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Tyrosine kinase modulation of protein kinase C activity regulates G protein-linked Ca^{2+} signaling in leukemic hematopoietic cells

Anton Vichalkovski, Ivana Kotevic, Niculina Gebhardt, Reto Kaderli, Hartmut Porzig *

Institute of Pharmacology, University of Bern, Friedbuehlstrasse 49, CH-3010 Bern, Switzerland

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Abstract

We have used a recombinant mouse pre-B cell line (TonB210.1, expressing Bcr/Abl under the control of an inducible promoter) and several human leukemia cell lines to study the effect of high tyrosine kinase activity on G protein-coupled receptor (GPCR) agonist-stimulated cellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE). After induction of Bcr/Abl expression, GPCR-linked SOCE increased. The effect was reverted in the presence of the specific Abl inhibitor imatinib (1 μM) and the Src inhibitor PP2 (10 μM). In leukemic cell lines constitutively expressing high tyrosine kinase activity, Ca^{2+} transients were reduced by imatinib and/or PP2. Ca^{2+} transients were enhanced by specific inhibitors of PKC subtypes and this effect was amplified by tyrosine kinase inhibition in Bcr/Abl expressing TonB210.1 and K562 cells. Under all conditions Ca^{2+} transients were essentially blocked by the PKC activator PMA. In Bcr/Abl expressing (but not in native) TonB210.1 cells, tyrosine kinase inhibitors enhanced PKCα catalytic activity and PKCα co-immunoprecipitated with Bcr/Abl.

Unlike native TonB210.1 cells, Bcr/Abl expressing cells showed a high rate of cell death if Ca^{2+} influx was reduced by complexing extracellular Ca^{2+} with BAPTA. Our data suggest that tonic inhibition of PKC represents a mechanism by which high tyrosine kinase activity can enhance cellular Ca^{2+} transients and thus exert profound effects on the proliferation, apoptosis and chemotaxis of leukemic cells.

Keywords: Tyrosine kinase; Protein kinase C; Store-operated calcium entry (SOCE); Leukemia cells; Bcr/Abl; G protein-coupled receptors

1. Introduction

Ca^{2+} ions are involved in the regulation of a host of cellular functions including proliferation, apoptosis or chemotaxis. In non-excitable cells, such as hematopoietic progenitors, stimulation of cellular Ca^{2+} transients is intimately coupled to the action of growth factors and cytokines [1–3]. Transiently enhanced cytosolic Ca^{2+} concentrations can be generated by activation of G protein-coupled receptors (GPCR) linked to phospholipase C-mediated Ca^{2+} release from intracellular stores or by activating Ca^{2+} influx from the extracellular space. These two processes are usually linked in that store depletion by an unknown mechanism initiates the transient opening of membrane Ca^{2+} channels ('store-operated calcium entry (SOCE)', for review see [4]). Ca^{2+} release and influx are both tightly controlled by intracellular signaling mechanisms including protein phosphorylation by serine/threonine or tyrosine kinases. Stimulation of protein kinase C (PKC) has been shown to reduce SOC fluxes [5–7]. Simultaneous inhibitory effects on Ca^{2+} release have also been observed [6]. On the other hand, activation of Src tyrosine kinases has been reported to promote SOC fluxes [8,9]. Conversely, blockers of Ca^{2+} entry inhibited the proliferation of several cancer cell lines [10,11]. However, in spite of a considerable research effort, the mechanisms involved in regulating the relevant Ca^{2+} entry pathways in non-excitable cells are still incompletely understood.

Hematopoietic progenitor cells possess an array of GPCR's, targeted by endogenous signaling compounds that elicit cellular Ca^{2+} signals [12,13]. Although the functional role of individual Ca^{2+} transients has not been defined in most cases, at least facilitation of chemotactic activity has been documented for Ca^{2+} signals induced by the chemokine CXCL12 interacting with its G_i-coupled receptor, CXCR4

* Corresponding author. Tel.: +41 31 632 3281; fax: +41 31 632 4992.
E-mail address: hartmut.porzig@pki.unibe.ch (H. Porzig).

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[3]. In a recent study in normal human erythroid progenitor cells, we could show that Src-type tyrosine kinases, via a negative feedback regulation, specifically inhibit the activity of PKCβ [14]. Since activation of Ca^{2+}-dependent PKC subtypes promotes cell proliferation in erythroid progenitors, this inhibitory effect results in a reduction of cell growth that can be antagonized by tyrosine kinase inhibitors. Given a strong link between PKC activity and SOC Ca^{2+} fluxes in hematopoietic cells, we reasoned that the same feedback mechanism would tend to enhance cellular Ca^{2+} transients after the stimulation of Src-type kinases. Consequently, the high level of tyrosine kinase activity in leukemic cells would be predicted to enhance Ca^{2+} transients by a tonic inhibitory effect on PKC and thus, to modulate survival, proliferation and the chemotactic behavior of the malignant cells.

In the present study, we have tested these predictions using normal human progenitor cells, three different human leukemia cell lines and a recombinant mouse promyelocytic cell line containing an inducible Bcr/Abl construct. Our results suggest that the tyrosine kinase–PKC feedback cycle represents one of the mechanisms that regulates the strength of cellular Ca^{2+} signals in response to endogenous G protein-linked stimuli in leukemic cells.

2. Materials and methods

2.1. Cells and tissue culture

The following cell lines and primary cells have been used: (1) EM-2 cells, a human chronic myeloic leukemia (CML) cell line showing myelocytic markers and constitutively expressing Bcr/Abl kinase (German Collection of Microorganisms and Cell Cultures, DSMZ). (2) K562 cells, a human CML cell line constitutively expressing Bcr/Abl and showing some erythroid–megakaryoid differentiation (American Type Culture Collection, ATCC). (3) HEL cells, a human erythroleukemia cell line (ATCC). These cells do not express Bcr/Abl. (4) TonB210.1 cells (TonB), a mouse promyelocytic BaF3-derived cell line, expressing Bcr/Abl under the control of a tetracycline-inducible promoter [15]. (5) Primary human hematopoietic progenitor cells isolated as described [14] from adult peripheral blood after G-CSF stimulation or from cord blood. Hematopoietic cell lines were generally grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM Glutamax 1, 50 U/ml penicillin and 50 μg/ml streptomycin. Non-induced TonB cells required mouse IL-3 (10 ng/ml) as a growth factor. After induction with doxycycline (2 μg/ml) the cells expressed Bcr/Abl and did no longer require IL-3 supplementation. As previously described [14] primary human progenitor cells were cultivated in serum-free BIT9500 medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented either with SCF, TPO, flt-3 and IL-3 or with SCF and Epo to promote the growth of multipotent or of erythroid progenitors, respectively.

2.2. Thymidine incorporation and determination of dead cell numbers

DNA incorporation of ^{3}H-thymidine was measured essentially as described in [14]. Individual cultures were kept for 10 h in the presence or absence of the calcium complexing agent BAPTA (1,2-bis-2-aminophenoxylethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.7 mM) dissolved in RPMI 1640 growth medium as described above, but with FCS reduced to 5%. At the end of the pretreatment period, the cells were washed and re-suspended either in the same medium or in 5% FCS-growth medium (containing 0.56 mM Ca^{2+}) supplemented with ^{3}H-thymidine (1 μCi/ml). Some cultures received CXCL12 (100 nM) or thrombin (3 U/ml) together with ^{3}H-thymidine. Incorporation of radioactivity was measured at the end of a further 12 h incubation period.

In some experiments the number of dead cells was determined in parallel by counting at predetermined time points the number of trypane blue-positive cells as percentage of the total cell count using a Terasaki chamber.

2.3. Ratiometric measurement of cellular Ca^{2+}

Changes in cellular free Ca^{2+} were recorded in fura-2-loaded cells with a Perkin-Elmer LS50 spectrofluorimeter as described before [20]. Intensity ratios of the fluorescent signals after excitation at 340 and 380 nm were converted to intracellular Ca^{2+} concentrations using the FL WinLab software (version 3.5) based on an apparent K_d value of 224 nM for the fura-2 Ca^{2+} complex. A standard protocol was applied to discriminate agonist-induced release from intracellular stores and the associated store-operated Ca^{2+} influx from the extracellular medium (see Section 3). GPCR agonist-induced cellular Ca^{2+} release and the corresponding SOCE were quantified as follows: for the release, we measured the area under the curve (AUC) from the addition of agonist to the end of the reaction. We subsequently followed, for 200 s, the cellular Ca^{2+} change resulting from a increase in the extracellular Ca^{2+} concentration to 2 mM. The agonist-dependent SOCE was then calculated as the difference in AUC between the curve in the presence of the agonist and a control curve obtained in its absence.

2.4. Immunoblotting and immunoprecipitation

Cellular proteins were separated in cytosolic and membrane fractions and subsequently solubilized as previously described [16]. After sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) the proteins were electrophoretically transferred to nitrocellulose membranes labeled with specific antibodies and visualized using horse radish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG together with an enhanced chemolumines-

cence assay (SuperSignal® Femto or Supersignal® Pico, Pierce/Perbio Science, Lausanne, Switzerland). Immunoprecipitation was performed during 3 h incubation of solubilized proteins at 4 °C with specific antibodies according to standard protocols. Immunoprecipitates were collected on Protein G plus agarose beads (Santa Cruz) and used for enzyme activity measurements or for SDS-PAGE and blotting procedures. For co-immunoprecipitation experiments with Bcr–Abl, we used an anti-c-Abl antibody coupled to agarose beads (Santa Cruz, Labforce AG, Nuenningen, Switzerland).

2.5. PKC activity measurements

Total PKC enzymatic activity was measured in cell lysates. Subtype-specific activity was determined after immunoprecipitation from cytosolic or membrane fractions with specific antibodies. The assay was based on a non-radioactive technique relying on the interaction of the enzyme with its substrate coated onto the wells of a 96-well culture dish. The reaction was quantified using an antibody specific for the phosphorylated substrate (Stressgen, AMS Biotechnology, Lugano, Switzerland).

2.6. Protein determination

Protein concentrations were measured using the bichinconic acid (BCA) method marketed as a kit by Pierce/Perbio Science.

2.7. Data analysis

The Prism 4 program (Graphpad software, San Diego CA) was used for statistical analyses, non-linear curve fitting, integration of Ca^{2+} transients and data presentation. Statistical significance of differences of group means were tested with Student's *t*-test. The scatter bars in plots of the data represent S.E.M. Since individual batches of cultured cells showed significant quantitative (though not qualitative) differences in agonist-induced Ca^{2+} transients, we used paired experiments whenever possible. p Values <0.05 were considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$).

2.8. Materials

Analytical grade biochemicals were obtained from Merck (Dietikon, Switzerland) and Sigma–Aldrich (Buchs, Switzerland). Cell culture media and supplements were purchased from Invitrogen, Basel, Switzerland or from Sigma–Aldrich. Granulocyte-macrophage colony-stimulating factor and interleukin-3 were generous gifts of Werthenstein Chemie (Schachen, Switzerland) and Novartis Pharma (Basel, Switzerland), respectively. Erythropoietin (Epro®) was purchased from CILAG, Schaffhausen, Switzerland). All other cytokines or chemokines were obtained from PeproTech EC, London, UK. PP2, PP3, bisindolylmaleimide I, Gö6976, the stable thromboxane A_2 analogue U-46619 were all supplied by Alexis Corp., Lausen, Switzerland. Imatinib mesylate was a gift from Novartis Pharma (Dr. E. Buchdunger). Sources of antibodies were Santa Cruz Biotechnology (PKCα, c-Abl agarose, Labforce, Nunningen, Switzerland), Cell Signaling Technology (c-Abl, phospho-Src, Crkl, phospho-Crkl, phospho-Lyn, Bioconcept, Allschwil, Switzerland), Abcam, Cambridge, UK (phospho-c-Abl) and BD Biosciences, Basel, Switzerland (PKCβ), Upstate (PKCα, Lucerna Chem AG, Lucerne, Switzerland).

3. Results

To quantify the effects and mutual regulatory interactions of Bcr/Abl and PKC activities, we first focused on GPCR-induced Ca^{2+} transients in the TonB cell model system. In the second part, we extended these experiments to three human leukemia cell lines in an attempt to assess possible effects of constitutively over-expressed tyrosine kinase activity on PKC-dependent Ca^{2+} transients.

Fig. 1(A)–(F) illustrates the PKC dependency of cellular Ca^{2+} signals in normal and leukemic human hematopoietic progenitors. In primary cells (Fig. 1(A)–(C)) PKC stimulation with PMA almost completely abolished both the cellular Ca^{2+} release as well as the store-operated Ca^{2+} entry while a specific PKC inhibitor, bisindolylmaleimide (BIM), caused a significant increase of these transients. SOCE is known to exhibit a rather slow inactivation rate [17]. Fig. 1(C) documents that PKC apparently acts by enhancing the inactivation rate of the Ca^{2+} influx channels. In leukemic cells (Fig. 1(D)–(F)), the effect of PKC inhibition on Ca^{2+} release from intracellular stores varied for different agonists and among cell lines between no effect and more than two-fold stimulation. These data are summarized in Table 1. By contrast, SOC entry was always significantly enhanced. To assess the contribution of Ca^{2+}-dependent PKC subtypes, we used the specific inhibitor Gö6976 [18]. Unfortunately, this compound caused a general inhibition of fura-2 fluorescence that varied in strength between cell lines but was considered unspecific, since it was also observed in digitonin-permeabilized cells. Therefore, fura-2 signals in presence of the two inhibitors could not be easily compared quantitatively. Nevertheless, at least in EM-2 and in HEL cells a stimulating effect of Gö6976 on SOCE could be established. (compare Fig. 1(F)). PKC activation by PMA essentially abolished Ca^{2+} release and SOCE in most cases. In HEL cells the strong thrombin-induced release reaction was reduced by 80% in the presence of PMA (10 nM, compare Fig. 1(F)). Similar data as in Fig. 1(D)–(F) were obtained with K562 cells (not shown). We conclude from these results that full activation of cellular Ca^{2+} release required lower levels of PKC activity than Ca^{2+} entry via store-operated pathways. The strong dependence of cellular Ca^{2+} transients on PKC in normal human hematopoietic cells is maintained in leukemic cells. Ca^{2+}-dependent subtypes of PKC appear to be at least partially responsible for this effect.

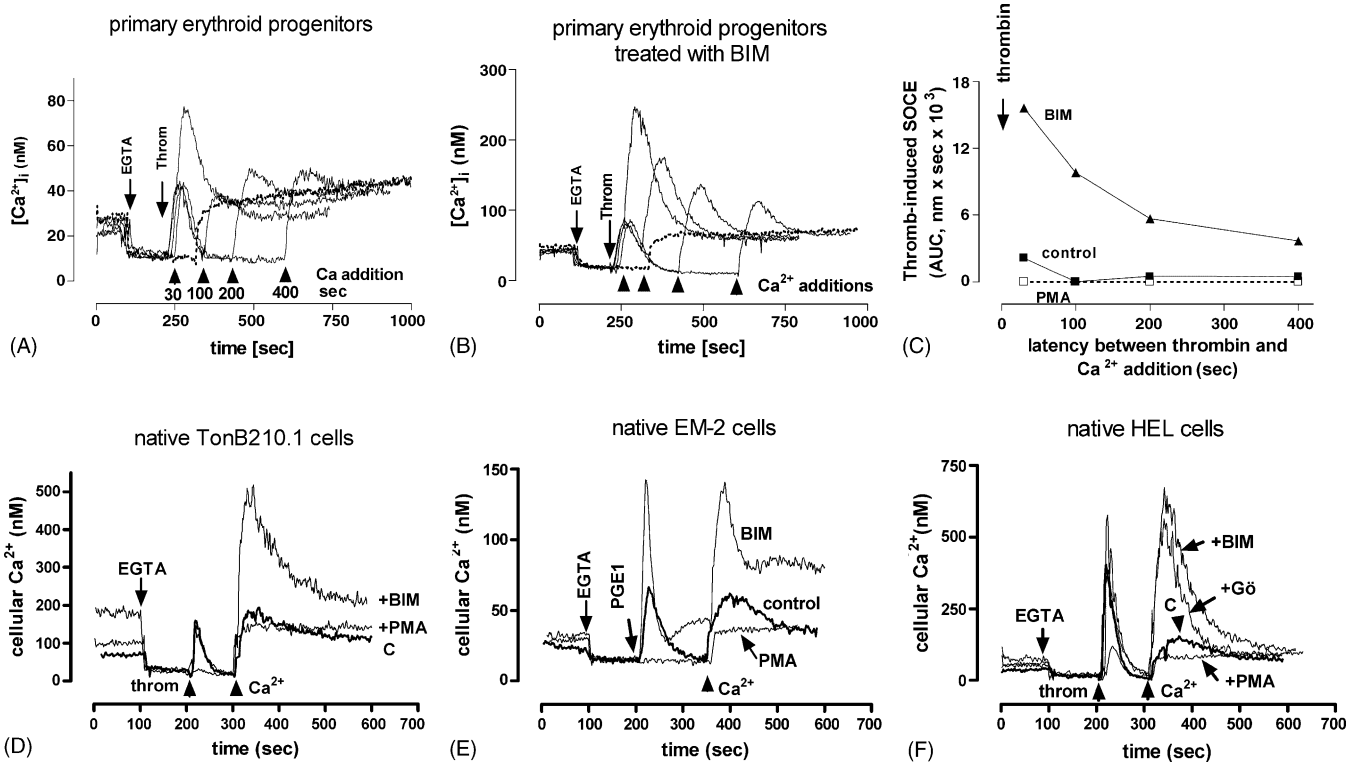

Fig. 1. PKC activity modulates Ca^{2+} transients in human hematopoietic progenitors and three different leukemic cell lines. (A–C) Human erythroid progenitors. (A) Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in native cells. The cells were suspended in a Ca^{2+}-free medium. After an equilibration period, 0.5 mM MgEGTA (EGTA) was added. Ca^{2+} release was initiated (100 s after EGTA) by adding thrombin (throm, 2 U/ml). (A) SOCE signal was elicited by adding $CaCl_2$ (2 mM) at four predetermined time intervals (arrowheads) after the start of the release reaction. (B) Same protocol as in (A), but cells pretreated for 5 min with the PKC inhibitor bisindolylmaleimide (BIM). (C) Comparison of the areas under the curve (AUC) for SOCE responses in native (control), BIM- or PMA-treated progenitors as a function of the time delay between the addition of thrombin and the addition of Ca^{2+}. (D–F) Leukemic cells treated according to the same protocol as in (A) and (B) but adding Ca^{2+} at a single fixed time. Curves labeled 'BIM' or 'PMA' were obtained after a 5 min pretreatment with the respective compounds. Curves labeled 'C' or 'control' show Ca^{2+} transients in cells without pretreatment. (D) Native TonB210.1 cells, Ca^{2+} was added 100 s after thrombin. (E) EM-2 cells, using prostaglandin E1 (PGE1) as G protein-coupled receptor agonist. Ca^{2+} was added after 150 s. (F) HEL cells with thrombin as agonist and Ca^{2+} added after 100 s. The curve labeled 'Gö' was obtained after the cells had been pretreated for 5 min with the PKC inhibitor Gö6976 (10 μM). Data in each panel are representative for atleast three independent experiments.

3.1. Ca^{2+} transients and tyrosine kinase activity in TonB cells

In these cells, we first studied Ca^{2+} transients prior and after induction of Bcr/Abl expression. In the absence of doxycycline, TonB cells, growing in the presence of IL-3, express modest levels of c-Abl but no or only traces of Bcr/Abl (Fig. 2(A)). Treatment with doxycycline (2 μg/ml) resulted in the loss of IL-3 dependence and in the expression of Bcr/Abl. The active, phosphorylated form of Bcr/Abl as measured with a phospho-Abl-specific antibody was clearly detected after 48 h but increased further between day 2 and 9 of treatment while the change in total protein expression during this time period was less marked (Fig. 2(A)). In parallel, also the level of phosphorylated Src family kinases increased significantly. On the other hand, phosphorylation of the adaptor protein Crkl, a well defined substrate of Abl tyrosine kinase reached its maximal level already after 2 days of induction. Treatment with imatinib (1 μM, 1 day), a specific inhibitor of Abl catalytic activity, largely reversed Bcr/Abl and Src phos-

Table 1
Effect of PKC inhibition on cellular Ca^{2+} release in human progenitors and in leukemic cell lines

Cell line	PGE1 (20 μM) BIM/control	Thrombin (2 U/ml) BIM/control	CXCL12 (50–100 ng/ml) BIM/control
Human progenitors	1.811 ± 0.396 (3)	1.618 ± 0.199 (5)	1.441 (2)
TonB210.1	NA	1.205 ± 0.069 (5)	1.565 ± 0.617 (4)
HEL	1.151 ± 0.075 (7)	1.256 ± 0.109 (3)	1.889 ± 0.379 (5)
K562	1.835 ± 0.221 (9)	NA	NA
EM-2	1.671 ± 0.322 (9)	NA	2.924 ± 0.811 (9)

The data give ratios between AUC's for the release reaction in the presence and in the absence (control) of the PKC inhibitor BIM (20 μM). A value of '1.0' results if BIM does not affect the release reaction. NA: not available because no Ca^{2+} transient could be elicited with this particular agonist.

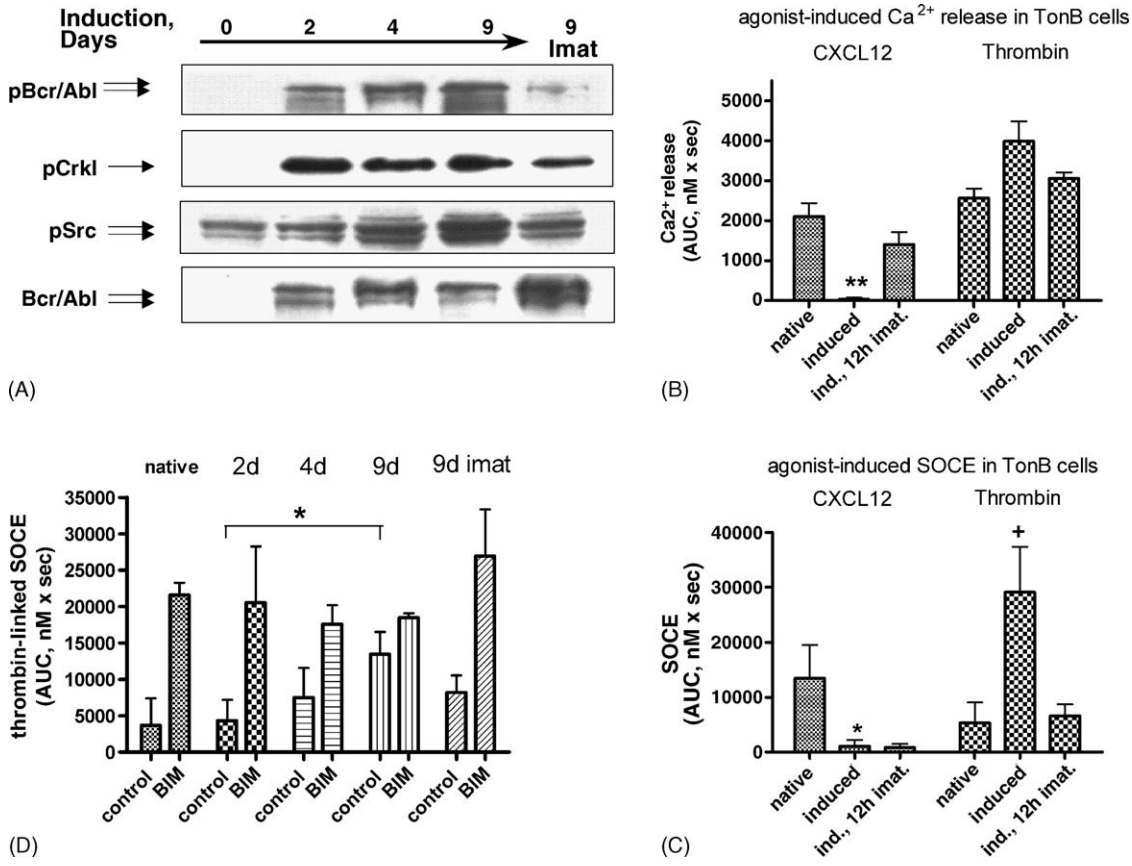

Fig. 2. Expression of Bcr/Abl changes Ca^{2+} transients in TonB210.1 cells. (A) Levels of phospho-Bcr/Abl, phospho-Src and phospho-Crkl in immunoblots of TonB cell lysates prior and after induction of Bcr/Abl expression for 2–9 days. For day 9, the changes induced by a 12 h treatment with the specific Abl inhibitor imatinib (imat, 1 μM) are also documented. Note the increase in pBcr/Abl and pSrc immunoreactivity between day 2 and day 9 and its reversal by imatinib. Data from 2–3 independent experiments. (B and C) CXCL12- and thrombin-induced cellular Ca^{2+} release (B) and SOCE (C) in TonB cells prior and after induction of Bcr/Abl expression for 9 days. Means of 3–8 independent experiments. Cells induced for 9 days with doxycyclin and then exposed for 12 h to imatinib (1 μM) are labeled 'ind.12 h imat.'. The data for this latter group give means of three independent experiments, except for the release with CXCL12 that is based on two independent experiments. Note that the loss of CXCL12-mediated Ca^{2+} transients in Bcr/Abl expressing cells could be reverted by imatinib. (D) SOCE transients in the absence (control) and presence of the PKC inhibitor BIM as a function of time of induction and after treatment with imatinib (imat). Means of 3–4 independent experiments. Note that SOCE increases in controls but stays constant in BIM-treated cells.

phorylation while the phosphorylation of Crkl was partially maintained.

Ca^{2+} transients in native and in doxycycline-induced cells were elicited by the chemokine CXCL12 and by thrombin. Fig. 2(B) and (C) shows the results for the release of intracellular Ca^{2+} and for the agonist-induced influx of extracellular Ca^{2+} (SOCE) at maximally effective concentrations of the agonists. Both agonists failed to elicit any changes in cellular Ca^{2+} concentrations after overnight treatment of the cells with PTX (150 ng/ml, not shown). Hence, the signals all require transduction via a G_i-type G protein. As has been observed earlier [19], Bcr/Abl activity caused a loss of CXCL12-induced Ca^{2+} transients that was partial after 2 days and complete after 9 days of induction. Unlike the suppression of SOCE, the loss of CXCL12-mediated Ca^{2+} release was reversible after a 12 h treatment with imatinib (Fig. 2(B) and (C)), but it was no longer present in imatinib-resistent, doxycyclin-induced cells growing in the presence of IL-3. The mechanism of the suppression of CXCL12-mediated Ca^{2+} responses is still unclear but it is not generally observed in Bcr/Abl expressing leukemic cells. In the human leukemia cell line EM-2 that expresses Bcr/Abl constitutively, CXCL12 induced significant, PTX-sensitive Ca^{2+} signals (see below).

On the other hand, thrombin-mediated Ca^{2+} transients remained unchanged or were significantly enhanced after doxycycline induction of TonB cells. After 9 days of induction, cellular Ca^{2+} release resulting from a maximally effective concentration of thrombin (3–5 U/ml) showed an increase that did not reach statistical significance, while the mean SOCE signal was six-fold larger. This stimulatory effect was reversed by short-term imatinib treatment. Like in human cells, the extent of cellular Ca^{2+} changes and, in particular, SOCE depended on PKC activity (Fig. 1(D)). If the mechanism of this increase in Ca^{2+} transients involved a Bcr/Abl-dependent inhibition of PKC, we would expect that the progressive increase in Bcr/Abl activity is associated with a parallel decrease in the efficiency of PKC inhibitors to enhance

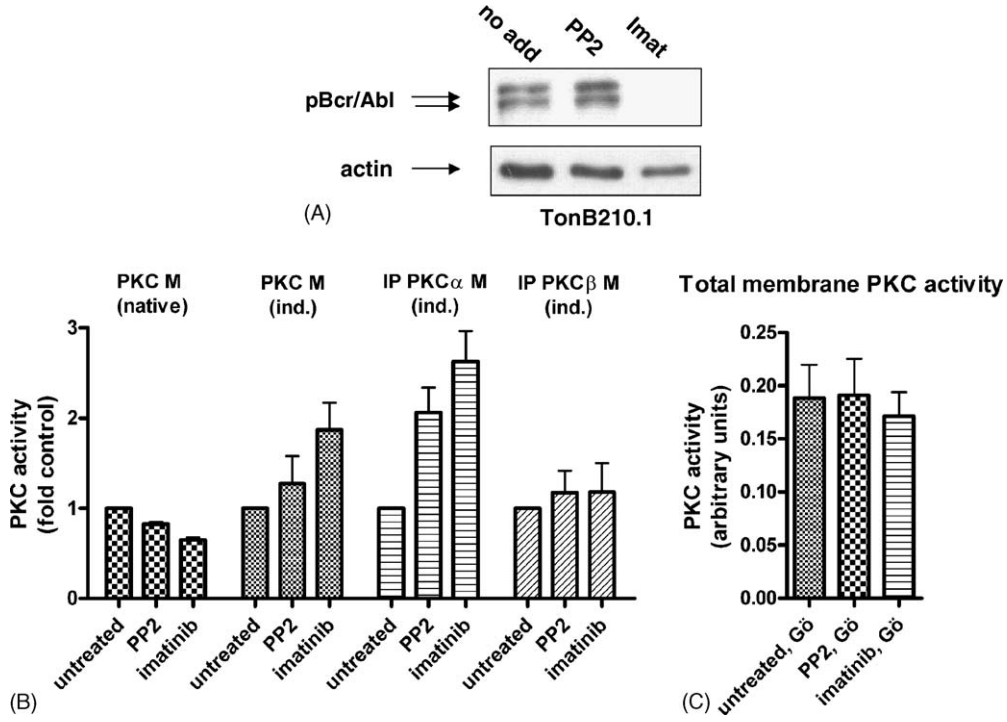

Fig. 3. Src and Abl tyrosine kinase inhibitors stimulate PKCα in Bcr/Abl expressing TonB cells. (A) Phospho-Bcr/Abl (pBcr/Abl) immunoreactivity in western blots after induction of Bcr/Abl expression for 10 days (no add., line 1). Treatment with imatinib (imat), but not with PP2, for 12 h blocked the generation of pBcr/Abl. Actin signals in the sample provide a loading control. (B) PKC activity, measured as described in Section 2, was determined in untreated cells and in cells treated with tyrosine kinase inhibitors PP2 and imatinib for 14 h. PKC M (native): total PKC activity in the membrane fraction (M) of native cells (means of two independent experiments). PKC M (ind.): total PKC activity in the membrane fraction of cells induced (ind.) for 10 days to express Bcr/Abl (means of two independent experiments). IP PKCα M (ind.): activity of PKCα in immunoprecipitates from the membrane fraction of induced cells (means of three independent experiments). IP PKCβ M (ind.): activity of PKCβ in immunoprecipitates from membrane fractions of induced cells (means of three independent experiments). Activity is quantified as fold increase over control (set to 1). Note that tyrosine kinase inhibitors have no stimulating effect in native cells. (C) Total membrane PKC activity measured in the presence of Gö6976 (means of four assays from two independent experiments).

cellular Ca^{2+} signals. This was indeed observed. In Fig. 2(D), we compare the BIM-induced increase in thrombin-activated SOCE in native cells and after 2, 4 and 9 days of doxycycline induction. BIM had a marked stimulating effect in native cells where very little SOCE was observed in the absence of BIM. A strong effect was maintained in freshly induced cells, but was essentially lost after 9 days. BIM responsiveness could be largely recovered after inactivation of Bcr/Abl by a 12 h treatment with imatinib. It should be noted that the total level of SOCE observed after PKC inhibition remained essentially constant during Bcr/Abl induction. Hence, the apparent increase under control conditions could be largely ascribed to progressive endogenous PKC inhibition.

To obtain more direct information on PKC catalytic activity in Bcr/Abl expressing TonB cells, we measured the effect of tyrosine kinase inhibitors on PKC substrate phosphorylation by an antibody-based technique (see Section 2). In parallel, we monitored Bcr/Abl activation by measuring the level of the enzyme in its phosphorylated form (Fig. 3(A)). PP2, a Src family kinase inhibitor had no significant effect on the formation of phospho-Bcr/Abl while imatinib essentially eliminated this active form of the enzyme. By contrast, the phosphorylation of the Src family kinase Lyn was blocked by PP2 under these conditions (not shown). Control experiments showed that a specific inhibitor of Ca^{2+}-dependent PKC subtypes (Gö6976) completely suppressed PKC activation in membranes of induced TonB cells (Fig. 3(C)) and hence, we did not screen systematically any Ca^{2+}-independent PKC subtypes. Enzymatic activity of PKC was determined by using either total PKC or subtype-specific immunoprecipitates of PKCα or PKCβ from solubilized membrane preparations. The results documented in Fig. 3(B) indicate that imatinib, the specific inhibitor of Bcr/Abl is a potent activator of PKCα. Similarly, the Src family tyrosine kinase inhibitor PP2, stimulated PKCα activity, though somewhat less effectively. None of the inhibitors had a significant effect on PKCβ. The stimulating effect of tyrosine kinase inhibitors was strictly limited to doxycyclin-induced cells. By contrast, PKC activity in native TonB cells tended to decrease in the presence of PP2 or of imatinib (Fig. 3(B)). Hence, the inhibitors do not cause a direct activation of PKC.

The mechanism linking Src or Bcr/Abl activity to an inhibition of PKCα in TonB cells could not be analyzed on the basis of these experiments alone. In particular, it remained unclear whether functional interaction of these kinases was based on their physical association within a common sig-

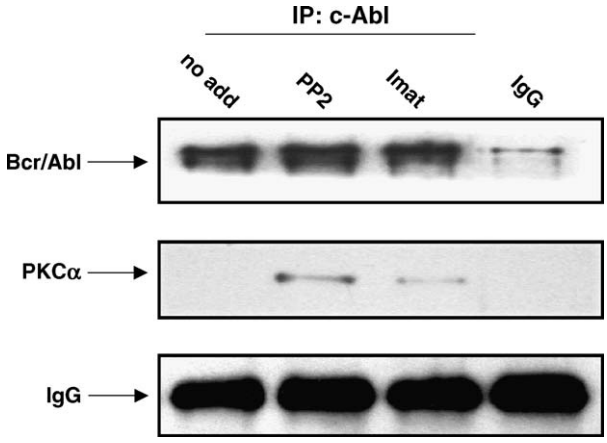

Fig. 4. Co-immunoprecipitation of PKCα with Bcr/Abl in TonB cells. Cells ($1–2 \times 10^7$/sample) induced to express Bcr/Abl for 10 days were lysed as described in Section 2 either without treatment (no add) or after a 12 h cultivation in the presence of PP2 or imatinib (Imat). For immunoprecipitation, the lysates were incubated with a Bcr/Abl antibody coupled to agarose beads. Upper row, Bcr/Abl immunoprecipitate, middle row, the corresponding PKCα co-immunoprecipitate, lower row, immunoreactive IgG in the sample providing a surrogate loading control. One of three independent experiments. IgG: Specific antibody replaced by rabbit IgG. Note that co-immunoprecipitation was observed only in PP2 or imatinib-treated cells.

naling complex. This was tested in co-immunoprecipitation studies. As documented in Fig. 4, PKCα was clearly detected in the Abl immunoprecipitates from whole cell lysate of imatinib- or PP2-treated cells but not in those of untreated controls. A similar pattern was obtained when a lysate prepared from only the membrane fraction of TonB cells was used for Abl co-immunoprecipitation. Conversely, an antibody directed against PKCα immunoprecipitated Bcr/Abl, though not very efficiently (not shown).

We conclude from these results that association with the Abl kinase promotes PKCα activation. However, this activation is unlikely to require Abl-catalyzed phosphorylation because physical interaction seems to occur preferentially with the catalytically inactive enzyme.

To assess the overall significance of Ca^{2+} influx in native and in Bcr/Abl expressing cells, we used two different approaches: Survival of cells in the presence and absence of Ca^{2+} was followed by measuring the number of trypane blue-positive (dead) cells as a function of time during a 24 h observation period (Fig. 5(A)). Ca^{2+}-dependent proliferation was tested by comparing ^{3}H-thymidine incorporation in Ca^{2+}-depleted cells to the one in cells supplemented with Ca^{2+} after a period of Ca^{2+} depletion (see Section 2). In each case Ca^{2+} depletion was achieved by adding BAPTA to the culture medium, resulting in a free extracellular Ca^{2+} concentration close to 1 μM. As shown in Fig. 5(A), doxycyclin-induced cells appeared significantly more sensitive to Ca^{2+} depletion than native TonB cells. Eighteen hours after the start of the experiment, native, BAPTA-treated cell cultures contained $12.5 \pm 1.7\%$ ($n = 4$) trypane blue-positive cells while doxycyclin-induced cultures reached $31.0 \pm 3.5\%$ ($n = 4$). By contrast, in both, native and doxycycline-induced cells, ^{3}H-thymidine incorporation was stimulated five- to six-fold upon Ca^{2+} addition after a period of Ca^{2+}-starvation. The addition of thrombin, together with Ca^{2+}, resulted in a small additional stimulating effect in native but not in induced cells (Fig. 5(B) and (C)). Neither in native nor in induced cells thrombin had any stimulatory effect in the presence of BAPTA. We conclude from these results that Bcr/Abl expressing TonB cells, unlike native cells, rely on Ca^{2+} as a survival factor while proliferation is stimulated to the same extent in native and induced cells.

3.2. Effect of tyrosine kinase inhibitors on Ca^{2+} transients in human leukemia cell lines

Additional experiments were designed to test the possibility that analogous tyrosine kinase–PKC interactions as described above for TonB cells, might affect Ca^{2+} transients in constitutively Bcr/Abl expressing CML-derived human leukemia cells. K562 cells and EM-2 cells were chosen as model systems. For comparison, we also used HEL cells a human erythroleukemia cell line that does not express Bcr/Abl.

3.3. EM-2 cells

Unlike TonB cells, EM-2 cells express a variety of receptors linked to G_i and/or non-G_i (most likely G_q) -type G proteins that mediate agonist-induced cellular Ca^{2+} transients. We selected three agonists acting exclusively via G_i (CXCL12), via G_i and non-G_i (PGE1) and exclusively via G_q (UTP) -coupled receptors. The extent of G_i-dependence was established by determining concentration–response curves for cellular Ca^{2+} release prior and after pretreatment with maximally effective concentrations (150 ng/ml) of pertussis toxin (PTX, not shown). Ca^{2+} release (Fig. 6(A)) and SOC influx (not shown) reached very different levels at maximally effective concentrations of the three agonists. The most prominent responses were obtained with UTP interacting with a $P2Y_2$ purinergic receptor that is also expressed in primary hematopoietic cells and in many human leukemic cell lines [12,20,21]. Treatment of EM-2 cells with imatinib resulted in a significant reduction of UTP-stimulated cellular Ca^{2+} release. With all GPCR agonists, the decrease in SOC influx did not reach statistical significance.

As with TonB cells, the tyrosine kinase inhibitors imatinib and PP2 both stimulated the membrane-associated PKC activity (Fig. 6(B)). However, there was no preference for PKCα over PKCβ activation. The basal level of catalytic activity and the extent of the stimulation both remained markedly lower than the corresponding values in TonB cells. Unlike with TonB cells, tyrosine kinase inhibitors did not increase the potency of BIM to amplify cellular Ca^{2+} transients (not shown).

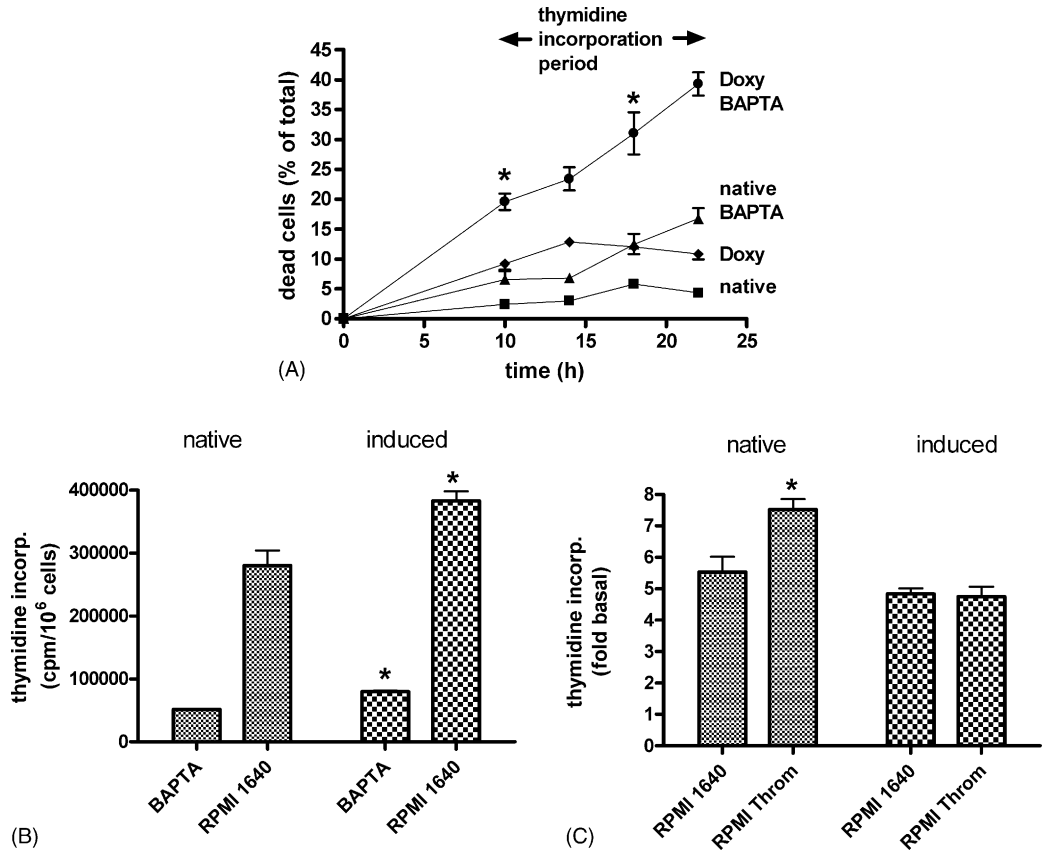

Fig. 5. Effect of extracellular Ca^{2+} on survival and proliferation of native and Bcr/Abl expressing TonB210.1 cells. (A) Number of trypane blue-positive cells as a function of time. 'native': cells in RPMI 1640 medium supplemented with 5% FCS and 10 ng/ml mouse IL-3. 'Doxy': Doxycyclin-induced cells expressing Bcr/Abl for 6–9 days growing in RPMI 1640 medium as above without IL-3. 'native BAPTA': cells maintained in the same medium as native cells but containing in addition 0.7 mM BAPTA (free Ca^{2+} close to 1 μM). 'Doxy BAPTA': Doxycyclin induced cells maintained as Doxy cells above but in the additional presence of 0.7 μM BAPTA. The data points give means of two or four (10, 18 h) independent cultures. Stars mark significant differences compared to 'native BAPTA'. (B) Comparison of thymidine incorporation in parallel cultures either kept in BAPTA (0.7 mM)-containing medium or switched after 10 h from BAPTA medium to normal RPMI 1640 medium. Stars indicate significant differences between corresponding columns in native and induced cells. (C) Effect of thrombin (Throm) on thymidine incorporation after the switch from BAPTA- to Ca^{2+}-containing medium. Note the significant difference induced by adding thrombin to RPMI 1640 medium (star). (B) and (C) give mean values from six separate cultures from two different batches of cells.

3.4. K562 cells

PGE1 and the stable thromboxane A_2 analog U46619 were the only GPCR agonists that we found capable of eliciting cellular Ca^{2+} transients in K562 cells. The effect of PGE1 was reduced but not eliminated in the presence of PTX while the effect of U46619 remained unaffected. Pretreatment with U46619 increased the effect of a subsequent challenge with PGE1 by more than two-fold. Therefore, we tested the effect of this combination on Ca^{2+} release and SOCE. A 12 h treatment with imatinib (1 μM) or PP2 (10 μM) reduced the SOCE signal under all conditions, but this decrease varied in extent and did not reach significance (not shown). Compared to untreated cells, BIM amplified the U46619/PGE1-stimulated SOCE in PP2-treated cells while the effect of imatinib remained small (Fig. 6(C)). The intracellular Ca^{2+} release in response to the two agonists remained essentially unchanged (not shown). Co-immunoprecipitation experiments in K562 cells prior or after treatment with imatinib or PP2 failed to show a significant association between PKCα or β with Bcr/Abl. Together, these observations suggest that a regulatory, though no tight physical, coupling between tyrosine kinase and PKC appears to exist in K562 cells.

3.5. HEL cells

These cells were chosen to represent a human erythroleukemia line that does not constitutively express Bcr/Abl while c-Abl expression is well maintained. We studied Ca^{2+} transients elicited by CXCL12, PGE1 and UTP. Cellular Ca^{2+} release with most GPCR agonists was insensitive to PKC inhibition but was inhibited by PMA. SOCE was markedly stimulated or inhibited with BIM and PMA, respectively (compare Fig. 1(F)). The one exception was CXCL12 which initiated a PKC-dependent Ca^{2+} release reaction but failed to activate any SOCE, irrespective of the level of PKC activity and also irrespective of the size of the release signal. The lack of a SOCE signal for CXCL12 was confirmed in a recombinant HEL cell line that we prepared to over-

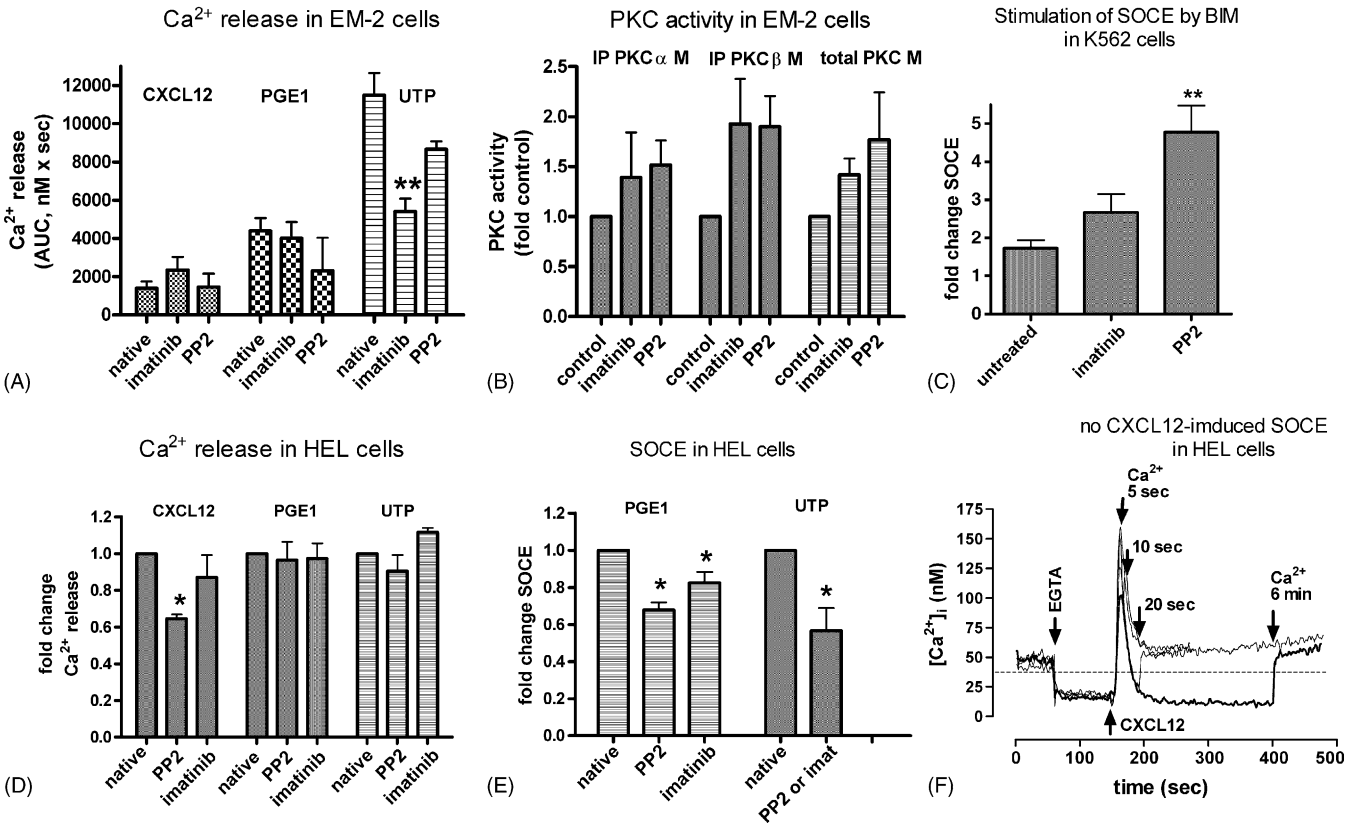

Fig. 6. Ca^{2+} transients and PKC activation in human leukemia cells. (A) Ca^{2+} transients for cellular release and PKC activity in EM-2 cells. Area under the curve (AUC) for Ca^{2+} release upon stimulation with CXCL12, PGE1 and UTP in native cells and after 12 h treatment with PP2 or imatinib. Data give means of 3–11 independent experiments. (B) PKC activity in native and tyrosine kinase inhibitor treated EM-2 cells, measured as fold increase of activity in controls (set to 1). IP PKCα M: activity of PKCα in immunoprecipitates from the membrane fraction (means of three independent experiments). IP PKCβ M: PKCβ activity in the same fractions (means of two independent experiments). Total PKC M: total PKC activity in the membrane fraction (means of two independent experiments). Note that PKCα stimulation did not reach significance. (C) K562 cells. Relative efficacy of BIM to stimulate SOCE transients induced by the combination of the thromboxane agonist U49916 with PGE1. BIM efficacy was significantly enhanced in the presence of PP2. (D) Effect of tyrosine kinase inhibitors on CXCL12-, PGE1- and UTP-induced Ca^{2+} release in HEL cells quantified as fold change of the values in native cells rather than as AUC due to the large variability of absolute AUC values. Note that only the CXCL12-induced release is affected by PP2. (E) SOCE transients corresponding to the experiment in (C). Data in (C)–(E) represent means of 3–9 independent experiments. (F) Ca^{2+} transients in HEL cells over-expressing CXCR4. Same protocol as in Fig. 1(A). The time arrows indicate Ca^{2+} additions. As in native HEL cells, even after a strong CXCL12-mediated release of intracellular Ca^{2+}, no SOCE could be elicited by addition of extracellular Ca^{2+} at any time point.

express the CXCL12 receptor CXCR4. Unlike with thrombin and in spite of producing a large Ca^{2+} release signal upon stimulation with CXCL12, almost no SOCE could be observed at any time point after the release reaction (Fig. 6(F), Vichalkovski et al., Unpublished results). Treatment of native HEL cells with imatinib (1 μM) or PP2 (10 μM), did not alter PGE1- or UTP-induced cellular Ca^{2+} release. By contrast, PP2 (but not imatinib) caused a significant reduction in CXCL12-mediated Ca^{2+} release (Fig. 6(D)). The PKC-sensitive SOC influx induced by stimulation with PGE1 or UTP was similarly inhibited by PP2 and imatinib (Fig. 6(E)). The potency of BIM to stimulate SOCE was enhanced by PP2 but this increase did not reach statistical significance (not shown). We conclude from these observations that in HEL cells, as in Bcr/Abl expressing cells, high tyrosine kinase activity is associated with an increase in Ca^{2+} transients. Yet, only a weak coupling to PKC activity appears to be maintained.

4. Discussion

In leukemic cells, high levels of tyrosine kinase activity appear to be associated with an increase in GPCR-mediated Ca^{2+} transients. This is probably a causal relationship because induction of Bcr/Abl expression and tyrosine kinase inhibitors exerted antagonistic effects on SOCE. In the present study, we have systematically tested to what extent a specific inhibitory interaction between tyrosine kinases and PKC might contribute to the regulation of GPCR agonist-induced cellular Ca^{2+} transients. It is known that Src- and Abl-type tyrosine kinases can interact both physically and functionally with PKC, in particular with the α and δ subtypes [22–25]. However, this interaction usually resulted in PKC activation. A negative feedback regulation of PKC by Abl- or Src-type tyrosine kinases was first described in normal human erythroid progenitors [14]. This latter observation provides a possible functional link between the effects of PKC and of

tyrosine kinases on Ca^{2+} transients observed in hematopoietic cells. Yet, it is unknown whether such mechanism would extend beyond the erythroid progenitor population and be active at the high levels of tyrosine kinase activity encountered in leukemic cells.

Expression of individual GPCRs in different leukemic cell lines proved to be highly variable. Therefore, it was not possible to compare an identical set of agonists with each cell line. Even for PGE1 that acted as an agonist in all human leukemia cells, we did not find any response in TonB cells. Similarly, the receptor-G protein coupling appeared heterogeneous among cell lines. Thus, thrombin, which is known to couple to G_i and G_q in human cells, exclusively interacted with G_i in TonB cells. An earlier report [19] had shown that cellular Ca^{2+} transients induced by the chemokine CXCL12 are lost after expression of the Bcr/Abl tyrosine kinase in TonB cells. Our data are not compatible with a generalized suppression of GPCR-mediated Ca^{2+} signals in Bcr/Abl expressing cells. Rather, they suggest a TonB cell-specific interference of Bcr/Abl with CXCL12 signal transduction downstream of G_i activation. Ca^{2+} responses induced by thrombin, another GPCR ligand coupled to G_i in these cells, were enhanced rather than reduced after Bcr/Abl expression. Moreover, there was probably no change in membrane Ca^{2+} channel expression pattern, since upon inhibition of Abl activity with imatinib, the loss of the Ca^{2+} response to CXCL12 was fully reversible. EM-2 cells, expressing Bcr/Abl constitutively, retained the CXCL12-induced Ca^{2+} release even though the signal was also exclusively transduced by G_i.

Like with primary human progenitors, in all leukemic cells, SOCE was almost completely obliterated upon stimulation of PKC with PMA and significantly enhanced in the presence of PKC inhibitors. By contrast, the response of cellular Ca^{2+} release to PKC inhibition varied between no effect and a marked increase, while PKC stimulation with PMA always reduced Ca^{2+} release. Hence, the Ca^{2+} release reaction appeared to be less sensitive than SOCE to changes in basal PKC activity. Correspondingly, cellular Ca^{2+} release in response to GPCR agonists was reduced by tyrosine kinase inhibitors only in those cases where BIM had a strong stimulating effect (as for UTP in EM-2 cells and for CXCL12 in HEL cells). On the other hand, a significant inhibition of SOCE by tyrosine kinase inhibitors was observed only in TonB and in HEL cells. The reduction in EM-2 and in K562 cells was very variable and did not reach statistical significance. Nevertheless, a significant increase in BIM efficacy upon inhibition of tyrosine kinase activity was observed in TonB as well as in K562 cells.

Taken together, these observations are consistent with the assumption that a feedback regulation between Abl- and/or Src-type kinases and PKC, analogous to the one described in human erythroid progenitors [14], is activated upon expression of Bcr/Abl in TonB cells and is also revealed after Src kinase inhibition in K562 cells. In general, the close functional coupling between PKC and tyrosine kinase activities appears to be weakened significantly if high tyrosine kinase activity is constitutively rather than transiently expressed in leukemic cells. As exemplified in EM-2 cells, a low basal PKC activity and its modest tyrosine kinase-dependent inhibition may be insufficient in some human leukemia cells to translate into a marked stimulation of Ca^{2+} transients.

The gradual loss of BIM sensitivity for thrombin-induced Ca^{2+} transients and the marked stimulation of PKCα in imatinib-treated TonB cells indicated that establishing strong coupling between PKCα and tyrosine kinase required some cellular adaptation. Such time-dependent cellular adaptations of signaling pathways in response to permanently elevated Bcr/Abl activity have been observed previously [26]. Conceivably, a similar adaptive process may be responsible for the partial loss of PKC–tyrosine kinase feedback coupling in the course of leukemic cell development. Hence, treatment with imatinib will not necessarily revert the cellular Ca^{2+} signaling network of Bcr/Abl expressing leukemic cells into the one of normal hematopoietic progenitors. Although, in TonB cells, the response to CXCL12 could be re-established in the presence of imatinib, CXCL12 sensitivity was again lost after the cells developed imatinib resistance (Vichalkovski et al., Unpublished observation).

Our results in TonB cells suggest a novel and somewhat surprising mechanism as possible basis for the interaction of PKC with Abl- and Src-type kinases in TonB cells. Co-immunoprecipitation experiments imply that the stimulation of PKCα is paralleled by its association with Bcr/Abl, irrespective of whether the enzyme is fully inhibited (as with imatinib) or not (as with PP2, compare Fig. 3). Hence, PKC activation may not obligatorily require tyrosine kinase activity. This finding has an analogy in the Bcr/Abl-mediated activation of p38 MAPK that seems to occur via Abl-dependent and Abl-independent, Bcr-requiring, mechanisms [26]. Since co-immunoprecipitation was also possible from the solubilized membrane fraction, the complex appeared to contain activated PKCα. However, additional experiments will be needed to definitely establish a causal link between the stimulation of PKCα and its physical interaction with Bcr/Abl. While PKCα dominated the interaction with tyrosine kinases in murine cells, it was PKCβ in human erythroid progenitors. This may represent a cell type and/or species-specific difference because the preference for PKCα was also lost in the human EM-2 leukemic cell line.

Few studies have assessed explicitly an interference with PKC, with or without tyrosine kinase activation, if cellular Ca^{2+} transients were observed to change in the course of pharmacological or pathophysiological interventions [27,28]. However, it is clear, also from our results in EM-2 cells, that tyrosine kinases may effect GPCR agonist-induced Ca^{2+} signals by mechanisms that do not involve PKC. Recent evidence suggests that a heterogeneous population of membrane Ca^{2+} channels may contribute to generate SOCE [29–33]. These different entities are subject to individual regulatory mechanisms and tissue-specific expression patterns [34]. This heterogeneity may explain the large quantitative differ-

ences in SOC fluxes among different hematopoietic cell lines and the poor correlation between the amount of intracellular Ca^{2+} release and the size of SOCE. It has not been worked out in detail which of these channels may be targets of PKC- and/or tyrosine kinase-dependent phosphorylation and regulation [35].

Earlier observations suggest at least three ways by which leukemic cells may 'profit' from generating strong cellular Ca^{2+} responses upon stimulation with endogenous GPCR agonists. By modulating Ras GTPase activity or by stimulating the transcription factor NFAT, calcium may promote cell growth [36–38]. Also via NFAT activation or by stimulating the degradation of the apoptotic enzyme calpain, calcium may inhibit apoptosis [1,39]. Finally, CXCL12-mediated chemotaxis of hematopoietic progenitor cells seems to rely on cellular Ca^{2+} transients [3]. High chemotactic mobility associated with elevated expression of CXCR4, the CXCL12 receptor, may result in a poor prognosis in acute myeloid or lymphoid leukemia [40,41]. Specifically, our observations in TonB cells imply that upon Bcr/Abl expression Ca^{2+} can replace IL-3 as a survival factor. To this end, the cells appear to rely on an increase in Ca^{2+} entry via a Bcr/Abl-mediated upregulation of membrane Ca^{2+} channel open probability or channel density rather than on an unspecific increase in Ca^{2+} permeability. Most likely, the enhanced SOCE transients reflect the same changes in channel properties. Similarly, earlier studies, using more indirect methods, have suggested that survival of leukemic cells may critically depend on high levels of SOCE [42,43]. Under the conditions of high Ca^{2+} influx PKC inhibition by tyrosine kinases, as described in this study, will be required to balance the negative feedback regulation of SOCE by Ca^{2+}-dependent PKC subtypes. Further studies will be necessary to establish whether and to what extent GPCR agonists contribute to promoting cell survival in Bcr/Abl expressing leukemic cells.

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