Biochem. J. (1992) 281, 73–80 (Printed in Great Britain)

Dual regulation of cyclic AMP formation by thrombin in HEL cells, a leukaemic cell line with megakaryocytic properties

Lawrence F. BRASS*†§ and Marilyn J. WOOLKALIS‡

Departments of *Medicine, †Pathology and ‡Pharmacology, University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, U.S.A.

Thrombin is thought to stimulate responsive cells by cleaving cell-surface receptors coupled to intracellular second-messenger-generating enzymes via G-proteins. In order to understand this process better, we have examined the regulation of adenylate cyclase by thrombin in the megakaryoblastic HEL cell line and compared it with platelets. A notable difference was found. In HEL-cell membrane preparations, thrombin inhibited cyclic AMP (cAMP) formation by a pertussis-toxin-sensitive mechanism comparable with that observed in platelets. In contrast, when added to intact HEL cells, thrombin activated adenylate cyclase and caused an increase in cAMP formation synergistic with that produced by forskolin and prostaglandin I_2. This increase, which was not seen with platelets, was accompanied by an increase in cAMP metabolism by phosphodiesterase. Like other responses to thrombin, the increase in cAMP formation required proteolytically active thrombin and was subject to homologous desensitization. An equivalent response could be evoked by the addition of a polypeptide, derived from the *N*-terminus of the thrombin receptor, that has been shown to activate the receptor. The effects of thrombin could not, however, be reproduced by the addition of phorbol ester and the Ca^{2+} ionophore, A23187, nor be prevented with inhibitors of arachidonate metabolism. Preincubation of the cells with adrenaline, which inhibited G_s-mediated activation of adenylate cyclase, or pertussis toxin, which inhibited phospholipase C activation, had no effect on thrombin-induced cAMP formation. These results suggest that thrombin can regulate cAMP formation by two different mechanisms. First, thrombin can inhibit adenylate cyclase in a G_i-dependent manner. This effect predominates in HEL-cell membrane preparations, as it does in platelets, but is not detectable when thrombin is added to intact HEL cells. Instead, in intact HEL cells thrombin activates adenylate cyclase. Although clearly receptor-mediated, this response does not appear to involve G_i, G_s, protein kinase C, eicosanoid formation or changes in the cytosolic Ca^{2+} concentration.

INTRODUCTION

Thrombin evokes biological responses from a variety of vascular and perivascular cells, including platelets, endothelial cells, fibroblasts and smooth muscle. The outcomes of these interactions vary, but in each case the responses are thought to be mediated by cell-surface receptors coupled via GTP-binding regulatory proteins (G-proteins) to the intracellular enzymes that generate second-messenger molecules. For example, in most responsive cells thrombin activates phospholipase C, leading to the formation of Ins(1,4,5)P_3 and diacylglycerol, both of which are second messengers. Thrombin can also activate phospholipase A_2, releasing arachidonate from membrane phospholipids and leading to the formation of one or more eicosanoids. Which eicosanoids are formed depends on the type of cell involved. In platelets arachidonate is metabolized primarily to thromboxane A_2, which promotes platelet activation. In endothelial cells the end-product is prostaglandin I_2 (PGI_2), which inhibits platelet function by activating adenylate cyclase via the G-protein G_s, causing an increase in cyclic AMP (cAMP). In some cells thrombin can also decrease cAMP formation by inhibiting adenylate cyclase activity via the G-protein, G_i. In platelets this inhibition has little, if any, effect on basal cAMP levels, but diminishes the stimulatory effects of PGI_2 and forskolin [1].

Until recently, comparatively little was known about the thrombin receptor. However, recently published evidence suggests that it resembles other receptors whose effects are mediated by G-proteins [2]. Notable structural features predicted from its nucleic acid sequence include seven transmembrane domains and an extracellular *N*-terminus that is cleaved by thrombin. Proteolysis by thrombin or a related proteinase creates a new *N*-terminus, which is thought to be the actual ligand for the receptor. Notably, a polypeptide containing the first 14 amino acids of the *N*-terminus following its cleavage by thrombin has been shown by Vu *et al.* [2] to be capable of aggregating platelets and activating *Xenopus laevis* oocytes expressing the thrombin-receptor clone, thereby providing a non-proteolytic mechanism for evoking thrombin responses from cells containing the receptor.

The G-proteins which mediate the interactions between thrombin receptors and intracellular enzymes are heterotrimers in which the α subunit contains the guanine nucleotide binding site and is frequently a substrate for ADP-ribosylation by bacterial toxins such as pertussis toxin and cholera toxin [3]. G-proteins are thought to be involved in at least two responses to thrombin, the inhibition of cAMP formation and the stimulation of phosphoinositide hydrolysis, and may be involved in the activation of phospholipase A_2 as well. In platelets, both the inhibition of adenylate cyclase and the activation of phospholipase C are sensitive to pertussis toxin [1], whereas in endothelial cells thrombin-induced activation of phospholipase C occurs via a pertussis-toxin-resistant mechanism [4]. Thus, even among the cells known to be responsive to thrombin, there are differences in the mechanism, as well as the consequences, of interaction with thrombin.

In order to understand better the basis for the diversity of cellular responses to thrombin we have compared the regulation of cAMP formation by thrombin in platelets and human erythro-

Abbreviations used: cAMP, cyclic AMP; G-protein, GTP-binding regulatory protein; PGI_2, prostaglandin I_2; IBMX, isobutylmethylxanthine; PPACK, D-phenylalanyl-L-prolyl-L-arginylchloromethane; NDGA, nordihydroguaiaretic acid.

§ To whom correspondence should be addressed, at the Hematology–Oncology Section, Silverstein 7, University of Pennsylvania.

leukaemia (HEL) cells. HEL cells possess a number of features common to the megakaryocyte/platelet lineage, including the ability to synthesize characteristic membrane glycoproteins and secretory proteins [5–9] and to undergo phosphoinositide hydrolysis and changes in cytosolic Ca^{2+} in response to thrombin [10,11]. HEL cells have also been shown to possess the same complement of pertussis-toxin-sensitive G-proteins that are present in platelets [12]. However, the present studies show that the effects of thrombin on cAMP formation in HEL cells are different from those observed in platelets, and include the capacity to stimulate, as well as to inhibit, adenylate cyclase activity. This stimulation results in an increase in cAMP levels which is receptor-mediated, but does not appear to involve G_s.

METHODS

Cell culture

HEL cells were maintained at 37 °C in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal-calf serum at a cell count between 2×10^5 and 1×10^6/ml.

cAMP formation

HEL-cell membranes were prepared by Dounce homogenization and resuspended in 1 mM-EGTA/50 mM-Hepes, pH 7.0 [11]. [α-^{32}P]ATP was used as the substrate for cAMP formation [13]. The 125 μl reaction mixture typically contained 45 μl of membranes (0.5 mg/ml) and 65 μl of reaction buffer [20 mM-$MgCl_2$, 0.4 mg of BSA/ml, 12 mM-phosphoenolpyruvate, 1.7 mM-ATP, 1.9 μCi of [α-^{32}P]ATP, 15 μM-GTP, 1.7 mM-isobutylmethylxanthine (IBMX), 1.7 mM-EDTA, 26 μg of pyruvate kinase/ml and 133 mM-Hepes, pH 8.0]. Any further additions, such as thrombin or forskolin, were made in the remaining 15 μl.

The amount of cAMP present in intact HEL cells was measured by radioimmunoassay (DuPont–New England Nuclear). Typically, HEL cells (3.5×10^5/ml) were preincubated with 1 mM-IBMX 10–30 min before addition of an agonist. For studies on the effects of Ca^{2+} and the ionophore A23187, the HEL cells were resuspended in buffer containing 137 mM-NaCl, 2.7 mM-KCl, 1 mM-$MgCl_2$, 3.3 mM-NaH_2PO_4 and 20 mM-Hepes, pH 7.35, supplemented with either 0.4 mM-$CaCl_2$ or 1 mM-EGTA.

cAMP phosphodiesterase activity

HEL cells (2×10^6/ml) were resuspended in RPMI 1640 supplemented with 10 mM-Hepes, pH 7.3, and incubated for 3 min at 37°. with thrombin (50 nM), IBMX (1 mM) or vehicle before being disrupted by sequential freezing and thawing. The resulting lysate was incubated at 37 °C with 10 μM-cAMP plus [^{3}H]cAMP (final concn. 24–48 nCi/ml). At times up to 30 min, the reaction was stopped by the addition of $HClO_4$ (final concn. 4%). Known quantities of non-radioactive adenosine, AMP and cAMP were added as markers and used to monitor recovery. After removal of any precipitate by centrifugation and neutralization with KOH, the samples were analysed by h.p.l.c. on a Whatman Partisil-SAX column by using a linear gradient of 0 to 1.5 M-ammonium formate, pH 3.7. Peaks containing [^{3}H]adenosine, [^{3}H]AMP and [^{3}H]cAMP were identified and quantified by on-line liquid-scintillation counting.

Inositol phosphate formation

HEL cells were resuspended in inositol-free RPMI medium (Gibco) containing 10% dialysed fetal-calf serum and incubated for approx. 22 h with *myo*-[^{3}H]inositol (10 μCi/ml; American Radiolabelled Chemicals, St. Louis, MO, U.S.A.). Afterwards, the cells were resuspended in RPMI without serum at a final cell concentration of 1×10^6/ml and allowed to equilibrate for 1 h at 37 °C before exposure to thrombin. A typical reaction mixture had a final volume of 600 μl, of which the HEL-cell suspension represented 540 μl and any additions such as thrombin were made in the remaining 60 μl. Reactions were terminated by addition of 300 μl of 12% $HClO_4$ containing 3 mM-EDTA, 1 mM-diethylenetriaminepenta-acetic acid and 15 μg of phytic acid. Adenosine, AMP, ADP and ATP were added as internal markers and to allow for correction of relative recoveries between samples. The $HClO_4$ extract was neutralized and the [^{3}H]inositol phosphates present were analysed by h.p.l.c. on a Whatman Partisil-SAX column by using a linear gradient of 0–1.5 M-ammonium formate, pH 3.7 [10,11]. Peaks of radioactivity corresponding to the various inositol phosphates were detected by scintillation counting and identified by using authentic standards.

Cytosolic Ca^{2+} measurements

HEL cells were resuspended at a concentration of 1.75×10^6 cells/ml in RPMI containing 10% fetal-calf serum and 20 mM-Hepes, pH 7.3, and allowed to equilibrate for 30 min. Afterwards, 5 μM-fura-2/AM (Molecular Probes) was added for 1 h at 37 °C before the cells were washed and resuspended in RPMI medium plus 10% fetal-calf serum. After an additional 1 h equilibration period, the cells were washed again and resuspended in RPMI at 3×10^6 cells/ml. Fluorescence was detected in a Perkin–Elmer spectrophotometer with excitation set at 340 nm and emission set at 510 nm. Approximate values for cytosolic [Ca^{2+}] were calculated by using an assumed k_D of 224 nM [14].

Other materials

Pertussis toxin was obtained from List Biological Laboratories. Highly purified human α-thrombin and D-phenylalanyl-L-prolyl-L-arginylchloromethane (PPACK)-treated thrombin were gifts from Dr. John Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A. Trypsin was obtained from Worthington Biochemicals and Sigma Chemical Co. Hirudin, indomethacin and nordihydroguaiaretic acid (NDGA) were obtained from Sigma. BW755c was a gift from Dr. Eduardo Lapetina, Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A. The thrombin-receptor activation peptide referred to as $TRP^{42/55}$ (SFLLRNPNDKYEPF) was synthesized in the protein core facility of the Hematology Division of Children's Hospital of Philadelphia.

RESULTS

The data in Fig. 1 compare the effects of thrombin on cAMP formation in intact HEL cells and HEL-cell membranes. When added to membranes, thrombin inhibited forskolin-stimulated cAMP formation in a concentration-dependent manner, half-maximal inhibition requiring approx. 1 nM-thrombin (Fig. 1*a*). However, when added to intact HEL cells, thrombin caused an increase in cAMP. This increase was readily detectable when thrombin was added by itself (Fig. 1*b*), but the magnitude was even greater when thrombin was combined with PGI_2 or forskolin (Figs. 1*c* and 1*d*). Notably, this difference between the results obtained with membranes and intact HEL cells was not observed with other agonists or with other responses to thrombin. For example, adrenaline, which inhibits forskolin-stimulated cAMP formation by HEL-cell membranes, also does so in intact HEL cells (see below), and data presented elsewhere show that thrombin stimulates phosphoinositide hydrolysis when added to either intact HEL cells or membranes [11].

To determine whether the increase in cAMP was due to increased synthesis or to decreased metabolism of cAMP, HEL cells were preincubated with thrombin and then lysed. Phospho-

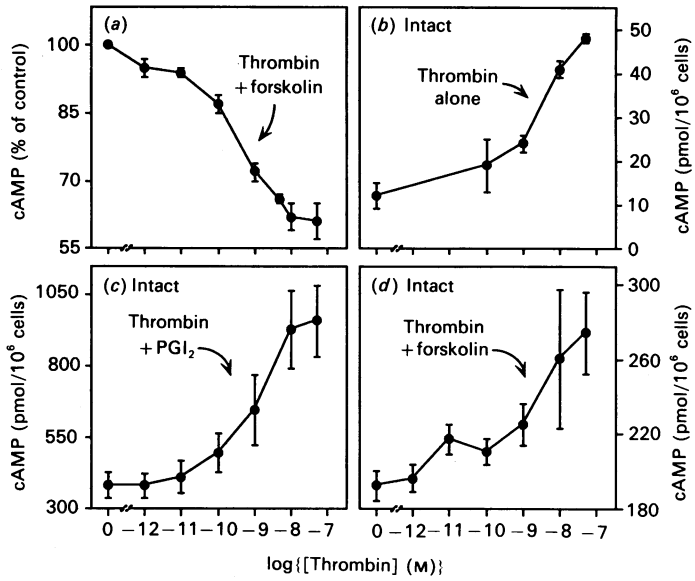

Fig. 1. Effect of thrombin on cAMP formation: intact HEL cells versus membranes

(*a*) HEL-cell membranes were incubated with thrombin for 30 s before addition of 20 μM-forskolin; cAMP formation was measured 30 min later. The results are expressed as percentages of forskolin-stimulated cAMP formation in the absence of thrombin (1808 ± 133 pmol/min per mg; $n = 3$); the basal rate of cAMP formation was 97 ± 5 pmol/min per mg. (*b*, *c*, *d*) Intact HEL cells were incubated with thrombin at the concentrations shown for 30 s before addition of forskolin (20 μM) or PGI_2 (10 μM). The cAMP concentration was measured 10 min later. The results are means ± S.E.M. of three studies.

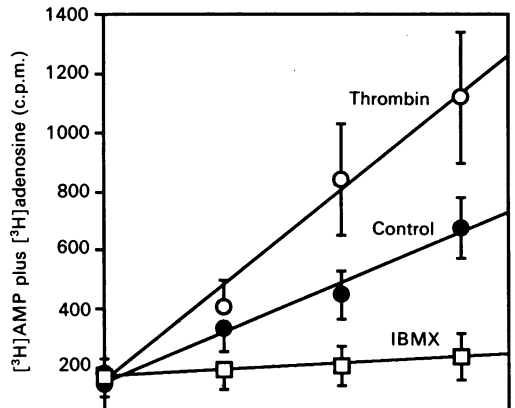

Fig. 2. cAMP phosphodiesterase activity

HEL cells were incubated with thrombin (50 nM), IBMX (1 mM) or buffer for 3 min before being lysed and incubated with [^{3}H]cAMP. Metabolites of the [^{3}H]cAMP were identified by h.p.l.c. The results shown are means ± S.E.M. from five studies.

diesterase activity was measured as an increase in metabolites formed from [^{3}H]cAMP. In control lysates, there was a linear increase in [^{3}H]AMP and [^{3}H]adenosine which was abolished by IBMX. In the presence of thrombin, the rate of metabolite formation increased (Fig. 2), an effect which may be due to thrombin-induced increases in the cytosolic free Ca^{2+}concentration, as has been shown to be the case when muscarinic

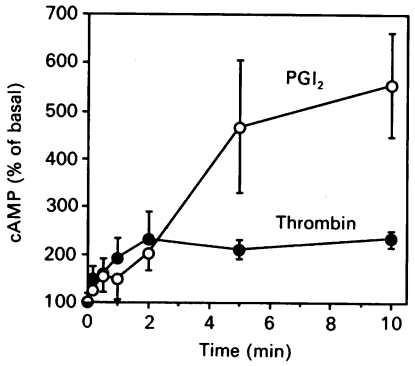

Fig. 3. Time course of cAMP formation in intact HEL cells

HEL cells were incubated with 50 nM-thrombin or 10 μM-PGI_2 for the times indicated. The results shown are expressed as percentages of basal cAMP levels and are means ± S.E.M. of three to five studies.

agonists are added to human astrocytoma cells [15]. Although the thrombin was not routinely removed before lysing the HEL cells, inactivating it with a 4-fold excess of hirudin had no effect on the results shown in Fig. 2. These results suggest that the increased cAMP concentration found in thrombin-treated HEL cells is due not to decreased metabolism, but to an increase in cAMP synthesis.

In order to characterize further the increase in HEL-cell cAMP levels caused by thrombin, the rate of cAMP accumulation in thrombin-treated cells was compared with the response to PGI_2. The results are shown in Fig. 3. The initial rates of cAMP accumulation were similar with both agonists. However, although the response to thrombin became maximal after approx. 2 min, cAMP levels in the PGI_2-stimulated cells continued to rise for several more minutes, yielding a higher final cAMP concentration.

Agents which elevate cAMP levels have been shown to inhibit many of the effects of thrombin on platelets [16–19]. Since thrombin stimulates cAMP formation in HEL cells, [^{3}H]inositol-loaded HEL cells were used to determine whether a prior increase in cAMP levels would prevent thrombin-induced phosphoinositide hydrolysis, as it does in platelets. The results are shown in Fig. 4. For these studies, HEL-cell cAMP levels were increased by adding 10 μM-PGI_2 2 min before thrombin, a concentration and duration that produced an increase in cAMP comparable with that caused by thrombin. In the absence of PGI_2 [^{3}H]Ins(1,4,5)P_3 formation was detectable within 10 s of thrombin addition and was followed by sequential increases in the other [^{3}H]inositol phosphates. These kinetics are similar to those reported in platelets [20]. Preincubation with PGI_2 inhibited thrombin-induced InsP_3 formation, suggesting that the cAMP-dependent inhibitory mechanisms described in platelets are also present in HEL cells, despite the differences between these cells in the effects of thrombin on cAMP formation.

Receptor mediation

The recent publication of the complete sequence of the thrombin receptor not only confirmed the prediction that it would resemble other receptors that are coupled to G-proteins, but also provided the unique observation that a peptide containing the amino acids proximal to the site at which the receptor is cleaved by thrombin can activate the receptor [2]. In order to determine whether the disparate effects of thrombin on cAMP formation are receptor-mediated, the 14-amino-acid polypeptide described by Vu *et al.* [2] was synthesized: SFLLRNPNDKYEPF, referred to here as thrombin-receptor

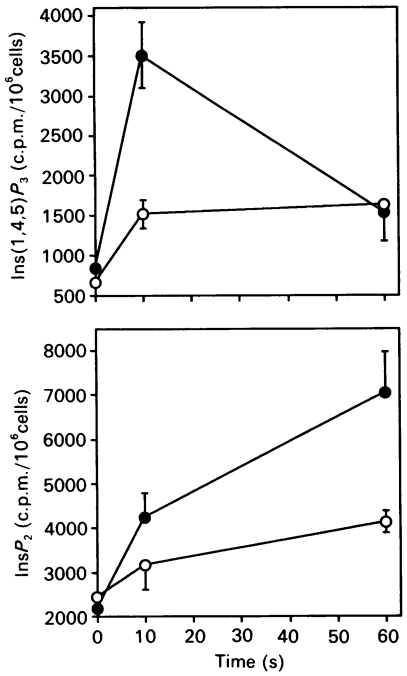

Fig. 4. Effect of raising cAMP levels with PGI_2 on thrombin-induced phosphoinositide hydrolysis

HEL cells radiolabelled with [^{3}H]inositol were incubated with thrombin (50 nM) either in the absence of PGI_2 (●) or in the presence of PGI_2 (10 μM) added 2 min before thrombin (○). The results shown are means ± S.E.M. of three studies.

activation peptide number 1 or '$TRP^{42/55}$'. Initial studies confirmed the observations by Vu *et al.* [2], and additionally showed that the peptide raises the cytosolic free Ca^{2+} concentration and causes pertussis-toxin-sensitive phosphoinositide hydrolysis in HEL cells (L. F. Brass, unpublished work). Table 1 shows the effects of $TRP^{42/55}$ on cAMP formation in HEL cells and platelets. The results parallel those obtained with thrombin. When added to intact platelets, platelet membranes or HEL-cell membranes, $TRP^{42/55}$ inhibited cAMP formation. When added to intact HEL cells, $TRP^{42/55}$ caused an increase in cAMP formation, both in the presence and in the absence of PGI_2. As noted previously [2], the molar concentration of $TRP^{42/55}$ required was considerably higher than for thrombin.

Several other characteristics of receptor-mediated interactions between thrombin and responsive cells were also examined in the context of the thrombin-induced increase in cAMP formation in HEL cells. These include a requirement for proteolytically active thrombin, the ability of other serine proteinases to mimic some of the effects of thrombin and the creation by thrombin of a state of homologous desensitization which persists for as long as 20 h after exposure to thrombin [11,21–24]. These properties have previously been shown to apply to thrombin-induced phosphoinositide hydrolysis in HEL cells [11] and were used in the present studies to test whether the increase in cAMP levels caused by thrombin is also a receptor-mediated event. First, a comparison was made between HEL-cell responses to native thrombin, thrombin inactivated with PPACK, and trypsin. PPACK-thrombin had no effect on cAMP formation even when added at concentrations as high as 50 nM (results not shown). Trypsin, like thrombin, stimulated cAMP formation in intact HEL cells (Fig. 5*a*).

Desensitization of the cAMP response to thrombin was tested by using HEL cells that were incubated briefly with thrombin, washed and the re-challenged 2 h later. Loss of the characteristic thrombin-induced increase in the cytosolic free Ca^{2+} concentration in fura-2-loaded HEL cells confirmed that desensitization had occurred (Fig. 5*b*). There was also a decrease in the magnitude of the increase in cAMP levels caused by thrombin, but no decrease in either the stimulation caused by PGI_2 or the inhibition caused by adrenaline.

Collectively, these results, and the observations with pertussis toxin described below, suggest that both the stimulation of cAMP formation caused by thrombin in intact HEL cells and the inhibition of cAMP formation observed when thrombin is added to HEL-cell membrane preparations are receptor-mediated events and do not involve the proteolysis of a substrate other than the thrombin receptor.

G-proteins

Agonist effects on adenylate cyclase are typically mediated by G_s, which stimulates cAMP formation, and G_i, which inhibits it [3]. An interaction between thrombin receptors and G_s might account for the thrombin-induced increase in cAMP levels, but has not previously been described. Since there are no inactivators of G_s with specificity comparable with that of pertussis toxin for G_i, adrenaline was used. Adrenaline inhibits G_s by activating G_i via α_2-adrenergic receptors. HEL cells were incubated with thrombin or PGI_2 in the absence and presence of adrenaline. Adrenaline inhibited PGI_2-stimulated cAMP formation by

Table 1. The thrombin-receptor activation peptide $TRP^{42/55}$ reproduces the effects of thrombin on cAMP formation

For the intact-cell studies, HEL cells or platelets were incubated with $TRP^{42/55}$ (25 μM) or thrombin (50 nM) for 30 s before the addition of PGI_2 (10 μM). The cAMP content of the cells was measured 10 min later; the results shown are means ± S.E.M. of five studies. In the studies with membranes, HEL-cell or platelet membranes were incubated with $TRP^{42/55}$ or thrombin for 30 s before addition of 20 μM-forskolin. cAMP formation was measured 30 min later; the results shown are means ± S.E.M. of four studies. In all cases, IBMX was present at a final concentration of approx. 1 mM.

	cAMP content		cAMP formation	
	HEL cells (pmol/10^6 cells)	Platelets (pmol/10^8 cells)	HEL membranes (pmol/min per mg)	Platelet membranes (pmol/min per mg)
Basal	32±3	28±2	27±2	36±6
$TRP^{42/55}$	61±12	29±3	22±1	34±4
Thrombin	72±8	31±6	24±4	24±4
PGI_2 or forskolin	744±58	453±65	559±22	1299±232
+$TRP^{42/55}$	2671±412	342±52	432±14	891±167
+Thrombin	3039±414	200±19	407±24	762±128

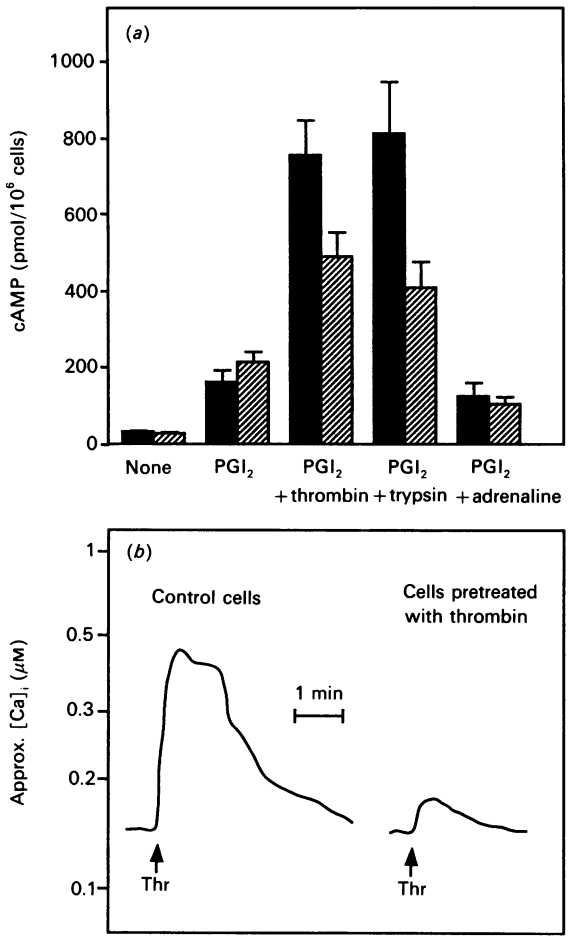

Fig. 5. Desensitization of thrombin and trypsin responses by a previous exposure to thrombin

(*a*) HEL cells were incubated for 10 min without (■) or with (▨) thrombin (50 nM) and then washed and resuspended; 2 h later, cAMP levels were measured after a 10 min incubation with the combinations of thrombin (50 nM), trypsin (50 μg/ml), adrenaline (20 μM) and PGI_2 (10 μM) shown. The results shown are means ± S.E.M. of three studies. (*b*) With the same protocol, HEL cells loaded with fura-2 were incubated without or with 50 nM-thrombin for 10 min, washed and resuspended in medium containing 1 mM-Ca^{2+}; 2 h later the change in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) evoked by 50 nM-thrombin (Thr) was determined. The results shown are representative of three studies.

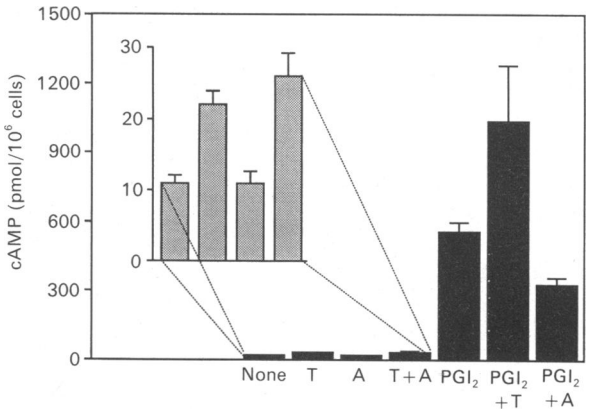

Fig. 6. Effect of adrenaline on thrombin-induced cAMP formation

HEL cells were incubated with combinations of thrombin (T; 50 nM) and adrenaline (A; 10 μM) with or without PGI_2 (10 μM). The cAMP concentration was measured 10 min later. The results shown are means ± S.E.M. of three studies. The inset shows an enlarged view of the data obtained in the absence of PGI_2.

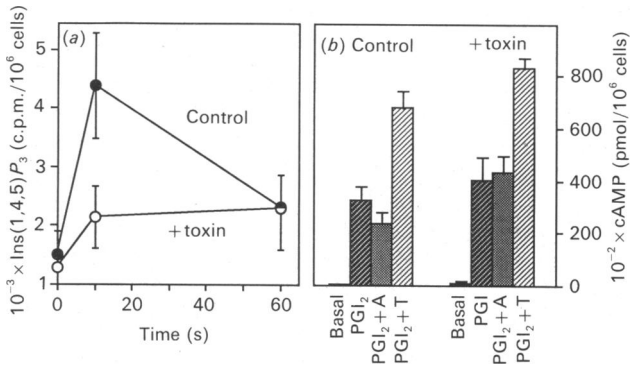

Fig. 7. Effects of pertussis toxin

HEL cells were incubated for 16 h with or without pertussis toxin (0.2 μg/ml). (*a*) HEL cells radiolabelled with [^{3}H]inositol were stimulated with 50 nM-thrombin. The results shown are means ± S.E.M. of three studies. (*b*) Thrombin (T; 50 nM) and/or adrenaline (A; 10 μM) were added to intact HEL cells 30 s before the addition of 10 μM-PGI_2. The cAMP concentration was measured 10 min later. The results shown are means ± S.E.M. of four studies.

approx. 50%, but had no effect on thrombin-stimulated cAMP formation (Fig. 6). This suggests that the increase in cAMP levels caused by thrombin is not mediated by G_s.

Other HEL-cell responses to thrombin have been shown to be inhibited by pertussis toxin, and are presumed to involve G_i or another G-protein whose α subunit is ADP-ribosylated by the toxin. To assess the role of a pertussis-toxin-sensitive G-protein in thrombin-induced cAMP formation, HEL cells were incubated overnight with a concentration of the toxin sufficient to ADP-ribosylate completely all of the members of the $G_i\alpha$ family present in HEL cells [11,12]. Under these conditions, pertussis toxin (1) completely suppressed the inhibition of forskolin-stimulated cAMP formation otherwise caused by thrombin in HEL-cell membrane preparations (results not shown), (2) inhibited thrombin-induced $InsP_3$ formation (Fig. 7*a*) and (3) abolished the inhibition of cAMP formation otherwise caused by adrenaline (Fig. 7*b*). It had no effect, however, on thrombin-stimulated cAMP formation (Fig. 7*b*). This suggests that none of the pertussis-toxin-sensitive G proteins in HEL cells are involved in the activation of adenylate cyclase by thrombin.

Other mediators

In addition to an interaction with G_s, several other mechanisms have been described by which agonists can stimulate adenylate cyclase activity. Typically, these involve changes in cytosolic Ca^{2+} or the release of other secondary messengers. The inability of pertussis toxin to prevent thrombin-induced cAMP formation under conditions in which phospholipase C activation is inhibited suggests that the products of phosphoinositide hydrolysis are not involved. However, this point was tested further. Preincubating HEL cells with 0.1 μM-phorbol 12-myristate 13-acetate to activate protein kinase C had no effect on thrombin-induced cAMP formation (results not shown). Similarly, replacing extracellular Ca^{2+} with EGTA greatly diminished the increase in intracellular Ca^{2+} caused by thrombin, but did not prevent the stimulation of cAMP formation caused by thrombin; nor did raising the cytosolic free Ca^{2+} concentration with the Ca^{2+} ionophore A23187 reproduce the effects of thrombin (results not shown). It

Table 2. Effects of cyclo-oxygenase and lipoxygenase inhibitors on cAMP formation in HEL cells

HEL cells were preincubated for 30 min with 1 mM-IBMX in the presence of the cyclo-oxygenase and lipoxygenase inhibitors indomethacin, NDGA or BW755c at the final concentrations shown. Afterwards, 50 nM-thrombin was added for 30 s, followed by an additional 10 min incubation with 10 μM-PGI_2. The results shown are means ± S.E.M. for nine studies with indomethacin, six studies with NDGA and two studies with BW755c.

	cAMP (pmol/10^6 cells)		
	Basal	PGI_2	PGI_2 + thrombin
Control	33 ± 8	669 ± 108	1387 ± 136
Indomethacin (10 μM)	35 ± 7	494 ± 42	1203 ± 145
Control	45 ± 2	539 ± 52	1535 ± 21
NDGA (10 μM)	44 ± 4	484 ± 32	1791 ± 446
NDGA (100 μM)	41 ± 4	44 ± 2	43 ± 5
Control	52 ± 21	676 ± 194	1272 ± 444
BW755c (200 μM)	72 ± 7	607 ± 174	1440 ± 532

therefore seems unlikely that either the activation of protein kinase C or changes in cytosolic Ca^{2+} can be solely responsible for the stimulation of cAMP formation by thrombin.

In addition to phospholipase C, thrombin typically activates phospholipase A_2, releasing arachidonate from membrane phospholipids. Several inhibitors of cyclo-oxygenase (indomethacin and BW755c) and lipoxygenase (NDGA and BW755c) were used at concentrations well above their reported K_i values in platelets and leucocytes [25] to test whether one or more metabolites of arachidonate might serve as mediators of the thrombin-induced increase in cAMP formation in HEL cells. The results are summarized in Table 2. Except for NDGA, none of the inhibitors diminished the response to thrombin. NDGA at 10 μM had no effect, but at a very high concentration (100 μM) abolished the cAMP increase caused by both thrombin and PGI_2, an effect taken to be non-specific.

Finally, a cell-free supernatant was prepared from thrombin-stimulated HEL cells and added to fresh HEL cells. Although a small increase in cAMP levels was observed, it was completely blocked by hirudin, suggesting that it was due to residual thrombin and not to the release of an unidentified mediator. In general, disrupting the HEL cells in any of several ways, including saponin, Dounce homogenization or freeze/thawing abolished the stimulatory effects of thrombin on cAMP formation. Re-addition of the cytosol fraction to freshly isolated membranes failed to reverse the inhibition of cAMP formation normally seen when thrombin was added to membrane preparations (results not shown).

DISCUSSION

In general, biochemical responses to thrombin occur rapidly, requiring only seconds to become detectable. These immediate responses are then followed by events such as platelet aggregation and secretion, which are complete within minutes, or mitogenesis, which may require hours. Irrespective of the time course, all of these events are thought to be initiated by the interaction of thrombin with small numbers of high-affinity cell-surface receptors, most, if not all, of which are thought to be coupled to intracellular second-messenger-generating enzymes via G-proteins. In the present studies we have used HEL cells, a cell line with megakaryoblastic features, to examine the regulation of cAMP formation by thrombin. The results were compared with those that we and others have previously obtained with human platelets.

Three distinct modes of regulation were observed. In HEL-cell membrane preparations, as in platelets, thrombin inhibited forskolin-stimulated cAMP formation by as much as 40%. This inhibition could be suppressed with pertussis toxin and is presumably mediated by G_i. Thrombin also caused an increase in cAMP phosphodiesterase activity, accelerating the metabolism of cAMP. Both of these effects would be expected to favour a decrease in cAMP, such as is seen in platelets. However, when added to intact HEL cells, thrombin caused a substantial increase in basal cAMP levels that reflected increased cAMP formation. This effect of thrombin was synergistic with the increase in adenylate cyclase activity caused by PGI_2 and forskolin, occurred with a time course similar to that of thrombin-induced phosphoinositide hydrolysis and had an initial rate that was at least equal to that of PGI_2-stimulated cAMP formation. Other effects of thrombin did not show this apparent difference between the results obtained with membranes and intact cells, nor did another agonist, adrenaline.

In platelets, agents which increase cAMP levels inhibit platelet responses to thrombin, and the same appears to be true in HEL cells. Thus, even though some of the consequences of an increase in cAMP levels are the same, the regulation of cAMP formation by thrombin appears to be an exception in an otherwise similar set of responses to thrombin in platelets and HEL cells. There are a number of possible explanations for this difference. One is that the responses of HEL cells to thrombin reflect normal events at an early stage of megakaryocyte development, whereas the responses of platelets reflect those which develop at a later stage. Stimulation of adenylate cyclase by thrombin is not, however, unique to HEL cells. We have found that thrombin causes a pertussis-toxin-sensitive increase in cAMP formation in early-passage human umbilical-vein endothelial cells [26], and Gordon *et al.* [27] reported that thrombin stimulates cAMP formation in hamster fibroblasts. However, in another study with hamster fibroblasts the effects of thrombin were biphasic with respect to concentration [28]. The stimulation of cAMP formation seen at high thrombin concentrations was attributed to the activation of protein kinase C overcoming the G_i-mediated inhibition that occurred at lower concentrations. Since in HEL cells pertussis toxin had no effect on thrombin-stimulated cAMP formation and the dose/response curves were not biphasic, a different mechanism appears to be involved from that present in either endothelial cells or fibroblasts. Several questions were posed in order to define this mechanism. First, is the increase in adenylate cyclase activity mediated by thrombin receptors, or is it due to a direct interaction between thrombin and adenylate cyclase? Second, does the increase involve any of the G-proteins known to be present in HEL cells, and third, is the increase in cAMP levels due to any of the other second messengers produced in response to thrombin?

Receptors

Since thrombin is a proteinase and adenylate cyclase is a transmembrane protein, a proteolytic interaction between the two is theoretically possible with either intact HEL cells or membranes [29]. However, several observations suggest that this probably does not occur. First, a direct interaction between thrombin and extracellular domains of adenylate cyclase would not account for the differences observed between intact cells and membranes, since such extracellular domains would be available in either situation. It also would not account for the ability of pertussis toxin to suppress the inhibition of cAMP formation seen when thrombin is added to HEL-cell membranes.

On the other hand, a direct interaction with adenylate cyclase

or G_s probably does account for some of the effects of trypsin and other proteinases when added to cell membranes [30–32]. Typically, these proteinases stimulate cAMP formation at low concentrations and inhibit cAMP formation at higher concentrations. Either effect could be due to proteolysis of G_s, but proteolysis of adenylate cyclase can also occur [33]. With intact HEL cells, as with platelets, trypsin mimics many of the effects of thrombin ([11]; the present work). When added to HEL-cell membranes, trypsin inhibits forskolin-stimulated adenylate cyclase activity to a far greater extent than does thrombin, even when the membranes have been pretreated with pertussis toxin [11]. This difference between thrombin and trypsin is presumably due to the proteolysis by trypsin of either G_s, adenylate cyclase or both.

If a direct interaction between thrombin and adenylate cyclase is unlikely, can it be concluded that thrombin receptors are involved? Several characteristics of receptor-mediated responses to thrombin were used to characterize thrombin's effects on cAMP formation. First, as with other effects of thrombin, the proteolytically active form of the enzyme was required. Second, as discussed above, with intact cells trypsin reproduced this effect of thrombin, just as it does other responses to thrombin. Third, the thrombin-induced increase in cAMP formation was subject to the same prolonged agonist-specific desensitization that is observed for other receptor-mediated responses to thrombin. Finally, and perhaps most notably, the 14-amino-acid polypeptide that has been shown to activate the cloned thrombin receptor [2] reproduced the diverse effects of thrombin on cAMP formation in platelets and HEL cells. Collectively, these observations suggest that the effects of thrombin on adenylate cyclase are receptor-mediated in both intact HEL cells and membranes.

G-proteins

HEL cells contain G_s and G_i [12,34]. Although G_s mediates the effects of other agonists that stimulate cAMP formation in HEL cells, it does not appear to do so for thrombin, since adrenaline, which inhibited the activation of adenylate cyclase by PGI_2, had no effect on thrombin-stimulated cAMP formation. Could another G-protein be involved? A recent study in which purified adenylate cyclase was reconstituted with various brain G-proteins suggests that at least some of the members of the G_i family can stimulate cAMP formation [35]. To test whether this type of 'atypical' interaction could account for the increase in cAMP synthesis caused by thrombin, HEL cells were incubated with pertussis toxin under conditions that cause $> 90\%$ ADP-ribosylation of G_i [11]. Under these conditions, pertussis toxin inhibited thrombin-induced phosphoinositide hydrolysis, but had no effect on thrombin-induced cAMP formation. Therefore, the involvement of G_i, like G_s, appears unlikely.

Second messengers

In theory, receptor-dependent activation of adenylate cyclase by thrombin could be a secondary phenomenon mediated by phosphoinositide hydrolysis, eicosanoid formation, protein kinase C activation or changes in the cytosolic free Ca^{2+} concentration. Such mechanisms have been used to account for the stimulation of adenylate cyclase activity seen in other cells [28,36], including lymphocytes and vascular smooth muscle, where activators of protein kinase C have been shown to enhance agonist-stimulated cAMP synthesis [37–39]. Each of these possibilities was examined. Since thrombin-induced cAMP formation continued even when phosphoinositide hydrolysis was blocked by pertussis toxin, phospholipase C appears not to be involved. Similarly, since neither the addition of phorbol ester nor the Ca^{2+} ionophore A23187 mimicked the effects of thrombin, protein kinase C and changes in cytosolic Ca^{2+} appear not to be involved. The failure of several inhibitors of cyclo-oxygenase and lipoxygenase to inhibit thrombin-stimulated cAMP formation suggests that the metabolites of arachidonate produced via these enzymes are not involved.

In conclusion, therefore, the regulation of cAMP formation by thrombin in HEL cells departs from the effects seen in platelets. In platelets thrombin inhibits cAMP formation, an effect commensurate with the fact that elevated cAMP levels depress platelet responsiveness. In HEL cells thrombin stimulates cAMP formation, raising the intracellular cAMP concentration to a level where inhibition of thrombin-induced phosphoinositide hydrolysis can be observed. This response appears to be receptor-mediated, but does not appear to involve G_s, G_i, protein kinase C, changes in cytosolic Ca^{2+}, or the formation of second messengers derived from arachidonate.

We express our gratitude to Elizabeth Belmonte, Calvin Shaller and Marie Elena Hudzicki for their expert technical assistance. These studies were supported in part by funds from the N.I.H. (P01 HL40387), the American Heart Association and the W. W. Smith Charitable Trust.

REFERENCES

1. Brass, L. F., Manning, D. R. & Shattil, S. J. (1990) Prog. Hemostasis Thromb. **10**, 127–174
2. Vu, T.-K. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991) Cell **64**, 1057–1068
3. Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
4. Brock, T. A. & Capasso, E. L. (1989) Am. Rev. Respir. Dis. **140**, 1121–1125
5. Poncz, M., Surrey, S., LaRocco, P., Weiss, M., Rappaport, E., Conway, T. & Schwartz, E. (1987) Blood **69**, 219–223
6. Kieffer, N., Debili, N., Wicki, A., Titeux, M., Henri, A., Mishal, Z., Breton-Gorius, J., Vainchenker, W. & Clemetson, K. J. (1986) J. Biol. Chem. **261**, 15854–15862
7. Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E. & Bennett, J. S. (1987) J. Biol. Chem. **262**, 8476–8482
8. Zimrin, A. B., Eisman, R., Vilaire, G., Schwartz, E., Bennett, J. S. & Poncz, M. (1988) J. Clin. Invest. **81**, 1470–1475
9. Yeo, E., Furie, B. C. & Furie, B. (1989) Blood **73**, 722–728
10. Michel, M. C., Brass, L. F., Williams, A., Bokoch, G., LaMorte, V. J. & Motulsky, H. J. (1989) J. Biol. Chem. **264**, 4986–4991
11. Brass, L. F., Manning, D. R., Williams, A., Woolkalis, M. J. & Poncz, M. (1991) J. Biol. Chem. **266**, 958–965
12. Williams, A., Woolkalis, M. J., Poncz, M., Manning, D. R., Gewirtz, A. & Brass, L. F. (1990) Blood **76**, 721–730
13. Salomon, Y., Londos, C. & Rodbell, M. (1974) Anal. Biochem. **58**, 541–548
14. Grynkiewicz, G., Poenie, M. & Tsien, R. (1985) J. Biol. Chem. **260**, 3440–3450
15. Harden, T. K., Evans, T., Hepler, J. R., Hughes, A. R., Martin, M. W., Meeker, R. B., Smith, M. M. & Tanner, L. I. (1985) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **19**, 207–220
16. Lerea, K. M., Glomset, J. A. & Krebs, E. G. (1987) J. Biol. Chem. **262**, 282–288
17. Watson, S. P., McConnell, R. T. & Lapetina, E. G. (1984) J. Biol. Chem. **259**, 13199–13203
18. Feinstein, M. B., Egan, J. J., Shaafi, R. I. & White, J. (1983) Biochem. Biophys. Res. Commun. **113**, 598–604
19. Sage, S. O. & Rink, T. J. (1985) FEBS Lett. **188**, 135–140
20. Rittenhouse, S. E. & Sasson, J. P. (1985) J. Biol. Chem. **260**, 8657–8660
21. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) J. Biol. Chem. **262**, 8557–8565
22. Paris, S., Magnaldo, I. & Pouyssegur, J. (1988) J. Biol. Chem. **263**, 11250–11256
23. Martin, B. M., Feinman, R. D. & Detwiler, T. C. (1975) Biochemistry **14**, 1308–1314
24. Jones, L. G., McDonough, P. M. & Brown, J. H. (1989) Mol. Pharmacol. **36**, 142–149
25. Salari, H., Braquet, P. & Borgeat, P. (1984) Prostaglandins Leukotrienes Med. **13**, 53–60
26. Woolkalis, M. J., Manning, D. R. & Brass, L. F. (1990) J. Cell Biol. **111**, 215a

27. Gordon, E. A., Fenton, J. W., II & Carney, D. H. (1986) Ann. N.Y. Acad. Sci. **485**, 249–263
28. Magnaldo, I., Pouysségur, J. & Paris, S. (1988) Biochem. J. **253**, 711–719
29. Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R. & Gilman, A. G. (1989) Science **244**, 1558–1564
30. Lacombe, M. L., Stengel, D. & Hanoune, J. (1977) FEBS Lett. **77**, 159–163
31. Anderson, W. B., Jaworski, C. J. & Vlahakis, G. (1978) J. Biol. Chem. **253**, 2921–2926
32. Marshak, D. & Neer, E. J. (1980) J. Biol. Chem. **255**, 4781–4785
33. Stengel, D., Lad, P. M., Nielsen, T. B., Rodbell, M. & Hanoune, J. (1980) FEBS Lett. **115**, 260–264
34. Gagnon, A. W., Manning, D. R., Catani, L., Gewirtz, A., Poncz, M. & Brass, L. F. (1991) Blood **78**, 1247–1253
35. Newton, D. L. & Klee, W. A. (1990) FEBS Lett. **271**, 207–210
36. Felder, C. C., Kanterman, R. Y., Ma, A. L. & Axelrod, J. (1989) J. Biol. Chem. **264**, 20356–20362
37. Sugden, D., Vanacek, J., Klein, D. C., Thomas, T. P. & Anderson, W. B. (1985) Nature (London) **314**, 359–361
38. Cronin, M. J., Summers, S. T., Sortino, M. A. & Hewlett, E. L. (1986) J. Biol. Chem. **262**, 13932–13935
39. Wiener, E. & Scarpa, A. (1989) J. Biol. Chem. **264**, 4324–4328

Received 18 June 1991; accepted 18 July 1991