer was added (final concentrations: 0.001% (w/v) bromphenol blue, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8). Samples were boiled, and 5 μg of protein per lane was subjected to SDS-PAGE (10% gel) and electroblotted onto a nitrocellulose membrane. The blots were stained with Ponceau S and scanned, and bands were quantified using ImageQuant from Molecular Dynamics. In all experiments the amount of protein per lane, based on the pixel density of all of the proteins, varied less than 10%. Subsequently, bands were destained with water and blocked in Tris-buffer saline with 0.2% (w/v) Tween 20 (TBST) supplemented with 2% (w/v) Protifar and 0.5% BSA for 1 h at room temperature. Incubation with antibodies against PKA-α, PKA-RIαβ, PKA-RIIα, or PKA-RIIβ was performed overnight in TBST at 4 °C. The membranes were washed six times with TBST and incubated with horseradish peroxidase-linked anti-rabbit, anti-goat, or anti-mouse antibody for 1 h at 4 °C in TBST. Bands on blot were visualized by enhanced chemiluminescence, and the intensity of the bands on the film was quantified using ImageQuant. Relative intensities were calculated, and the value for MEG-01 cells (for comparison of different cell lines) or stem cells (for comparison of different stages of megakaryopoiesis) was set at 1.00.

Transient Transfection and FACS Sorting of Transfected CHRF Cells—CHRF cells (1 × 10⁶) were resuspended in 300 μl of Fischer’s medium (without serum and penicillin/streptomycin/β-glutamine), transferred to a Gene Pulser cuvette (0.4-cm electrode gap; Bio-Rad), and incubated for 17 min with 12 μg of EGFP-N1 and 41 μg of pCevin while gently mixing after 10 min. For mock transfection, cells were incubated with 12 μg of EGFP-N1 and 41 μg of pCevin while gently mixing after 10 min. For mock transfection, cells were incubated with 12 μg of EGFP-N1 and 41 μg of ZnSO₄ to induce PKA-C activity. The activity of PKA at different time points was deduced from the mobility shift of VASP on a Western blot caused by PKA-mediated phosphorylation on Ser577, as described elsewhere (30).

Measurement of Ca²⁺ Responses in Transfected CHRF Cells—Ca²⁺ responses were measured in transfected CHRF cells by single cell fluorescence imaging microscopy as described in Ref. 27. In short, transfected CHRF cells were adhered to anti-GPIIIa (7F12) antibody-coated coverslips and loaded with Fura-2/AM. Fura-2 fluorescence was recorded upon stimulation of the cells with iloprost (1 μM) and thrombin (1 unit/ml) as indicated. Subsequently, EGFP fluorescence was determined with a 485-nm excitation filter (half-bandwidth, 22 nm), a 505-nm dichroic long pass filter, and a 530-nm emission filter (half-bandwidth, 30 nm). Ca²⁺ tracings of EGFP-positive cells were analyzed and averaged (n = 17).

RESULTS

Inhibition of Ca²⁺ Signaling by Iloprost in Platelets and Mks—Fig. 1A illustrates the rapid increase in [Ca²⁺], in platelets after stimulation with thrombin (1 unit/ml). Following a subsequent slight decrease, a stable level was maintained for at least 2 min. The addition of the prostacyclin analog iloprost (1 μM) 30 s after the thrombin addition immediately lowered the elevated Ca²⁺ level to almost basal levels. When platelets were first incubated with iloprost for 1 min and thereafter stimulated with thrombin, the thrombin-induced Ca²⁺ increase was almost completely abolished (Fig. 1B). These data illustrate the potent inhibition of [Ca²⁺], increases by iloprost and confirm earlier observations (5). Similar experiments in megakaryocytic cell lines (Fig. 2A), stem cells, and immature and mature Mks (Fig. 2B) revealed that the effect of iloprost on thrombin-induced Ca²⁺ signaling was different in these cells. When megakaryocytic cell lines were first stimulated with thrombin (1 unit/ml) and after 3 min treated with iloprost (1 μM), different responses were observed among MEG-01, DAMI, and CHRF 288-11 cells. In MEG-01 cells, thrombin induced a Ca²⁺ increase of 153 ± 50 nM (mean peak height of Ca²⁺ increase ± S.D.; n = 3). The subsequent addition of iloprost failed to reduce [Ca²⁺], but instead triggered a further rise in the Ca²⁺ level of 165 ± 30 nM. As we described in detail in another report (27), this iloprost-induced Ca²⁺ increase is also found in stem cells. It is caused by an increase in cAMP and further signal generation to the sacro/endothelial reticulum via a mechanism that is independent of PKA. In DAMI cells, a first addition of thrombin raised [Ca²⁺], by 210 ± 28 nM, which is about 140% of the response observed in MEG-01. The subsequent addition of iloprost again induced a rise in [Ca²⁺], but the increase was only 75 ± 17 nM, which is 45% of the response found in MEG-01. CHRF-288-11 cells also responded to thrombin with a rise in [Ca²⁺], which reached 512 ± 73 nM or 335% of the response by MEG-01 cells. The subsequent addition of iloprost lowered the [Ca²⁺], from 200 ± 34 nM to 150 ± 18 nM, which is a reduction of 25%. Together, these findings showed that the maturation of Mks is accompanied by a gradual increase in the capacity of thrombin to raise [Ca²⁺], as well as a gradual change in the effect of iloprost shifting from an inducer of [Ca²⁺], elevation to an inhibitor of elevated [Ca²⁺]. This inhibition is also observed in platelets.

A similar increase in the thrombin-induced elevation of [Ca²⁺], and the change in the effect of iloprost was found when stem cells were compared with immature and mature Mks. In stem cells, thrombin induced a Ca²⁺ rise of 220 ± 55 nM, which was followed by a further increase induced by iloprost of 78 ± 15 nM. In immature Mks, the thrombin-induced rise of [Ca²⁺], had increased to 285 ± 34 nM, which is 130% of the response
Inhibition by cAMP during Megakaryocytogenesis

**Fig. 2. Effect of thrombin and iloprost on $[\text{Ca}^{2+}]_i$ in megakaryocytic cell lines, stem cells, and Mks.** A, increases in $[\text{Ca}^{2+}]_i$, after the addition of thrombin (1 unit/ml) and 3 min later iloprost (1 $\mu$M) in the human megakaryocytic cell lines MEG-01, DAMI, and CHRF-288-11, representing stages of increasing maturation. B, increases in $[\text{Ca}^{2+}]_i$, after the addition of thrombin and iloprost in purified human stem cells, immature Mks, and mature Mks. Experimental conditions are the same as described in the legend to Fig. 1.

seen in stem cells. The subsequent addition of iloprost raised $[\text{Ca}^{2+}]_i$, by 83 ± 22 nM or 113% of the response by stem cells. In mature Mks, thrombin raised $[\text{Ca}^{2+}]_i$, by 440 ± 35 nM or 200% compared with stem cells, which was followed by a 30% decrease induced by iloprost. Thus, these results in Mk cultures were qualitatively similar to those found in megakaryocytic cell lines and show that the inhibition of $\text{Ca}^{2+}$ signaling by iloprost appears at a late stage in the maturation of Mks.

To investigate the inhibition by iloprost prior to stimulation with thrombin, the different cell populations were first treated with iloprost (1 $\mu$M) and 1 min later stimulated with thrombin (1 unit/ml). In MEG-01 and DAMI cells (Fig. 3A) and stem cells and immature Mks (Fig. 3B), iloprost induced a rise in $[\text{Ca}^{2+}]_i$. The subsequent addition of thrombin (1 unit/ml) induced a further elevation of $[\text{Ca}^{2+}]_i$. Thus, iloprost failed to inhibit the thrombin-induced $\text{Ca}^{2+}$ increase in these immature cells. In contrast, in CHRF-288-11 cells and mature Mks there was a weak inhibition of thrombin-induced $\text{Ca}^{2+}$ increases by iloprost amounting to 16 ± 5 and 18 ± 6%, respectively (Fig. 3A and B). A stronger inhibition by iloprost was observed at lower concentrations of thrombin (not shown). Thus, iloprost inhibited $\text{Ca}^{2+}$ increases both before and after thrombin stimulation in CHRF-288-11 cells and mature Mks but not in the immature stages of Mk maturation.

**Iloprost Inhibition of IP$_3$-induced $\text{Ca}^{2+}$ Release during Mk Maturation**—Multiple steps in agonist-induced increases in $[\text{Ca}^{2+}]_i$ are known to be inhibited by cAMP-mediated activation of PKA. Common steps in the signaling pathways that trigger the mobilization of $\text{Ca}^{2+}$ ions from storage sites in the sarcoplasmic endoplasmic reticulum are the generation of IP$_3$, and the subsequent activation of IP$_3$ receptors, which form tetrameric ligand-gated $\text{Ca}^{2+}$ channels that release $\text{Ca}^{2+}$ from intracellular stores upon the binding of IP$_3$. To investigate the contribution of these steps in the development of iloprost-inhibition, MEG-01 cells, CHRF-288-11 cells, and platelets were permeabilized with saponin to enable the entry of IP$_3$, and the release of $\text{Ca}^{2+}$ was determined as described elsewhere (29). The addition of IP$_3$ (50 nM) to MEG-01 and CHRF-288-11 cells and platelets induced an increase in the initial $\text{Ca}^{2+}$ concentration of 210 ± 18 nM and 500 ± 34 nM per $3 \times 10^7$ MEG-01 and $1.5 \times 10^7$ CHRF-288-11 cells in 1 ml, respectively, and of 110 ± 9 nM per $1 \times 10^9$ platelets in 1 ml. A preincubation with iloprost (1 $\mu$M, 1 min) had no effect on the IP$_3$-induced $\text{Ca}^{2+}$ increase in MEG-01 cells (Fig. 4A). In CHRF-288-11 cells, pretreatment with iloprost suppressed the IP$_3$-induced $\text{Ca}^{2+}$ increase by 21 ± 5%, and in platelets this inhibition was 52 ± 6% (Fig. 4, B and C). The results for platelets correspond well with findings by other investigators (12). Thus, the increased inhibition of $\text{Ca}^{2+}$ mobilization is one of the mechanisms that contribute to the development of $\text{Ca}^{2+}$ inhibition by iloprost.

**Role of PKA in the Inhibition of $\text{Ca}^{2+}$ Signaling by Iloprost**—The observation that iloprost fails to inhibit thrombin-induced $\text{Ca}^{2+}$ signaling in stem cells and the early stages of megakaryocytogenesis might have different causes. First, iloprost might fail to induce cAMP accumulation because of the absence of IP receptors, the trimeric G-protein $G_s$, or adenyl cyclase. Second, cAMP accumulation might be prevented by enhanced removal by phosphodiesterases. Third, PKA or certain PKA subtypes might not have been fully expressed in these immature cells. In earlier studies, we showed that the megakaryocytic cell lines MEG-01, DAMI, and CHRF-288-11 respond to iloprost with a rise in cAMP. Iloprost raised the cAMP concentration in MEG-01 and DAMI cells from 10 to 50 pmol/10$^6$ cells (15). This is in the range found in platelets that are 1000× smaller and accumulate 90 pmol cAMP/10$^9$ cells following stimulation with prostaglandin E$_1$ (PGE$_1$), which also activates $G_s$ (13). At this cAMP level in platelets, thrombin-induced increases in $[\text{Ca}^{2+}]_i$, are almost completely abolished (29). Thus, differences in iloprost sensitivity between different maturation stages cannot be explained by insufficient cAMP accumulation.

To investigate a possible defect in the expression or function of PKA, the different cell populations were screened for their
capacity to phosphorylate VASP, which is phosphorylated on Ser\textsuperscript{157} by PKA, leading to a mobility shift on SDS-PAGE (30). All cell populations revealed a mobility shift of VASP upon the addition of iloprost (1 \textmu M) as shown in Fig. 5\textit{A} for MEG-01 and CHRF-288-11 cells, stem cells, and mature Mks. Iloprost-induced VASP phosphorylation in these cells was rapid, reaching 50\% after 1 min and remaining high for at least 10 min. A similar degree of VASP phosphorylation has been observed in prostaglandin E\textsubscript{1}-treated platelets, and this is accompanied by the complete inhibition of thrombin-induced Ca\textsuperscript{2+} increases (13, 29). Furthermore, the mobility shift of VASP was strongly inhibited by H89, a specific inhibitor of PKA. These results are in agreement with the inhibition of VASP-Ser\textsuperscript{157} phosphorylation by H89 in platelets (31). Together, these data indicate that PKA is present and functional at all stages of Mk maturation.

To investigate how PKA contributed to the Ca\textsuperscript{2+} inhibition by iloprost, the effect of the PKA inhibitor H89 on thrombin-induced rises in Ca\textsuperscript{2+}, was studied in cells showing a partial (CHRF-288-11 cells and mature Mks) or complete (platelets) inhibition by iloprost. Both CHRF-288-11 cells (Fig. 5\textit{B}) and mature Mks (not shown) fully recovered from a pretreatment with iloprost in the presence of H89, reaching increases in [Ca\textsuperscript{2+}], of 180 \pm 24\% of cells stimulated in the absence of iloprost and H89. A complete recovery of thrombin-induced Ca\textsuperscript{2+} signaling was found in platelets reaching 100 \pm 13\% of untreated samples (Fig. 5\textit{C}). Thus, the inhibition of Ca\textsuperscript{2+} by iloprost in these more mature cell populations was mediated by PKA.

Changes in PKA Subunit Expression during Megakaryocytopenesis—To understand why PKA was present and functional in stem cells and all stages of Mk maturation but inhibited Ca\textsuperscript{2+} signaling only in mature Mks and especially in platelets, the expression of PKA subunits was analyzed by Western blotting. As shown in Fig. 6, A and B, PKA-C subunits with molecular masses of 39 and 42 kDa were present in MEG-01 and CHRF-288-11 cells. A 44-kDa PKA-C isoform was weakly expressed in MEG-01 and DAMI, and CHRF-288-11 cells but was abundantly present in platelets. The expression of PKA subunits was analyzed by Western blotting. As shown in Fig. 6, A and B, PKA-C subunits with molecular masses of 39 and 42 kDa were present in all cell types. A 44-kDa PKA-C isoform was weakly expressed in MEG-01, DAMI, and CHRF-288-11 cells but was abundantly present in platelets. Quantitation of the total pixel density of the three PKA-C subunits revealed a 1.4-fold increase in the megakaryocytic cell lines and a further increase to 1.9-fold in platelets. In the cultured megakaryocytes this expression was more or less constant, but platelet formation was accompanied by a 1.9-fold rise in the expression of PKA-C. Hence, the appearance of Ca\textsuperscript{2+}.
inhibition by prostacyclin observed especially in platelets was accompanied by up-regulation of PKA-C.

An antibody against the PKA-R subunits Iα and Iβ revealed a band at an apparent molecular mass of 49 kDa in cell lines, cultured Mks, and platelets, showing that they express the PKA-R1α subunit. PKA-R1α expression varied little among these cell populations (Fig. 6, A and B). Immature Mks expressed the 54–55-kDa PKA-R1β isof orm, but this isof orm was not present at more mature stages. Also, the expression of PKA-R1Iα remained more or less the same. In contrast, the expression of PKA-R1Iβ showed a stepwise increase when cell lines and Mks were compared at different maturation stages, reaching a 15-fold increase in CHRF-288-11 cells compared with MEG-01 cells and a 4-fold increase in mature Mks compared with stem cells. No further increase was observed in platelets. Thus, changes in PKA-R1Iβ expression did not correlate with the appearance of Ca2+ inhibition by prostacyclin.

PKA-Cα Overexpression in CHRF-288-11 Cells Enhances Ca2+ Inhibition by Iloprost—To confirm that increases in PKA-C expression contribute to the inhibition of Ca2+ increases by iloprost, PKA-Cα and PKA-Cβ were transiently overexpressed in CHRF-288-11 cells, and Ca2+ responses were determined. Co-expression of EGFP and Ca2+ analysis in single, immobilized cells allowed specific screening of transfected (EGFP+) cells, which constituted only 10–15% of the total cell population.

The transfection of CHRF-288-11 cells with PKA-Cα introduced a 39-kDa PKA-C subtype (Fig. 6, inset). Together with the basal expression of a 42-kDa subtype, this resulted in a 1.5-fold increase in total PKA-C. A similar transfection with PKA-Cβ had less effect and together with the 42-kDa subtype led to an expression that was only 1.2-fold higher than in mock-transfected cells (data not shown). Strikingly, the introduction of PKA-Cα led to a 50% increase in Ca2+ inhibition by prostacyclin (Fig. 6). No change in Ca2+ inhibition was induced by PKA-Cβ overexpression, possibly because of the minor increase in total PKA-C observed in these cells. These findings support the concept that an increase in PKA-C expression causes Ca2+ inhibition by prostacyclin.

DISCUSSION

The present study shows that the inhibition of Ca2+ increases by prostacyclin develops at a late stage during megakaryocytopoiesis. The inhibition is absent in MEG-01 and DAMI cells and in CD34+ stem cells and immature Mks but becomes apparent in CHRF-288-11 cells, mature Mks, and especially in platelets. Each of these cell populations is capable of raising cAMP levels and contains PKA capable of inducing VASP phosphorylation. Thus, the signaling elements required for the activation of PKA are present in these immature cell populations, including receptors for prostacyclin (IP receptors), the stimulatory G-protein Gs, and adenylyl cyclase. PKA is present and induces the phosphorylation of VASP, one of its major substrates (13). Apparently this is not sufficient to suppress Ca2+ rises. Two explanations may account for this discrepancy. First, the PKA-mediated inhibition is present but is overruled by the cAMP-induced Ca2+ increase in these cells. Second, the inhibition by PKA is too weak because of the incomplete expression of the components of the PKA complex. Evidence for the first explanation comes from the observation that in the presence of the PKA inhibitor H89, cAMP- and thrombin-induced Ca2+ increases are higher than in the absence of this inhibitor (Ref. 27 and the present study). Evidence for the second explanation comes from the observation that the maturation of Mks and platelet formation are accompanied by changes in the expression of the PKA catalytic and regulatory subunits.

The present data show that the appearance of Ca2+ inhibi-
tion by prostacyclin is accompanied by a sharp up-regulation of PKA-C. Western blots show the presence of two major PKA-C subtypes with molecular masses of 39, 42, and 44 kDa (PKA-C) and the PKA regulatory subunits Iα (49 kDa) and Iβ (54 kDa) (PKA-Rliα/β) in megakaryocytic cell lines and platelets (A) and in purified stem cells, immature and mature Mks, and platelets (B). Cell lysates (5 µg of protein/lane) were subjected to SDS-PAGE. Bands representing PKA-C (39-, 42-, and 44-kDa isoforms), PKA-Rliα, PKA-Rliα, and PKA-Rliβ were scanned, and pixel densities were determined using ImageQuant software. For PKA-C the cumulative pixel density of the 39-, 42-, and 44-kDa bands is depicted. The pixel density of the bands in MEG-01 cells and stem cells was set to 1.00, and the density of other bands on the same blot was related to these internal standards. Relative intensities are indicated below the blots. A representative blot for three observations with similar results is shown. C, CHRF-288-11 cells were co-transfected with EGFP (as control for transfection) and Zem3 (empty control vector) or pCαEV (PKA-Cα-encoding construct) as described under "Experimental Procedures." Averaged single cell Ca²⁺ tracings from 17 EGFP⁺ CHRF-288-11 cells are shown for Zem3- and pCαEV-transfected cells. The inset shows a Western blot analysis of 1 µg of total protein for PKA-C expression in EGFP⁺ FACS-sorted cells. The cumulative pixel density of the 39-, 42-, and 44-kDa PKA-C bands is depicted below the blot.
The expression of PKA-RIβ changes during Mk maturation, but the up-regulation starts at a much earlier stage and appears to precede the development of Ca²⁺ mobilization. Nevertheless, up-regulation of PKA-RIβ expression might be important for PKA activity in platelets. PKA-RIβ has been shown to mediate the targeting of the PKA holoenzyme to different subcellular compartments via the binding to protein kinase A anchoring proteins (AKAPs) (34). Type II PKA containing the regulatory subunit IIβ binds to the sarco/endoplasmic reticulum via δ-AKAP1 (34–37). This might be a prerequisite for PKA to come in close proximity to IP₃ receptors and facilitate their phosphorylation and suppression of Ca²⁺ mobilization. Also, the steps in signaling cascades initiated by surface receptors may be subject to PKA inhibition via anchorage to AKAPs. In myometrial cells, the anchoring of PKA to the plasma membrane via AKAP79 was necessary to inhibit phosphatidylinositol turnover (38). PKA is targeted to phospholipase Cβ via its regulatory subunit IIβ and AKAP79 in myometrial cells (38) and to receptors in the plasma membrane via AKAP250/gravin in endothelial cells (39, 40).

Apart from changing PKA subunit composition as part of the formation of an important inhibitory mechanism in platelets, alterations in subunit expression may serve a role in the proliferation of Ms. In human neuroblastoma cells, the overexpression of PKA-RIβ correlated with the inhibition of cell growth (41). By analogy, up-regulation of PKA-RIβ might be a mechanism by which immature Ms shift from a proliferating to a maturing phenotype.

Multiple steps in signaling cascades from surface receptors to Ca²⁺ mobilization are inhibited by PKA. A common downstream step is the release of Ca²⁺ ions from the sarco/endoplasmic reticulum via the activation of IP₃ receptors. Our experiments in permeabilized cells indicate that the Ca²⁺ inhibition by prostacyclin observed in mature Ms and especially in platelets is also found at the level of IP₃-induced Ca²⁺ release. These results correspond with studies on Ms derived from Wistar rats, which showed 80% reduction in IP₃-induced Ca²⁺ in increases by the prostacyclin analog carbachol (42). This indicates that in fully mature Ms CAMP-mediated inhibition of IP₃-induced Ca²⁺ release has completely developed. Thus, the mobilization of Ca²⁺ from internal stores might be at least one of the steps responsible for the changes in Ca²⁺ signaling seen during Mk maturation and a direct consequence of the up-regulation of PKA-C. In addition, the appearance of iloprost inhibition might reflect a change in IP₃ receptor subtypes. Type IV IP₃ receptors have been identified in platelets and are known as IP₃-G, -RI, -RII, and -RIII (43). Type I IP₃ receptors are better phosphorylated by PKA than type II or type III receptors (44), making this receptor the most sensitive target for CAMP-mediated inhibition. Expression studies in DAMI cells and platelets revealed up-regulation of IP₃-RI and down-regulation of IP₃-RII with IP₃-RII expression being the same in the two cell types (45). Other observations are in conflict with these data and show down-regulation of IP₃-R-I and up-regulation of IP₃-R-II (46). Thus, a better insight in the role of IP₃ receptor subtypes in the development of Ca²⁺ inhibition by PKA awaits more detailed studies in Ms at different stages of maturation.

In conclusion, our data reveal that the appearance of Ca²⁺ inhibition by prostacyclin starts at a late stage of megakaryocyte maturation when platelets are formed. This inhibition is accompanied by a sharp up-regulation of PKA-C expression, which appears to be a key step in the development of Ca²⁺ inhibition by prostacyclin in platelets.
Inhibition by cAMP during Megakaryocytopoiesis

37. Huang, L. J., Durick, K., Weiner, J. A., Chun, J., and Taylor, S. S. (1997) *J. Biol. Chem.* 272, 8057–8064
38. Dodge, K. L., Carr, D. W., and Sanborn, B. M. (1999) *Endocrinology* 140, 5165–5170
39. Herberg, F. W., Maleszka, A., Eide, T., Vossebein, L., and Tasken, K. (2000) *J. Mol. Biol.* 298, 329–339
40. Fan, G., Shumay, E., Wang, H., and Malbon, C. C. (2001) *J. Biol. Chem.* 276, 24005–24014
41. Kim, S. N., Lee, G. R., Hwang, E. S., Lee, J. H., Park, S. D., Cho-Chung, Y. S., and Hong, S. H. (1997) *Biochem. Biophys. Res. Commun.* 232, 469–473
42. Tertyshnikova, S., and Fein, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 1613–1617
43. El-Daher, S. S., Patel, Y., Siddiqua, A., Hassock, S., Edmunds, S., Maddison, B., Patel, G., Goulding, D., Lupu, F., Wojcikiewicz, R. J., and Authi, K. S. (2000) *Blood* 95, 3412–3422
44. Wilcox, R. A., Primrose, W. U., Nahorski, S. R., and Challiss, R. A. (1998) *Trends Pharmacol. Sci.* 19, 467–475
45. Siddiqua, A., El-Daher, S. S., Wuytack, F., and Authi, K. S. (1999) *Thromb. Haemostasis.* (suppl.) 635 (abstr.)
46. Lacabaratz-Porret, C., Launay, S., Corvazier, E., Bredoux, R., Papp, B., and Enouf, J. (2000) *Biochem. J.* 350, 723–734