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Thrombin and Phorbol Esters Potentiate G_s-Mediated cAMP Formation in Intact Human Erythroid Progenitors via Two Synergistic Signaling Pathways Converging on Adenylyl Cyclase Type VII

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ABSTRACT

In intact, but not in permeabilized, human erythroid progenitor cells, thrombin and phorbol esters potentiate cellular cAMP formation in response to G_s-coupled receptor agonists such as prostaglandin E_1 (PGE_1). We show here that the two agonists achieve their phenotypically similar effects by using distinctly different signaling pathways, both of which require protein kinase C (PKC) activation. After short term exposure (11 min), phorbol esters caused an alkaline shift of cellular pH by ~0.1 unit, resulting in a 1.5–2-fold increase in PGE_1-induced cAMP formation. The effect of phorbol esters was inhibited by 5-(*N*-ethyl-*N*-isopropyl)amiloride, a specific inhibitor of the Na^+/H^+ exchanger, and by the PKC inhibitors GF 109203X, Gö 6976, and staurosporine. Thrombin increased cellular pH by only 0.02–0.05 unit but seemed to potentiate PGE_1-stimulated cAMP formation by an effect on the G_s-activated adenylyl cyclase involving a Ca^{2+}-independent (novel) PKC. This effect was inhibited by GF 109203X and staurosporine but was resistant to 5-(*N*-ethyl-*N*-isopropyl)amiloride or Gö 6976. Inactivation of PKC by incubation of the cells in the presence of 10 nM phorbol-12-myristate-13-acetate for 18 hr completely abolished the potentiating effect of thrombin on cyclase activity, whereas the pH-dependent stimulation was fully retained. Northern blots with specific cDNA probes and a lack of Ca^{2+} sensitivity indicate that progenitor cells predominantly express adenylyl cyclase type VII. Our results suggest that in normal human erythroid progenitors, thrombin can activate pH-dependent and -independent, PKC-linked pathways converging on adenylyl cyclase type VII to potentiate cAMP formation in response to G_s-coupled receptor agonists.

Normal human erythroid progenitor cells (burst-forming unit and colony-forming unit stages) express a rich inventory of G protein-coupled receptors and of G proteins from all known subfamilies together with the corresponding effector systems (Porzig *et al.,* 1995a). Little is known about the mutual interactions and the functional significance of these different G protein-linked signaling systems. In previous studies, we observed a specific priming mechanism between G_s-coupled receptor agonists and thrombin that results in a marked potentiation of adenylyl cyclase stimulation by adenosine, PGE_1, or isoprenaline (Porzig *et al*., 1995b). Thrombin receptors may interact with G_i-, G_q-, and $G_{12/13}$-type G proteins (reviewed in Grand *et al*., 1996). However, because the potentiating effect of thrombin was resistant to pertussis toxin treatment and was not shared by any other putatively G_i-coupled receptor ligand in these cells such as PAF or neuropeptide Y, potentiation was attributed to a G_q-linked mechanism. A $G_{\alpha s}$-activated cyclase was tentatively identified as the target of the action of thrombin because thrombin alone or in combination with forskolin was ineffective. A similar interaction had been detected previously in HEL cells, a human erythroleukemia cell line (Brass and Woolkalis, 1992; Turner *et al*., 1992). From this earlier work, it also was clear that cyclase potentiation by thrombin in intact cells was converted into an inhibitory effect in permeabilized cells. The reason for this surprising result and the detailed mechanism and functional significance of the stimulating action of thrombin remain largely unknown. Thrombin acts as a growth factor in some tissues, in particular in vascular smooth muscle cells (reviewed in Grand *et al*., 1996). On the other hand, cAMP has been shown to inhibit megakaryocyte proliferation while enhancing differentiation (Tortora *et al*.,

ABBREVIATIONS: PGE_1, prostaglandin E_1; PKC, protein kinase C; GF-109203X, bisindolylmaleimide I; HEL, human erythroleukemia cell line; PMA, phorbol-12-myristate-13-acetate; CD, cluster of differentiation (surface antigen classification of blood cells); IDMEM, Iscove's modified Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; DMB-forskolin, 7β-deacetyl-7β-[γ-(morpholino)butyryl]-forskolin hydrochloride; FCS, fetal calf serum; PAF, platelet activating factor; AM, acetoxymethyl ester.

1989; Vittet *et al.*, 1995). Therefore, the prominent synergistic activation of the adenylyl cyclase by the two signaling pathways is of potential relevance for hematopoietic cell development. In the current study, we used normal human progenitor cells to analyze in more detail the *in vivo* signaling pathways that are involved in the thrombin-mediated potentiation of cAMP formation. In particular, we have identified a pH-dependent and a pH-independent pathway, which are both linked to PKC and converge on adenylyl cyclase type VII. This cyclase subtype seems to be prominently expressed in erythroid progenitors. In addition, we show that the signaling pathways activated by thrombin and by the PKC-stimulating phorbol ester β-PMA are different despite their phenotypically similar effects on cellular cAMP formation.

Experimental Procedures

Erythroid progenitor cell preparation and culture. In most experiments, fresh human blood obtained from healthy volunteers was used as a source for $CD34^+$ cells. Some additional blood samples were collected after informed consent from a patient treated for primary hemochromatosis with phlebotomy at monthly intervals. Peripheral blood $CD34^+$ cell counts of this patient were ~3-fold higher than those in healthy control subjects. Isolation of mononuclear cells by density gradient centrifugation in Ficoll-Paque and subsequent multistep enrichment of $CD34^+$ cells by a combination of discontinuous isotonic Percoll gradient centrifugation and negative antibody panning procedures followed an established method (Gabbianelli *et al.*, 1990) as described previously (Porzig *et al.*, 1995a). Briefly, nonprogenitor mononuclear cells were removed using monoclonal antibodies to CD2, CD11b, and CD45 as well as a polyclonal anti-human IgG. The resulting partially purified cell population (0.5–1% of the initial mononuclear cell number) was cultivated for 6–8 days at a density of 0.5–1 × 10^6 cells/ml in IDMEM supplemented with 5% lymphocyte-conditioned medium, 20% conditioned medium from an irradiated human bone marrow stroma cell line, 100 μmol/liter hemin, 25 ng/ml human recombinant (hr) interleukin-3, 20 ng/ml hr-granulocyte-macrophage colony-stimulating factor, 5 units/ml hr-erythropoietin, 50 ng/ml hr-stem cell factor, 300 μg/ml human transferrin (iron saturated), and 15% FCS. During a 7-day cultivation period in this medium, erythroid progenitor cell numbers increased ~50-fold while maturating into the CFU-E stage. Most contaminant cells had died by day 5, so erythroid progenitors usually formed 80–95% of the experimental cell suspension. Control experiments with cells maintained in the absence of erythroid growth factors showed that nonerythroid cells did not contribute significantly to the overall cAMP formation in the presence of agonists even in experiments in which the fraction of stimulated progenitors remained below 80%.

cAMP assay. cAMP synthesis was determined in intact cells according to standard methods (Johnson and Salomon, 1991). Briefly, the cells were loaded for 2 hr with [^{3}H]adenine (final concentration, 0.8 μM) to label the cellular adenine nucleotide pool. A stock suspension of cells (0.8–2 × 10^6/ml) was prepared in one of four serum-free standard incubation media [1, IDMEM; 2, KCl medium containing 140 mM KCl, 20 mM HEPES buffer, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 1 mM Na-pyruvate, pH 7.25, at 37°; 3, Na/K medium containing 100 mM KCl, 40 mM NaCl, other components as in 2; 4, NaCl medium (Ca^{2+}-free) containing 140 mM NaCl, 10 mM KCl, all other components (except Ca^{2+}) as in 2]. Experimental incubations (in duplicate; total volume, 200 μl) contained 90 μl of cell suspension, 100 μl of medium supplemented with the phosphodiesterase inhibitor Ro-201724 (final concentration 0.5 mM), and various additions in a total volume of 10–12 μl. The formation of [^{3}H]cAMP from labeled precursors was measured after an 11-min equilibration period followed by an 11-min stimulation period in the presence of various agonists at 37°. Reactions were stopped by adding 200 μl of ice-cold 5% perchloric acid containing 1 mM of nonlabeled carrier cAMP. After neutralization and removal of perchlorate, adenine nucleotides were chromatographically separated. Depending on experimental conditions, stimulation with agonists induced the conversion of 0.2–4% of the cellular ^{3}H-pool into [^{3}H]cAMP. Results are expressed as fold increase in basal cAMP formation (0.05–0.15% conversion).

Cellular Ca^{2+} measurements. Cellular Ca^{2+} transients in response to agonist stimulation were measured with the Fura-2 method (Grynkiewicz *et al.*, 1985; Baltensperger and Porzig, 1997). Precursor cells (10^7/ml) were loaded with membrane-permeable Fura-2 AM (9 μmol/liter) for 45 min at 37° in IDMEM containing 5% FCS. After washing, the cells were suspended in a buffer containing 140 mM NaCl, 10 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgCl_2$, 22 mM glucose, and 5% FBS, pH 7.4. Then, 100-μl aliquots of this cell suspension were diluted into 300 μl of the same medium in a semimicrocuvette and kept at 30° in the thermostat-equipped holder of a Perkin-Elmer Cetus (Norwalk, CT) LS-50B dual-wavelength spectrofluorometer. Emission intensities at 485 nm were determined at excitation wavelengths of 340 and 380 nm using the "fast-Fura" attachment at frequencies of 5–10 Hz. Fluorescence intensities were calibrated by determining maximum and minimum fluorescence ratios after cell lysis with digitonin (12.5 μmol/liter) in the presence of 1 mM $CaCl_2$ with or without 20 mM Tris-buffered EGTA. Free cellular Ca^{2+} was calculated using the WinLab 2.0 software (Perkin-Elmer Cetus) and assuming an apparent K_d value of 224 nM for the Fura-2/Ca^{2+} complex.

Cellular pH measurements. Changes in cellular pH were followed using the fluorescent indicator BCECF essentially as described by Grinstein *et al.* (1989). Cells (10^7/ml) were loaded with 2 μg/ml of the membrane-permeable BCECF AM for 30 min at 37° in IDMEM without serum. After washing, the cells were resuspended in KCl medium. Then, 100 μl of this stock suspension was added to 900 μl of NaCl medium in a well-stirred semimicrocuvette (Hellma, Müllheim, Germany) kept at 35°. pH-dependent fluorescence changes were determined after an equilibration time of ≥5 min at an emission wavelength of 530 nm using excitation wavelengths of 505 and 450 nm as suggested by the manufacturer (Molecular Probes, Eugene, OR). Because of the slow time course of agonist-induced pH changes, each effect was followed for ~15 min. In each experiment, a calibration curve was established after cell lysis with Triton X-100 (0.05%) using Tris-base and HEPES (free acid) to reach predetermined pH values. The calibration curve was linear for pH values ranging from 6.9 to 7.5.

Northern blot analysis. Standard techniques were used for the Northern blot analysis of adenylyl cyclase subtypes expressed in progenitor cells. Polyadenylated [poly(A)$^+$] RNA was prepared from proliferating human erythroid progenitor cells at days 6–8 of suspension culture as well as from HEL cell cultures using the Oligotex Direct mRNA Mini kit (Qiagen, Hilden, Germany). Then, 4 μg of poly(A)$^+$ RNA were electrophoretically separated and blotted onto Qiabrane nylon membranes (Qiagen). Hybridization followed an established protocol (Brown and Mackey, 1997) using the following adenylyl cyclase cDNA probes: rat type II (provided by Dr. W.-J. Tang, University of Chicago), rat and rabbit type V (provided by Dr. R. Iyengar, Mount Sinai School of Medicine, New York, and Dr. T. Pfeuffer, University of Düsseldorf, Germany, respectively), and human type VII (provided by Dr. B. Tabakoff, University of Colorado, Denver, CO). For DNA probe preparation, plasmids were extracted with the Wizard Plus SV miniprep purification system (Promega, Madison, WI), digested, electrophoretically separated, and isolated from the gel using the Qiaquick gel extraction kit (Qiagen). Random labeling of double-stranded DNA-fragments followed the protocol of the Prime-a-Gene labeling system (Promega). A fragment of the housekeeping gene *GAPDH* was used as a control. Specific hybridization was detected after a 5-hr exposure to storage phosphor screen (Kodak) with a Storm 840 PhosphorImager (Molecular Dynamics,

Sunnyvale, CA) and/or autoradiography at −80° for 48 hr on Kodak X-O-MAT film with intensifier screen.

Data analysis. The statistical significance of differences between mean values was assessed using Student's t test. $p < 0.05$ was considered significant. Data were analyzed and fitted, where applicable, with a nonlinear least-squares fitting routine using the Prism 2.04 and InStat2 programs (GraphPAD Software, San Diego, CA).

Materials. Analytical grade reagents were purchased from Merck ABS (Dietikon, Switzerland) or Fluka (Buchs, Switzerland). Materials for adenyl nucleotide column chromatography, human and bovine thrombin, PGE_1, hemin, phorbol esters, and polyclonal anti-mouse and anti-human IgG were from Sigma (Buchs, Switzerland). Tissue culture reagents and media were obtained from Gibco/Life Technologies (Basel, Switzerland) or from Sigma. Density gradient media (Percoll, Ficoll-Paque) were from Pharmacia (Dübendorf, Switzerland). Fluorescent indicators Fura-2 AM and BCECF AM were purchased from Molecular Probes. EIPA was a gift of Hoechst Pharma AG (Dr. H. Lang, Frankfurt-Hoechst, Germany). GF-109203X, Gö 6976 [12-(2-cyanoethyl)-6,7,12,13-terahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole], and staurosporine were from LC Laboratories/Alexis (Läufelfingen, Switzerland) Water-soluble DMB-forskolin was from Research Biochemicals (Natick, MA). SFLLRN peptide was from Bachem (Bubendorf, Switzerland). Erythropoietin, stem cell factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3 were generous gifts of Cilag (Schaffhausen, Switzerland), Immunex (Seattle, WA) Werthenstein Chemie (Schachen, Switzerland), and Sandoz AG (Basel, Switzerland), respectively. Monoclonal antibodies used for cell panning were purified by G protein affinity chromatography from culture supernatants of the respective hybridomas (American Type Culture Collection, Rockville, MD: 8027-CRL for anti-CD2, 8026-CRL for anti-CD11b, 10508-HB for anti-CD45).

Results

PKC dependence of thrombin- and PMA-enhanced cAMP formation. Preceding studies in our laboratory (Porzig *et al*., 1995b) had provided evidence that the potentiating effect of thrombin on cAMP formation in human progenitors was linked to the activation of one or several PKC isoforms. Part of this evidence was based on the observation of a similar, although less potent, stimulatory effect of the phorbol ester βPMA. However, a number of important questions concerning the mechanism of this interaction between G_s- and G_q- (or $G_{12/13}$)-coupled receptor agonists have remained unresolved. What are the functional characteristics of PKC isotypes involved in the reaction, and what is the target of the supposed PKC-dependent phosphorylation? Why does the potency of thrombin to stimulate cAMP formation exceed significantly the one of PMA? Does the analogy between the effects of PMA and of thrombin indeed reflect the activation of identical signaling pathways, or is the same target enzyme stimulated by two separate mechanisms? These questions have been addressed in the current study.

The overall contribution of PKC stimulation to the effect of thrombin in intact progenitor cells was first assessed using a panel of inhibitors, differing in selectivity and/or inhibitory mechanism. The results are summarized in Fig. 1. The stimulation of PGE_1-induced cAMP formation by maximum effective concentrations of PMA or thrombin in the absence of inhibitors is shown in Fig. 1A. Thrombin (2 units/ml) enhanced the PGE_1 effect by a factor of 3 from 10.1 ± 1.1- to 28.9 ± 4.1-fold stimulation of basal cAMP formation. PMA (10 nM) was significantly less effective ($p < 0.005$, paired t test). PMA concentrations up to 100 nM did not induce any additional effect. Control experiments showed that in the absence of a G_s-activating ligand, PMA and thrombin stimulated basal cAMP formation by a factor of <2. Moreover, the effects of thrombin could be qualitatively mimicked by the thrombin receptor peptide SFLLRN, an analog of the tethered ligand receptor agonist (data not shown). Therefore, receptor-independent effects of thrombin probably are not involved in its cyclase potentiating action. Three chemically related PKC inhibitors were tested (Fig. 1B) that are all known to interact with the ATP binding site of protein kinases while showing different subtype specificity. At maximum effective concentrations (20 nM), the nonspecific protein kinase inhibitor staurosporine (Meyer *et al*., 1989) inhibited the thrombin- and PMA-dependent increments of PGE_1-dependent cyclase activation by 68% and 84%, respectively, whereas the PGE_1-stimulated activity was not affected. GF-109203X, an inhibitor of most PKC subtypes (Toullec *et al*., 1991), but not of protein kinases from other families, reduced the effects of thrombin and PMA by 33% and 100%, respectively. Finally, the indolocarbazole Gö 6976 (Martiny-Baron *et al*., 1993), a putative specific blocker of Ca^{2+}-dependent PKC isotypes, was without any effect on the thrombin-mediated cAMP increase but caused a ~50% inhibition of the PMA response. The results with Gö 6976 were unexpected because in most responsive cells, including HEL cells, thrombin provokes a transient increase in cellular calcium levels ($[Ca^{2+}]_i$) (Baltensperger and Porzig, 1997), which is believed to mediate the activation of PKC. Therefore, we used the Fura-2 technique to quantify the thrombin-associated $[Ca^{2+}]_i$ response in progenitor cells. Contrary to our expectations, thrombin did not cause any significant change in erythroid progenitor cell $[Ca^{2+}]_i$ levels (Fig. 2). This was not due to a lack of releasable intracellular calcium because other G protein-coupled receptors agonists, such as ADP or PAF, induced typical transient increases in $[Ca^{2+}]_i$.

The differential efficacy of PKC inhibitors to block the effects of thrombin and of PMA suggest that the two com-

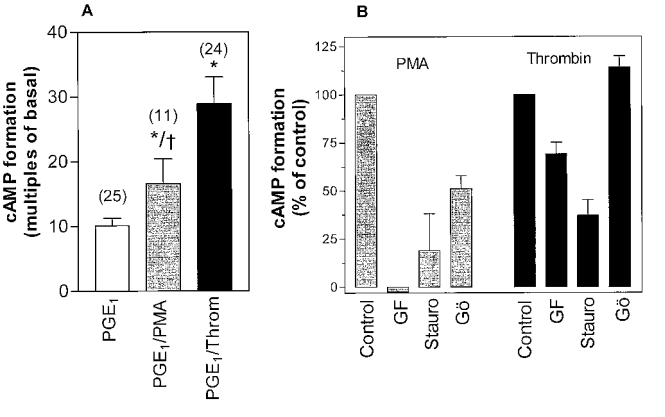

Fig. 1. cAMP formation in intact human erythroid progenitor cells in response to different stimuli (A) and the inhibitory effect of different PKC inhibitors (B). A, cAMP formation in the presence of agonists in multiples of nonstimulated (basal) cAMP formation (mean ± standard error). The combined effect of PGE_1/PMA is significantly different (*, paired t test) from both the effects of PGE_1 alone and PGE_1 + thrombin (*Throm*) (†). Brackets, number of experiments. B, *PMA*, effect of PKC inhibitors on the PMA-dependent fraction of the PMA/PGE_1-stimulated cAMP formation. *Thrombin*, effect of inhibitors on the thrombin-dependent fraction of PGE_1/thrombin-stimulated cAMP formation. The data are normalized with respect to the effect in the absence of inhibitors (*control*). Values are mean ± standard error from 3–12 independent experiments. *GF*, GF 109203X, *Gö*, Gö 6976, *Stauro*, Staurosporine.

pounds do not activate identical signaling pathways. In particular, $[Ca^{2+}]_i$ -independent mechanisms, such as Ca^{2+}-insensitive PKC subtypes, are likely to play a major role in thrombin-mediated cyclase activation. In addition to cellular Ca^{2+} release and PKC activation, primary effects of thrombin in sensitive cells usually include cytosolic alkalinization due to a stimulation of the ubiquitous membrane Na^+/H^+ exchanger (Grand *et al*., 1996; Wakabayashi *et al*., 1997). The same transport protein is a target for PKC-dependent activation. However, thrombin also may initiate a complementary, PKC-independent pathway that leads to exchanger activation via a G protein of the G_{12} family (Dhanasekaran *et al*., 1994).

Role of cellular pH in modulating cyclase activity. In subsequent experiments, we analyzed in more detail the contribution of an increase in cellular pH to the overall cyclase activation by thrombin and by PMA. Fig. 3 summarizes experiments in which we explored the involvement of the exchanger by measuring the extent to which the stimulatory effects of thrombin and PMA could be reduced with the specific inhibitor EIPA (L'Allemain *et al*., 1984). Concentrations of EIPA ranging from 3 to 30 μM were applied because higher levels caused an nonspecific general inhibition of cAMP synthesis. Moreover, all experiments were performed at reduced (40 mM) sodium concentrations (Na^+/K^+ medium; see Experimental Procedures) to diminish competitive interactions between Na^+ and EIPA. The results show that in short term experiments (Fig. 3, 11-min pretreatment with PMA), only the PMA-mediated potentiation was significantly inhibited in the presence of EIPA. Thrombin-induced potentiation was not significantly reduced. With 10 and 30 μM EIPA, the effect of PMA decreased by 58% and 78%, respectively. Thus, in short term experiments, the Na^+/H^+ exchanger seems to represent the main target of cellular PMA action, whereas it is involved to a lesser degree, if at all, in the effect of thrombin.

pH sensitivity of cAMP formation. The results with EIPA lead to the following predictions: 1) cAMP formation in human progenitor cells is strongly pH sensitive and 2) within the limits of physiological pH values, PKC stimulation causes a cellular pH shift that is sufficiently large to explain a major stimulation of cAMP. A stimulatory effect of increased cellular pH levels on cAMP formation has been observed previously in other cell types (Ho *et al*., 1992) but not in normal hematopoietic cells. The current experiments were designed to test these predictions in erythroid progenitors.

Defined cellular pH values were established by suspending progenitor cells in isotonic KCl medium in the presence of the K^+/H^+ ionophore nigericin (10 μM) to allow equilibration of intracellular and extracellular pH (Grinstein *et al*., 1989). Medium pH and consequently, cellular pH, were adjusted to values ranging between 6.9 and 7.8 at 37°. The effect of a stepwise change in pH from 6.9 to 7.8 on cAMP formation in the absence of G protein-coupled receptor agonists was first tested with the cyclase activator DMB-forskolin (Fig. 4). At a concentration (25 μM) slightly below its K_A value, cAMP synthesis increased almost linearly with increasing pH. Within the range of physiological cellular pH values (6.9–7.3) the relative change was ~1.5-fold, and a pH shift of ≥0.3 unit was required to reach a significant stimulation. However, the relationship between cAMP formation and pH became distinctly sigmoidal whenever a G_s-coupled receptor agonist was present. The steepest rise occurred usually between 7.0 and 7.3. Fig. 4 shows the relation between cellular pH and cAMP formation in the joint presence of PGE_1 and thrombin. Under these conditions, an alkaline shift of 0.1 unit resulted in an almost 2-fold increase in the rate of cAMP synthesis. Numerical values for pH-dependent activation of cAMP synthesis in the presence of different agonists are summarized in Table 1. It can also be inferred from Table 1 that unlike the absolute values, the relative potentiating effect of thrombin or of PMA (i.e., the percentage increase in PGE_1-dependent cAMP formation due to thrombin or PMA) remained constant (at 46.0 ± 2.0% and 148.2 ± 4.3%, respectively) with different pH values. The persistent effect of PMA at high pH values indicates that its overall stimulatory effect on cyclase activity cannot result entirely from the cellular pH shift but must include, in addition, a component that is independent of the intracellular pH. Basal cAMP formation in the absence of

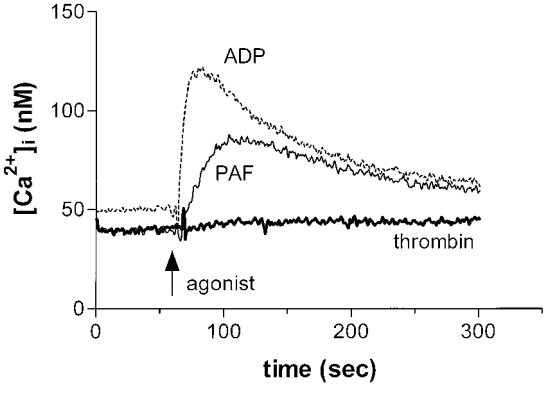

Fig. 2. Effect of G protein-coupled receptor agonists on cellular Ca^{2+} release in human erythroid progenitors. Ca^{2+} increase induced by ADP, PAF, and thrombin was measured with the Fura-2 technique. Note the near lack of a Ca^{2+} response after application of thrombin. See Experimental Procedures for a detailed description of the measuring technique. Results are from one of three independent experiments.

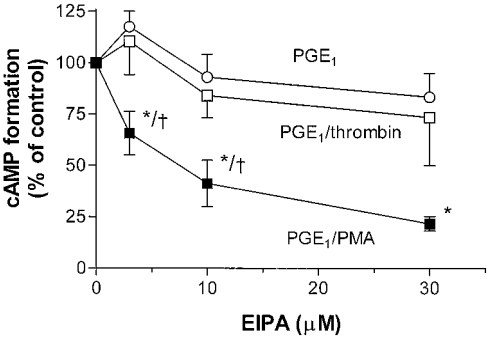

Fig. 3. Effect of EIPA, a specific inhibitor of the Na^+/H^+ exchanger, on PMA- and thrombin-dependent potentiation of PGE_1-stimulated cAMP formation. *PGE_1*, PGE_1 (10 μM) was added after an 11-min equilibration period with EIPA at 37°. cAMP formation was measured at the end of an 11-min incubation period with PGE_1. *PGE_1/thrombin,* thrombin-dependent fraction of total cAMP formation under these conditions (same protocol as above, except that PGE_1 and thrombin were added simultaneously at the end of the equilibration period with EIPA). *PGE_1/PMA*: PMA-dependent fraction of total cAMP formation [same protocol as above except that the cells were exposed to EIPA and PMA (10 nM) during the equilibration period]. cAMP values are normalized with respect to controls incubated in the absence of EIPA. *Data points,* mean ± standard error of three (PGE_1), four (PGE_1/thrombin), and five (PGE_1/PMA) independent experiments; *, significant difference from the corresponding value for PGE_1. †, Significant difference from the value for PGE_1/thrombin. EIPA had no effect on basal cAMP formation.

agonists did not show any systematic changes in the 6.9–7.7 pH range.

Magnitude of agonist-induced pH change. The shift in intracellular pH induced by thrombin or PMA was assessed in progenitor cells loaded with the fluorescent pH indicator BCECF and incubated in HEPES-buffered NaCl medium. The results of a representative experiment are shown in Fig. 5. Thrombin and PMA both induced a slow, sustained alkaline shift of cellular pH. However, the effect of PMA occurred faster and was significantly more pronounced with a ~ 0.1-unit increase during a 15-min treatment period. With thrombin, we observed an increase of 0.02–0.05 pH units. Simultaneous application of PGE_1 did not increase the response to thrombin (not shown). In control experiments with agonists that caused a transient increase in cellular Ca^{2+} (ADP, NPY, UTP), pH shifts were generally smaller than those with thrombin. All pH shifts were completely suppressed if the NaCl medium was replaced by an isotonic KCl medium to eliminate Na^+/H^+ exchange or in the presence of 10 μM EIPA (not shown). These results are consistent with the prominent inhibitory effect of EIPA on PMA-induced cyclase stimulation that was described above (Fig. 3).

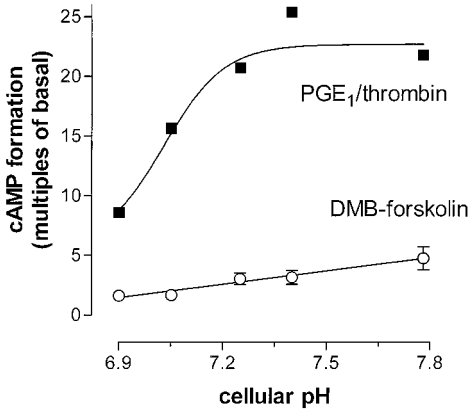

Fig. 4. pH-dependence of agonist-stimulated cAMP formation in erythroid progenitor cells. The cells were equilibrated for 11 min in KCl medium adjusted to different pH values and containing 10 μM of the H^+/K^+ ionophore nigericin. cAMP formation was measured at the end of an 11-min stimulation period. Cells were stimulated with DMB-forskolin (25 μM) or with PGE_1/thrombin (10 μM/2 units/ml). Data were fitted by linear regression (forskolin) or by nonlinear least-squares fitting (PGE_1/thrombin). *Data points* for forskolin are mean ± standard error values of five experiments; *data points* for PGE_1/thrombin represent mean values of two experiments (five additional experiments were performed at only three different pH values and are included in the respective mean values given in Table 1). Compare with Table 1 for mean ± standard error values of supplementary experiments.

Relation between PKC- and pH-dependent stimulation of cAMP formation. In principle, synergistic stimulation of cAMP formation by alkaline pH and thrombin-mediated PKC activation could result from pH sensitivity of either the PKC or the cyclase enzymatic activities (or both). To assess the contribution of PKC to the overall pH-induced stimulation, we studied the effect of pH changes in progenitor cells pretreated for 18 hr with 10 nM PMA to fully downregulate PKC activity. In the experiments shown in Fig. 6, we compared the pH dependency of PGE_1-activated cAMP formation in the presence or absence of thrombin after pretreatment of progenitor cells for 18 hr with 10 nM PMA. Under these conditions, thrombin-dependent potentiation of PGE_1-dependent cAMP synthesis was completely eliminated, whereas the stimulatory effects of alkaline pH values and of PGE_1 were both retained. We conclude from these results that pH-dependent activation of cAMP synthesis is primarily an intrinsic property of the adenylyl cyclase rather than being mediated by pH-dependent PKC activity.

Possible targets of the thrombin-activated protein kinase activity. The experiments described provide evidence that the potentiating effect of thrombin on cAMP formation is largely mediated by a protein kinase targeting a component of the G_s-linked signaling pathway. Moreover, the absolute requirement for the simultaneous presence of a G_s-coupled receptor ligand suggests that coactivation of a component of the cAMP pathway is a prerequisite for the action of thrombin. This conclusion was confirmed by experiments shown in Fig. 7A. Progenitor cells were pretreated for 5 min with thrombin before the addition of PGE_1. The resulting cAMP response was normalized to the value obtained when thrombin and PGE1 were added simultaneously. After preconditioning with thrombin, PGE_1 yielded a cAMP response that was significantly lower than the response reached on simultaneous application of the two ligands (Fig. 7A, ■ and ▨, respectively). Hence, thrombin-induced PKC activation before stimulation of the G_s-coupled receptor failed to effectively condition the system. Targets for a thrombin-triggered phosphorylation within the G_s-dependent signaling chain might, in principle, be located at the level of the receptor, the adenylyl cyclase, or some intermediary regulatory protein. An effect on the level of the receptor seems unlikely because the rate of receptor desensitization, which is known to be sensitive to receptor phosphorylation, was similar in the presence or absence of thrombin. As shown in Fig. 7B, cellular cAMP increased at an almost constant rate for 5–10 min after stimulation with PGE_1,. Thereafter, cAMP formation ceased resulting in a plateau level after 20–30 min.

TABLE 1

pH dependency of agonist-stimulated adenylyl cyclase activity in human erythroid progenitor cells

Progenitor cells were equilibrated with the indicated pH values in the presence of 10 μM nigericin at 36° in KCl medium. Stimulation was for 11 min with the listed agonists. The numbers give fold stimulation over basal. Basal cAMP formation was similar at all pH values tested. Note that the relative amplification of the PGE_1-induced cAMP signal by thrombin or PMA remained constant within the pH range tested.

Agonist	$[pH]_i$		
	6.9	7.26	7.7
	cAMP formation in multiples of basal activity		
PGE_1(10 μM)	3.01 ± 0.37 (9)	5.66 ± 1.10 (10)	7.44 ± 1.56 (12)
PGE_1/thrombin (10 μM/2 units/ml)	7.36 ± 0.93 (11)	14.53 ± 2.14 (11)	18.10 ± 3.39 (10)
PGE_1/PMA(10 μM/10 nM)	4.45 ± 0.71 (6)	8.04 ± 1.23 (6)	11.03 ± 2.21 (6)
Forskolin(25 μM)	1.64 ± 0.16 (6)	2.95 ± 0.38 (6)	4.69 ± 0.78 (6)

Values are mean ± standard error.

The same time course was observed in the presence of thrombin (even though a 2–3-fold higher absolute cAMP level was usually reached under this condition). Similar results were obtained when PGE_1 was replaced by adenosine (not shown). These observations suggest that thrombin- and G_s-linked signaling are integrated at a more downstream element of the cascade, possibly via a protein kinase-dependent phosphorylation of the adenylyl cyclase.

Screening of adenylyl cyclase subtypes in human progenitors. Direct activation of the adenylyl cyclase catalytic activity is well established for the type II and type V cyclase isoforms (Jacobowitz and Iyengar, 1994; Kawabe *et al*., 1994; Zimmermann and Taussig, 1996) and may also apply for type VII (Watson *et al*., 1994; Hellevuo *et al*., 1995). Therefore, we tested which of the PKC-sensitive cyclase subtypes were expressed in human erythroid progenitor cells. Using subtype-specific cDNA probes, we performed Northern blotting with poly(A)$^+$ RNA isolated from proliferating normal human progenitor cells, as well as from HEL cells, a human erythroleukemia cell line. As shown in Fig. 8, only the type VII probe showed prominent hybridization with progenitor or HEL cell RNA, suggesting that this subtype is dominantly expressed in human erythroid cells. Although the type II and V probes reacted with rat brain poly(A)$^+$ RNA, no hybridization occurred with RNA from either progenitor or HEL cells. Control experiments using commercially available human multiple tissue RNA blots (Clontech, Palo Alto, CA) confirmed that the cDNA probes used in these experiments also hybridized strongly with poly(A)$^+$ RNA of the appropriate size in human brain (types II and V), skeletal muscle (type II), and cardiac muscle (type V). Other control experiments showed that proliferating, phytohemagglutinin-treated lymphocytes from human peripheral blood also expressed low levels of type VII cyclase mRNA. In addition, we measured the sensitivity of the PGE_1/thrombin-stimulated cAMP formation to changes in $[Ca^{2+}]_i$ using the Ca^{2+} ionophore ionomycin. In the presence of ionomycin (5 μM), extracellular Ca^{2+} (20–50 μM) caused a modest increase in PGE_1/thrombin-stimulated cAMP synthesis by 37.9 ± 7.8%. This increase was no longer significant in presence of the PKC inhibitor GF-109203X. We concluded from these observations that the effect of Ca^{2+} was most probably due to cyclase activation via a Ca^{2+}-sensitive isoform of PKC, whereas the enzyme subtype that is stimulated by PGE_1/thrombin seems to be Ca^{2+} insensitive. Consequently, cyclase types I, III, V, VI, and VIII, which are either activated or inhibited by micromolar Ca^{2+} concentrations (Cooper *et al*., 1995), could be excluded.

On the basis of the Ca^{2+} insensitivity reported above and the results with Northern blots, we conclude that a type VII cyclase is the most likely target for a thrombin-mediated phosphorylation that may be catalyzed by a PKC isoform of the Ca^{2+}-independent novel family.

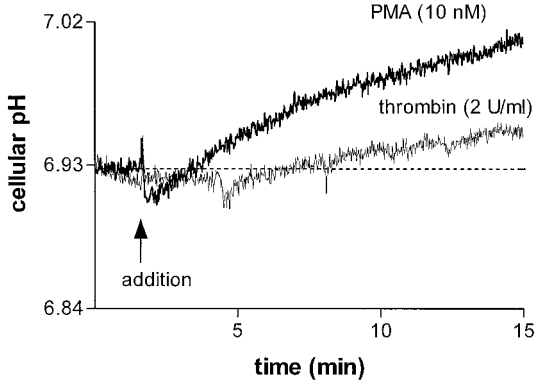

Fig. 5. Effects of PMA and of thrombin on cellular pH values. The cells were suspended in thermostated, stirred NaCl medium at 35° and supplemented with 14 mM KCl. PMA or thrombin was added after an equilibration period of ~5 min. The response was followed for 15 min. Data are shown from one of three independent experiments with similar results.

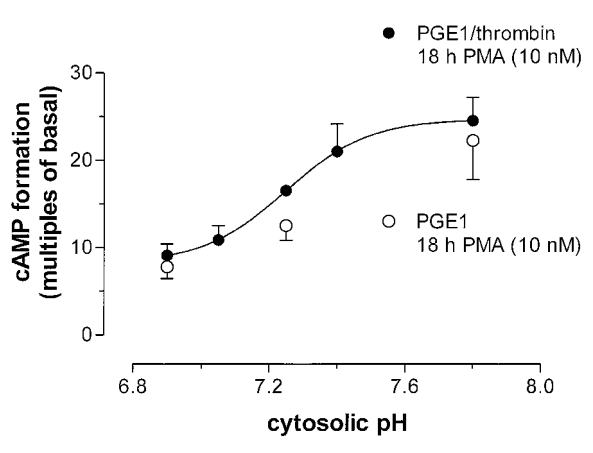

Fig. 6. pH dependency of PGE_1 (10 μM)-stimulated cellular cAMP formation in progenitor cells that were pretreated with 10 nM PMA for 18 hr in IMDEM containing 10% FCS. Experimental protocol as described in the legend for Fig. 4. Long term pretreatment with 10 nM PMA did not abolish pH dependency but resulted in the loss of thrombin (2 units/ml)-induced potentiation of the PGE_1 signal (compare *curve* with ● with the values obtained with PGE_1 alone, ○). Results are from three independent experiments. *Bars*, standard error.

Discussion

Regulation of adenylyl cyclase activity by different synergistic or antagonistic G protein-linked signaling pathways has been increasingly appreciated (Lustig *et al*., 1993; Tsu and Wong, 1996; Zimmermann and Taussig, 1996). However, few other studies have looked systematically at such cross-talk mechanisms in intact, nontransformed cells without disrupting spatially organized interactions and stoichiometries.

Our study in intact, primary human progenitor cells compares the PKC-mediated potentiation of adenylyl cyclase activity induced by a G protein-coupled receptor agonist (thrombin) with the stimulation of cAMP formation via receptor-independent direct activation of PKC with PMA. The latter phenomenon is well known and has usually been explained by PKC-mediated cyclase phosphorylation (Turner *et al*., 1992; Jacobowitz and Iyengar, 1994; Kawabe *et al*., 1994; Watson *et al*., 1994). The elements of the thrombin and PMA signaling cascades in our system are tentatively summarized in Fig. 9. The G protein subtype or subtypes coupling to the thrombin receptor in human erythroid progenitors have not been identified directly. Judging from results in other cell types (Grand *et al*., 1996) and from the fact that thrombin responses are resistant to pertussis toxin (Porzig *et al*., 1995b), $G_{\alpha q}$ and $G_{\alpha 12/13}$ are the most likely candidates. All of these proteins are well expressed in erythroid progenitors (Haslauer M and Porzig H, unpublished observations). Because activation of $G_{\alpha q}$ is usually linked to cellular Ca^{2+} release, which, under our conditions, is essentially absent with thrombin, we favor an involvement of $G_{\alpha 12/13}$. This

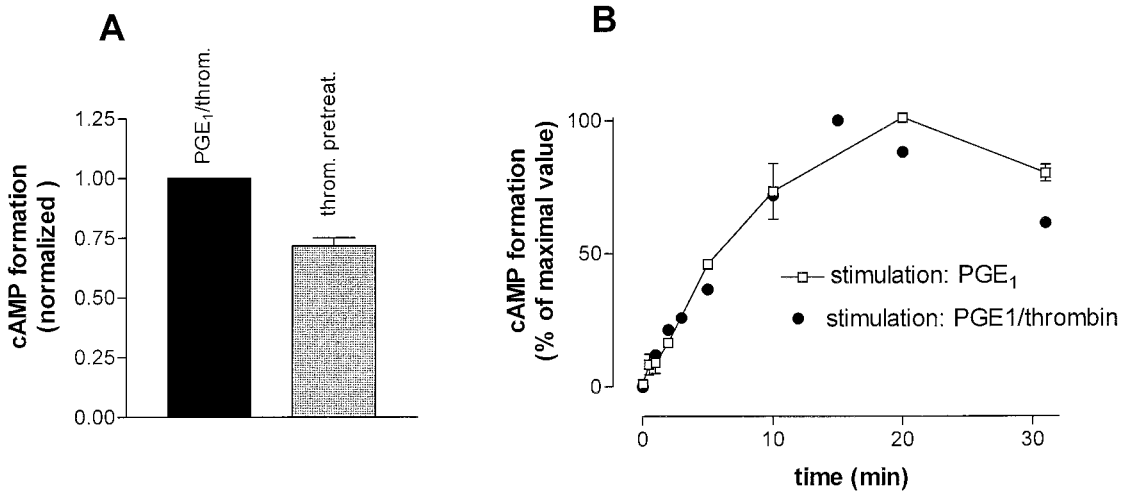

Fig. 7. A, Effect of conditioning by thrombin on PGE_1-induced cAMP formation in erythroid progenitors. Cells were pretreated for 5 min with thrombin (2 units/ml) before the addition of PGE_1 (10 μM). cAMP responses measured 11 min after the addition of PGE_1 were normalized to the response observed after simultaneous application of PGE1 and thrombin (■). Significantly less cAMP was formed after pretreatment with thrombin *(throm.*, ▨). Data show mean ± standard error values from three independent experiments. B, Time course of PGE_1-stimulated cAMP formation in progenitor cells in the absence or presence of thrombin. Data are normalized with respect to the maximal cAMP level reached under each condition. Note that the time courses are very similar (although the absolute maximal values differed). Data are from three experiments with PGE_1 and one experiment with PGE_1/thrombin. *Error bars*, standard error except where smaller than symbol.

assumption would also be consistent with our previous observation (Porzig, 1995b) that several other G_q-coupled receptor agonists (ADP, PAF, NPY), all of which induce a transient Ca^{2+} signal, could not mimic the cyclase potentiating effect of thrombin.

Differences between thrombin and PMA signaling. Several observations in the current study suggest that thrombin and PMA use distinct, although synergistic, pathways to potentiate cyclase stimulation by G_s-coupled receptor agonists: Thrombin has a significantly higher efficacy than PMA (Fig. 1). During short time exposures, PMA potentiates PGE_1-stimulated cyclase activity mainly indirectly via a Na^+/H^+ exchanger-mediated alkaline shift of cellular pH (Fig. 3), whereas thrombin seems to induce an activation of the enzyme by a different pathway. Nevertheless, other findings confirm a central role of PKC in the potentiating effects of both thrombin and PMA: The effect was completely eliminated after down-regulation of PKC by prolonged incubation with phorbol esters (Fig. 6), and it was partially (thrombin) or fully (PMA) inhibited by specific PKC inhibitors (Fig. 1). Pathways linking thrombin receptor activation to PKC stimulation via $G_{\alpha q}$ or $G_{\alpha 12}$ are well established (Dhanasekaran and Dermott, 1996).

Some adenylyl cyclase subtypes and the Na^+/H^+ exchangers have been identified as targets for PKC-dependent phosphorylation (reviewed in Sunahara *et al*., 1996, and Wakabayashi *et al*., 1997). In view of their differential activation by PMA and thrombin, one has to assume that a different pattern of activated PKC subtypes dominates the effects of these two agonists in intact cells. The expression of enzymes from all three families (conventional, novel, and atypical; for a review, see Nishizuka, 1995) in progenitor cells can be inferred from studies in human megakaryocytic or erythroleukemic cell lines (Ballen *et al*., 1996; Zauli *et al*., 1996). PMA is known to activate both conventional (Ca^{2+}-sensitive) and novel (Ca^{2+}-insensitive) subtypes (cPKC and nPKC, respectively), whereas thrombin in our system seems to stimulate predominantly Ca^{2+}-insensitive subtypes. In line with this conclusion, the nonspecific PKC inhibitor GF-109203X partially blocked cyclase activation by thrombin, whereas Gö 6976, a specific inhibitor of Ca^{2+}-sensitive PKC isotypes, was ineffective.

Phosphorylation-dependent activation of adenylyl cyclases. Up to now, it has not been clear which PKC subtypes are indeed able to phosphorylate the various cyclase isoforms (or the Na^+/H^+ exchanger) and whether direct phosphorylation can account for all PKC-induced changes in cyclase activity. Although stimulation by phorbol esters has been observed with most cyclase subtypes, direct phosphorylation has been established only for types II and V (Jacobowitz and Iyengar, 1994; Kawabe *et al*., 1994; Zimmermann and Taussig, 1996). A phenotypically similar coactivation by G_s-coupled receptor agonists and PKC-stimulating phorbol esters has been observed after transfection of human embryonic kidney 293 cells with type VII cyclase (Watson *et al*., 1994; Hellevuo *et al*., 1995). Using purified enzymes, it has been shown that phosphorylation by PKC type α will activate

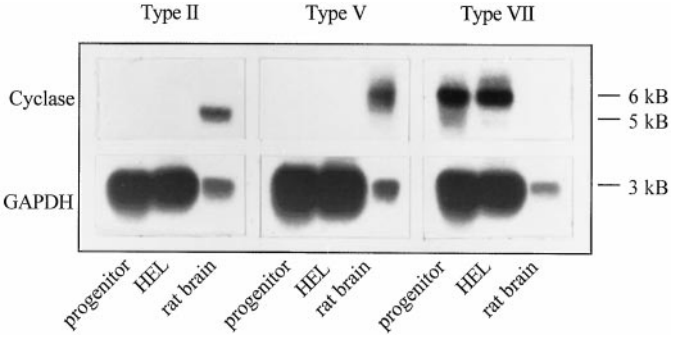

Fig. 8. Northern blots of human erythroid progenitor, HEL cell, and rat brain poly(A)$^+$ RNA hybridized with specific cDNA probes of different adenylyl cyclase subtypes (II, V, VII as indicated at the *top* of each group). Probes for types II and V were of rat origin, and type VII was of human origin. Rat probes hybridized with human brain mRNA at the appropriate position in commercially available human multiple tissue RNA blots (not shown). A probe for *GAPDH* was used as a mRNA control.

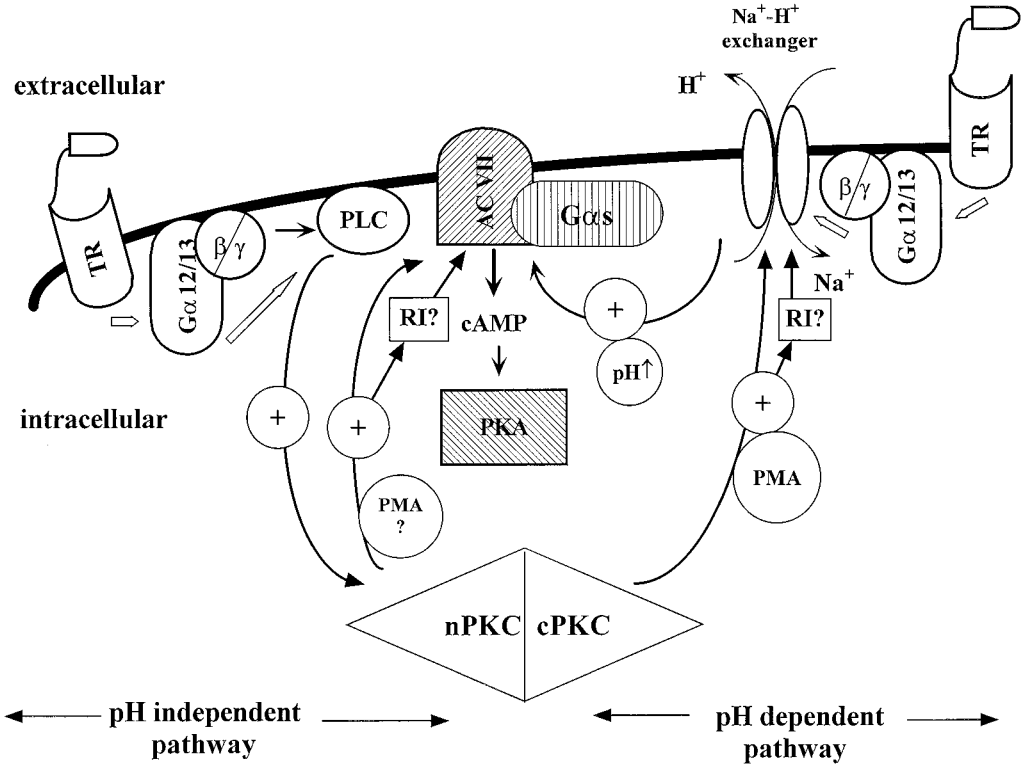

Fig. 9. Thrombin receptor (*TR*)-linked synergistic signaling pathways converging on $G_{\alpha s}$-activated adenylyl cyclase type VII (*AC VII*) in human erythroid progenitor cells. A pH-independent pathway is diagrammed (*left*). Receptor-mediated activation of a trimeric G protein ($G_{12/13}$ *family*) results in the stimulation of a Ca^{2+}-independent nPKC-subtype. cAMP formation is enhanced by phosphorylation of the cyclase or a hypothetical regulatory intermediate (*RI*). Activation of part of the same pathway by PMA may account for the pH-independent fraction of its effect. Stimulation of the $G_{\alpha s}$-activated cyclase via an increase of cellular pH is depicted (*right*). Cellular alkalinization is achieved either by a direct stimulation of the Na^+-H^+ exchanger via a G_{12}-type G protein or by a PMA-sensitive, PKC (cPKC or nPKC subtypes)-mediated pathway, possibly via a regulatory intermediate. *PLC*, phospholipase C; *PKA*, protein kinase A.

the type II and V enzymes (Kawabe *et al.,* 1994; Zimmermann and Taussig, 1996). The functional properties of type II and V cyclases that are activated by direct phosphorylation differ from our observations in progenitor cells in at least two important aspects. (1) The phosphorylated enzymes are synergistically stimulated by forskolin, whereas in progenitor cells, thrombin does not enhance forskolin-activated cyclase. (2) PMA has a significant cyclase stimulating effect on its own, whereas in progenitors, PMA is ineffective in the absence of G_s-coupled receptor agonists (Porzig *et al*., 1995a). A similar lack of synergism with forskolin and conditional activation of cAMP formation by phorbol esters also has been observed in a neuronal cell line (HT4) expressing type I and VI cyclases (Morimoto and Koshland, 1994), but a direct phosphorylation of these two cyclase subtypes has not been demonstrated. Our results (Fig. 7A) show that thrombin treatment of the cells before application of the G_s-coupled receptor agonist results in a potentiation of cAMP formation that is significantly lower than the response observed with simultaneous application of the two ligands. These observations favor the assumption that the $G_{\alpha s}$-activated, rather than the inactive enzyme, is the preferred target for the thrombin-stimulated kinase, although phosphorylation of the activated G protein α subunit (rather than the cyclase) cannot be excluded. We do not know whether the thrombin-activated PKC subtype phosphorylates its target directly or acts via an indirect pathway. In analogy to the PKC-mediated stimulation of the Na^+/H^+ exchanger (Wakabayashi *et al*., 1994), an indirect mechanism via phosphorylation of a regulatory protein could well be envisaged.

Possible functional significance of cAMP-linked signaling in erythroid progenitor cells. Synergistic coactivation of adenylyl cyclase by two different G protein-linked signaling systems in early erythroid progenitors but not in late precursors suggests that the regulation of cAMP levels by circulating G protein-coupled receptor ligands may be involved in the regulation of cell growth and development. From results in leukemia-derived human cell lines, it appears that both cAMP and thrombin may have a growth-inhibitory effect (Vittet *et al*., 1995; Vittet *et al*., 1992). Increased concentrations of cAMP block the activation of Raf-1, a protein kinase involved in growth factor-linked signal transduction pathways (Wu *et al*., 1993). On the other hand, induction of differentiation in leukemic cells has been associated with elevated cellular cAMP levels (Tortora *et al*., 1989; Vittet *et al*., 1995). These data together with evidence of extended cross-talk between tyrosine kinase-activating cytokines and serine/threonine kinase-mediated pathways (Mufson, 1997) point to an important modulatory function of G protein-coupled signaling in cytokine-controlled hematopoiesis. However, determination of the actual contribution of these different cAMP-dependent mechanisms to the regulation of erythroid cell development *in vivo* will require further studies in nontransformed progenitor cells.

Acknowledgments

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