Defective Nitrovasodilator-stimulated Protein Phosphorylation and Calcium Regulation in cGMP-dependent Protein Kinase-deficient Human Platelets of Chronic Myelocytic Leukemia*

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The presence and functional role of the cyclic nucleotide signal transduction system was investigated in platelets from patients with myeloproliferative disorders. Platelets from certain patients with chronic myelocytic leukemia showed decreased expression of cGMP-dependent protein kinase, and platelets from two such patients were studied in some detail. These platelets had very little if any cGMP-dependent protein kinase but a normal level of cAMP-dependent protein kinase. They also contained a normal level of VASP (vasodilator-stimulated phosphoprotein, a specific substrate of both cAMP- and cGMP-dependent protein kinase), as well as a functionally intact prostaglandin E₁-stimulated cAMP-mediated VASP phosphorylation. In contrast, sodium nitroprusside-stimulated VASP phosphorylation was severely impaired in these cGMP-dependent protein kinase-deficient platelets, despite an exaggerated cGMP response to sodium nitroprusside. Furthermore, whereas selective activation of the cGMP-dependent protein kinase by 8-(4-chlorophenylthio)-cGMP strongly inhibited the ADP- or thrombin-evoked calcium mobilization from intracellular stores in normal platelets, this agonist-evoked calcium response was not inhibited by the cGMP analog in cGMP-dependent protein kinase-deficient platelets. The results demonstrate a defect in the nitrovasodilator-/cGMP-regulated signal transduction system in human platelets from some patients with myeloproliferative disorders, and underscore that a cGMP-dependent protein kinase is involved in agonist-evoked calcium mobilization from intracellular stores. However, nitrovasodilators and cGMP are known to regulate several distinct signal transduction systems including cAMP-gated ion channels, cGMP-inhibited or -stimulated phosphodiesterases, cGMP-dependent protein kinases, and perhaps even cAMP-dependent protein kinases (1, 4, 15–17). Therefore it has been difficult to conclusively establish a functional role of cG-PK in platelet inhibition and other cellular functions. We now have studied the presence and regulation of cGMP-dependent protein kinase and its function in platelets from patients with myeloproliferative disorders including chronic myelocytic leukemia (CML). Patients with myeloproliferative disorders have been reported to exhibit distinct, but variable platelet abnormalities, such as changes in platelet morphology, reduced glycoprotein Ib levels, deficient α-adrenergic, prostaglandin D₉, or Fc receptors, altered arachidonic acid metabolism, and often abnormal platelet coagulant activity, all of which may cause both bleeding and thrombotic complications (18). CML is a chronic disease of bone marrow stem cells of variable duration which usually evolves into a more aggressive leukemia characterized by an accelerated phase and ultimately the often terminal blast crisis (19–21). The initial cell transformed in the chronic phase of CML is thought to be a pluripotent stem cell since all myeloid and often even lymphoid cell lineages are more or less affected. The Philadelphia (Ph¹) chromosomal anomaly...
involving reciprocal translocation of parts of the long arm of chromosomes 9 and 22, and more specifically the translocation of the c-ABL protooncogene from chromosome 9 to the bcr region of chromosome 22, has been identified as the pathogenetic molecular lesion responsible for the chronic phase of CML (22-24). In contrast, the molecular lesions causing the accelerated phase and/or blast crisis have not been unequivocally elucidated, although alterations in the p53 anti-oncogene and n-RAS genes have been detected (20, 21).

We investigated whether platelets from patients with myeloproliferative disorders have an intact cyclic nucleotide-regulated signal transduction system. Moderate alterations with respect to the content and regulation of cAMP, cGMP, and their respective protein kinases were frequently detected (13), however, severe reductions of cG-PK levels were observed in platelets from two patients with CML. Comparison of normal platelets and cG-PK-deficient platelets enabled us to investigate the functional role of cG-PK in agonist regulation of platelet protein phosphorylation and calcium responses. The data demonstrate that cG-PK is an essential component of nitrovasodilator and cGMP actions in human platelets. A preliminary account of this work has been presented in abstract form (13).

**cG-PK**

![Autoradiographs showing Western blot analysis of cG-PK, catalytic subunit (C) of cA-PK, or VASP in purified standards or human platelet homogenates.](image)

**Catalytic subunit of cA-PK**

![Autoradiographs showing Western blot analysis of cG-PK, catalytic subunit (C) of cA-PK, or VASP in purified standards or human platelet homogenates.](image)

**VASP**

![Autoradiographs showing Western blot analysis of cG-PK, catalytic subunit (C) of cA-PK, or VASP in purified standards or human platelet homogenates.](image)

**EXPERIMENTAL PROCEDURES**

**Materials**—$^{125}$I-Labeled protein A (30 mCi/mg) and the $^{125}$I-cAMP assay system were obtained from Amersham Buchler (Braunschweig, Germany). $^{125}$I-cGMP was purchased from Du Pont (Bad Homburg, Germany). Characterization and use of antisera against cGMP, cG-PK, catalytic subunit of cA-PK, and VASP have been reported (12). PG-E, and sodium nitroprusside were purchased from Sigma (Munich, Germany) and diluted as described in Ref. 12. 8-(4-Chlorophenylthio)-cGMP (8-pCPT-cGMP) was from Biolog (Bremen, Germany). Other chemicals and materials were from commercial sources as reported previously (7, 9).

**Characterization of CML Patients—**Diagnosis and classification of patients (based on standard clinical, hematological, and pathological criteria) were determined by our departments of medicine, transfusion medicine, and pathology. Peripheral blood from healthy donors and patients was collected and analyzed after obtaining informed consent.

**Isolation of Human Platelets and Measurement of cG-PK, Catalytic Subunit of cA-PK, and VASP—**Isolation of human platelets was performed as described in detail previously (12). The content of cyclic nucleotide-dependent protein kinases and their substrate VASP in platelets was determined by the Western blot technique (12). Radioactively labeled proteins were localized by autoradiography, and autoradiographs were analyzed with a laser densitometer (Pharmacia, Freiburg, Germany).

**Analysis of VASP Phosphorylation and Cyclic Nucleotide Levels in Intact Human Platelets—**Blood was obtained by cubital vein puncture and anticoagulated with a final concentration of 0.38% sodium citrate and 3 mM EDTA. Aliquots of 2 ml were centrifuged for 20 s at 2940 × g at room temperature using an Eppendorf centrifuge. The supernatant (platelet-rich plasma (PRP)) was transferred to a microcentrifuge tube and kept at 37°C for subsequent incubations. The PRP was either not treated (basal condition) or incubated with 5 μM PG-E, or 100 μM SNP for 5 min. This PRP was then centrifuged for 10 s at 8160 × g, and the pellet was resuspended in SDS containing stop solution or in 10% trichloroacetic acid solution for analysis of VASP phosphorylation or cyclic nucleotide levels, respectively. Determination of VASP phosphorylation by the Western blot technique or
cGMP-dependent Protein Kinase-deficient Platelets

FIG. 3. Analysis of the regulation of cAMP, cGMP, and VASP phosphorylation in platelets from healthy control persons and in two separate platelet preparations (dates indicated) from the CML patient F. B. An aliquot of platelet-rich plasma was incubated without additions (basal), with 100 μM SNP, or with 5 μM PG-E1 for 5 min. Platelets were then analyzed on Western blots for determination of VASP phosphorylation for which phosphorylated VASP (phospho-VASP, 50-kDa protein) was expressed as a percentage of total VASP (46 + 50-kDa proteins). Cyclic nucleotide levels in platelets were determined by radioimmunoassay (see "Experimental Procedures"). For normal platelets, means ± S.D. are shown for data obtained from seven individuals analyzed in separate experiments.

cyclic nucleotide levels by radioimmunoassay were carried out as described previously (12).

Fluorescence Measurements with Fura-2-loaded Platelets—Experiments were performed as described in detail previously (7). Briefly, PRP was incubated with 4 μM fura-2 at 37 °C for 45 min, then platelets were pelleted and resuspended in physiological buffer (pH 7.4, containing 10 mM Hepes and 0.1 unit/ml apyrase) to yield a final density of 1 × 10⁶ cells/ml. Samples were stirred and kept at 37 °C throughout the following experiments. Platelet aliquots were preincubated without additions (control) or with 0.5 mM 8-pCPT-cGMP, 100 μM sodium nitroprusside, or 10 μM prostaglandin E₁ for 10 min and then stimulated with agonists (20 μM ADP or 0.1 unit/ml thrombin). Fura-2 fluorescence was analyzed with a Perkin-Elmer luminescence spectrophotometer using excitation and emission wavelengths of 340 and 510 nm, respectively.

RESULTS

Western Blot Analysis of cG-PK, Catalytic Subunit of cA-PK, and VASP in Platelets from Healthy Control Persons or CML Patients—Specific well characterized antisera against cG-PK, catalytic subunit of cA-PK, and VASP were used to quantitate these proteins in platelets from healthy control persons and CML patients. Antisera specificity and the establishment of Western blot conditions for determining antigen concentrations in human platelets have been reported previously (12). Western blot analysis of cG-PK, catalytic subunit of cA-PK, and VASP present in platelets from a control person (Co) and CML patients (F. B. and H. F.) is shown in Fig. 1. The amount of cG-PK in platelets (prepared and analyzed on two different dates) from CML patient F. B. was very low, almost undetectable, although the amount in platelets from CML patient H. F. was quite similar to that of a normal healthy donor (F. F., Co). The cG-PK level in platelets from two additional CML patients was in one case normal (patient R. R.) and in another very low (patient C. S.) (data not shown). In contrast to the reduced level of cG-PK in platelets from the CML patient F. B., levels of catalytic subunit of cA-PK or VASP were normal or occasionally even slightly elevated (Fig. 1, middle and lower panels), as was the case for the other CML patients investigated (data not shown). These results indicate the loss of cG-PK in platelets from a subgroup of CML patients.

Cyclic Nucleotide-regulated VASP Phosphorylation and Cyclic Nucleotide Levels in Platelets from Healthy Control Persons and CML Patients—VASP phosphorylation in response to various platelet inhibitors is mediated by either cG-PK or cA-PK in intact human platelets (9, 12). The mobility change of VASP in SDS-polyacrylamide gel electrophoresis after phosphorylation (9) and the availability of a specific antiserum which recognizes both the dephospho form (46-kDa protein) and phospho form (50-kDa protein) of VASP enabled us to investigate cGMP- and cAMP-dependent VASP phosphorylation in normal platelets from control persons and in cG-PK-deficient platelets from CML patients F. B. and C. S. In these experiments, platelets were incubated in the absence or presence of 100 μM sodium nitroprusside or 5 μM PG-E₁ for 5 min. In Fig. 2, typical Western blot results of such experiments are demonstrated. In untreated platelets from both healthy donors and CML patients, most of VASP was detected as the 46-kDa dephospho form, but was converted to the 50-kDa phospho form by PG-E₁, which elevated platelet cAMP levels about 10-fold without affecting cGMP
levels (Figs. 2 and 3). In normal platelets, a 5-min incubation with sodium nitroprusside caused significant VASP phosphorylation (up to 30-40%) accompanied by a severalfold elevated cGMP level (Figs. 2 and 3). In contrast, sodium nitroprusside had very little or only moderate effects on VASP phosphorylation in the cG-PK-deficient platelets from CML patient F. B. (independently analyzed on two different dates) despite a very increased SNP-induced cGMP response (Figs. 2 and 3). Results similar to those demonstrated here with platelets from the CML patient F. B. were also observed with the cG-PK-deficient platelets from the CML patient C. S., whereas the effects of PG-EI and sodium nitroprusside on cyclic nucleotide levels and VASP phosphorylation in platelets from the CML patients H. F. and R. R (which had a normal cG-PK content) were similar to the responses observed for control platelets (data not shown).

**Regulation of Agonist-induced Calcium Mobilization by 8-pCPT-cGMP or PG-EI in Platelets from the CML Patient F. B. and a Control Person**—It has been reported previously (7, 25, 26) that the ADP- or thrombin-induced calcium mobilization from intracellular stores, but not the ADP-gated cation channel, can be inhibited by cAMP- and cGMP-elevating agents and cell membrane-permeant cAMP and cGMP analogs in intact human platelets. The cGMP analog 8-pCPT-cGMP is a potent and selective activator of cG-PK in intact cell preparations (6, 7). Therefore, we used this cGMP analog to investigate the regulation of calcium mobilization in control platelets and in the cG-PK-deficient platelets from the CML patient F. B. The ADP-induced calcium response of fura-2-loaded platelets from the control person, but not from CML patient F. B., was strongly inhibited when platelets were preincubated for 10 min with 0.5 mM 8-pCPT-cGMP (Fig. 4).

Similarly, a 10-min pretreatment with 8-pCPT-cGMP prevented the thrombin (0.1 unit/ml)-induced calcium response in normal platelets but not in the platelets from CML patient F. B. (Fig. 5). In contrast, a 10-min preincubation with the cAMP elevating PG-EI prevented the ADP-evoked calcium response both in normal platelets as well as in the cG-PK-deficient platelets from CML patient F. B. (data not shown). It is also interesting to note that the increased fura-2 fluorescence after thrombin and especially after ADP stimulation declined more rapidly in control platelets than in the cG-PK-deficient platelets from CML patient F. B. Unfortunately, more extensive experiments analyzing the regulation of the calcium response in platelets from the two CML patients F. B. and C. S. were not possible due to limited platelet samples.

**FIG. 4. Effects of 8-pCPT-cGMP on the ADP-induced calcium response in platelets from a healthy individual or the CML patient F. B.** Fura-2-loaded platelets from a healthy donor (A) or the CML patient F. B. (B) were preincubated for 10 min without additions (control) or with 0.5 mM 8-pCPT-cGMP. When indicated (arrows), platelets were activated by addition of 20 μM ADP (final concentration). Fura-2 fluorescence (I; arbitrary units) was recorded using an excitation wavelength of 340 nm with emission at 510 nm.
FIG. 5. Effects of 8-pCPT-cGMP on the thrombin-induced calcium response in platelets from a healthy individual or the CML patient F. B. Fura-2-loaded platelets from a healthy donor (A) or the CML patient F. B. (B) were preincubated for 10 min without additions (control) or with 0.5 mM 8-pCPT-cGMP. When indicated (arrows), platelets were activated by addition of 0.1 unit/ml thrombin (final concentration). Fura-2 fluorescence (I; arbitrary units) was recorded using an excitation wavelength of 340 nm with emission at 510 nm.

DISCUSSION

Elevation of cGMP and stimulation of cG-PK is closely associated with the inhibition of an early step in the human platelet activation cascade, most likely at the level of phospholipase C activation and subsequent mobilization of Ca²⁺ from intracellular stores (1-4, 7, 27, 28). However, in addition to cG-PK, cGMP is capable of regulating several other distinct targets including ion channels and phosphodiesterases (1, 4, 15-17) which may contribute to the overall cellular response to cGMP. The present study was initiated to obtain more conclusive experimental evidence for the physiological role of cG-PK as a mediator of agonist-stimulated cGMP in human platelets.

Modern genetic approaches to elucidating the physiological significance of proteins include production and analysis of mutants, or development of transgenic or targeted gene disruption organisms. Obviously these approaches for studying cG-PK roles in human platelets are not possible. In the study reported here, it was, however, possible to identify certain patients with chronic myeloproliferative disorders who demonstrated a reduction, almost elimination, of the normal platelet cG-PK. It was then possible to investigate whether this deficit led to the expected consequences, alteration of cellular functions ascribed to cG-PK.

Platelets from CML patients are known to develop variable but distinct defects at the level of receptors, integrins, and metabolism (18), but the expression and function of protein kinases and their substrates have not been previously analyzed. Initial screening of platelets from 10 control individuals and 24 patients with myeloproliferative diseases for their content of cyclic nucleotides, cA-PK, cG-PK, and VASP (12, 13) revealed platelets from two CML patients which had defective cGMP/cG-PK signal transduction. Platelets from CML patients F. B. and C. S. contained a very low, almost undetectable level of cG-PK, but in contrast, normal levels of cA-PK catalytic subunit and the substrate protein VASP (Fig. 1, see “Results”). Perhaps all platelets from these two patients expressed a very low level of cG-PK, or alternatively, there may have been a small number of normal healthy platelets among a majority of platelets deficient in cG-PK.

Defective cG-PK expression does not appear to be directly involved in the cause of CML disease, which occurs by c-ABL protooncogene activation following a reciprocal translocation of parts of chromosomes 9 and 22 (22-24), since the human gene for type I cG-PK has been recently localized to chromosome 10 (29). However, the underlying chromosomal anomaly in CML could nevertheless result in defective cG-
PK expression in platelets from some CML patients. Whether defective cG-PK expression might be an indicator of the development of the accelerated phase and/or blast crisis of CML needs to be investigated in greater detail in a prospective clinical study.

For the purposes of the present study, evaluation of the platelets from the two CML patients permitted a correlation to be made between platelet cG-PK deficiency and absent cellular functions. CML platelets, in contrast to normal platelets, demonstrated considerably reduced nitrovasodilator-stimulated cGMP-mediated VASP phosphorylation despite an exaggerated cGMP elevation in response to sodium nitroprusside (Figs. 2 and 3). In comparison, the PG-E1-stimulated cAMP-mediated VASP phosphorylation in CML platelets was essentially intact. It is possible that the low level of SNP-induced VASP phosphorylation was due to the activation of cA-PK by cGMP since SNP increased the intracellular level of cGMP in these CML platelets from 1 to 47-85 pmol/10^6 cells (Fig. 3). Based on a platelet cell volume of 5.2 fl and other information (12), these elevated cGMP levels may correspond to an intracellular concentration of 9-16 μM which would be sufficient to partially activate the calcium cA-PK system (6).

Whereas agonists which elevate cGMP have the potential to act on a number of effector systems, the analog 8-pCPT-cGMP is a selective activator of cG-PK in intact human platelets since it has no effect on cGMP-regulated phosphodiesterases and activates cA-PK only at very high concentrations (6). Selective activation of cG-PK in intact human platelets by 8-pCPT-cGMP has been shown to strongly inhibit agonist-evoked calcium mobilization from intracellular stores without affecting the ADP-gated cation channel (7). In the present study (Figs. 4 and 5) preincubation with 8-pCPT-cGMP prevented the ADP- and thrombin-induced calcium mobilization from intracellular stores in normal platelets, but not in cG-PK-deficient platelets. In contrast, PG-E1 (which prevents calcium mobilization in human platelets by a cAMP-dependent mechanism (25, 26)) inhibited the ADP- and thrombin-induced calcium mobilization in both normal platelets and platelets from the CML patient F. B. (data not shown).

Collectively, our present results with human CML platelets indicate that deficient cG-PK expression impairs nitrovasodilator-stimulated protein phosphorylation and cGMP regulation of calcium mobilization and strongly suggest that cG-PK normally plays an important role in these regulatory events. We do not exclude that CML platelets, including the ones studied here, may have additional defects in intracellular signal transduction systems. For example, the delayed decline of the calcium response after ADP or thrombin stimulation (Figs. 4 and 5) may be indicative of an altered calcium re-uptake and/or extrusion system. However, we provided functional evidence for the presence of ADP, thrombin, and pros-taglandin receptors, for guanylyl and adenylyllyclease activities, and for an intact cAMP signal transduction system.

Unfortunately, extensive additional studies were not possible due to the limited quantities of human CML platelets from individual patients.

Our present results emphasize the important role of the cG-PK signal transduction system in mediating the effects of cGMP-elevating platelet inhibitors, and also demonstrate that the cG-PK-dependent mechanism is distinct from that of cA-PK or other cGMP-dependent effectors. Additionally, our results show that normal human platelets are not only useful models for the elucidation of signal transduction systems, but that CML platelets can function as valuable adjuncts for substantiation of proposed physiological roles of specific components of these regulatory systems.

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