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The thrombin receptor in adrenal medullary microvascular endothelial cells is negatively coupled to adenylyl cyclase through a G_i protein

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Abstract

The effects of thrombin on adenylyl cyclase activity were examined in rat adrenal medullary microvascular endothelial cells (RAMEC). Confluent RAMEC monolayers were stimulated for 5 min with cAMP-generating agents in the absence and presence of thrombin, and intracellular cAMP was measured with a radioligand binding assay. Thrombin (0.001–0.25 U/ml) dose-dependently inhibited IBMX-, isoproterenol- and forskolin-stimulated cAMP accumulation. A peptide agonist of the thrombin receptor, γ-thrombin, and the serine proteases trypsin and plasmin, also inhibited agonist-stimulated cAMP levels, while proteolytically inactive PPACK- or DIP-α-thrombins were without effect. Moreover, the thrombin inhibitor hirudin abolished the inhibitory effect of thrombin but not of the peptide agonist. These results suggest that the inhibitory action of thrombin on cAMP accumulation is mediated by a proteolytically-activated thrombin receptor. The inhibitor of G_i-proteins pertussis toxin abolished the inhibitory effect of thrombin on isoproterenol- or IBMX-stimulated cAMP production, while the phorbol ester PMA partly impaired it. The protein kinase C inhibitors staurosporine or H7 and the intracellular Ca^{2+} chelator BAPTA-AM were without effect. Collectively, our data suggest that the thrombin receptor in RAMEC is negatively coupled to adenylyl cyclase through a pertussis toxin-sensitive G_i-protein.

Keywords: Thrombin receptor; cAMP; Adenylyl cyclase; Endothelial cell; Protein kinase C; G_i protein

1. Introduction

Thrombin is a plasma serine protease that induces blood coagulation and also stimulates immediate and long-term responses on various cell types that vary depending on the cell type [1,2]. In endothelial cells (EC) thrombin increases monolayer permeability, modulates proliferation, induces migration, and increases the expression of prostacyclin, interleukin-1, nitric oxide, endothelin, tissue factor, and platelet activating factor [3–8]. Most of the cellular actions of thrombin are mediated through activation of a cell surface receptor belonging to the 7-transmembrane spanning domain, G-protein coupled receptor family [9]. The mechanism of activation of this receptor involves proteolytic cleavage by thrombin after an arginine site of its NH_2-terminus. The newly exposed NH_2-terminus of the receptor then binds intramolecularly to the body of the receptor and triggers cellular signaling [9]. Synthetic peptides, mimicking the amino

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acid sequence of the new NH_2-terminus, are able to reproduce many of the vascular actions of thrombin [10]. The effects of thrombin are usually short-lived, as the thrombin receptor is rapidly desensitized either due to proteolysis, phosphorylation, or internalization [11].

The hormone-stimulated adenylyl cyclase pathway is a well described signaling system [12]. The enzyme adenylyl cyclase is coupled to cell surface receptors through both stimulatory (G_s) and inhibitory (G_i) GTP-binding proteins, it catalyzes the formation of the second messenger cyclic AMP (cAMP), and it is expressed in at least 8 distinct isoforms with different regulatory properties and tissue distributions [13–17]. Cyclic AMP participates in the regulation of diverse fundamental cellular processes such as proliferation and differentiation, as well as cell type-specific functions. Recently, it has become apparent that, in addition to its second messenger function, cAMP acts as a gate which regulates signal flow through other pathways [18]. The discovery of adenylyl cyclase isoform multiplicity and diversity, together with important new functions ascribed to cAMP, have renewed interest in this classical signaling pathway [17,18].

The interaction of thrombin with the adenylyl cyclase signaling system has been studied in several cell types. For example, in platelets it was shown that low (< 0.1 U/ml) concentrations of thrombin inhibit cyclic AMP accumulation through activation of a pertussis toxin (PTX)-sensitive G_i protein [19,20]. This observation was later confirmed in fibroblasts [21], and in human erythroleukemia (HEL) megakaryoblastic cell membranes [22]. However, in intact HEL cells, the same study found that similar doses of thrombin markedly potentiated the cAMP response to prostaglandin and cAMP-increasing agents [22]. A stimulatory effect of thrombin on cAMP levels was recently also reported in human erythroid progenitor cells [23]. In human umbilical vein endothelial cells (HUVEC), one study found a small but significant increase in cAMP levels following stimulation with 1 U/ml thrombin [24]. Another found a small effect of thrombin on cAMP levels that was significantly augmented by cholera toxin [25], while yet other studies reported that thrombin induces a several-fold increase in cAMP levels in the presence of the phosphodiesterase inhibitor IBMX [3,26]. Based on such evidence, a recent review concluded that thrombin increases cAMP levels in EC [11]. In another study, however, no effect of thrombin on intracellular cAMP levels was detected in bovine pulmonary artery EC [27]. To date, no inhibitory effects of thrombin on cAMP production have been reported in EC.

In addition to the rather ill-defined role of thrombin in regulating cAMP, EC activation by thrombin involves a number of other well-characterized signaling cascades. Most notably, binding of thrombin to its receptor activates phospholipase C_β, which in turn catalyzes the transient production of inositol trisphosphate and diacylglycerol from membrane phosphatidylinositols [10]. Inositol trisphosphate mobilizes the release of Ca^{2+} from internal stores and also contributes to the entry of extracellular Ca^{2+}, whereas diacylglycerol activates protein kinase C (PKC). Thrombin can also activate phospholipase D, which catalyzes the sustained formation of diacylglycerol from phosphatidylcholine and, thus, the subsequent sustained activation of PKC [10]. Interestingly, both PKC and intracellular Ca^{2+} can affect adenylyl cyclase activity through cross-talk interactions [17,28].

We previously reported the isolation and characterization of a microvascular EC type from the rat adrenal medulla, RAMEC [29]. These cells express a distinct set of adenylyl cyclase isoforms [30]. Here, we report that thrombin inhibits basal and agonist-stimulated cAMP accumulation in RAMEC; this inhibitory action requires proteolytic activation of the thrombin receptor and is sensitive to PTX. These findings suggest that the thrombin receptor in this cell type is negatively coupled to adenylyl cyclase through a G_i-protein. Further, we present evidence suggesting that the inhibitory effect of thrombin on cAMP in RAMEC is independent of second messengers generated downstream of the phospholipase C cascade.

2. Materials and methods

2.1. Materials

Human α- and γ-thrombins were prepared as previously described [31]. These preparations had fibrinogen clotting activities of 2712 and 10.24 U/mg protein, and active site titrations of 2.98×10^{-5} and

4.57×10^{-5} M, respectively. 1 U of α-thrombin corresponds to a concentration of approx. 10 nM. D-Phe-Pro-Arg-chloromethyl ketone (PPACK)-α-thrombin, and diisopropylphosphoryl (DIP)-α-thrombin were prepared as previously described [32], and were essentially enzymatically inactive. The synthetic thrombin receptor agonist peptide SFLLRNPNDKYEPF (TRAP) was obtained from Peninsula Laboratories (Belmont, CA). Bovine trypsin type 1, bovine plasmin and porcine pancreatic elastase were purchased from Sigma Chemical Co. (St. Louis, MO), and their proteolytic activity is defined in units as described in the Sigma catalog. Forskolin, isoproterenol, dibutyryl cyclic AMP (dbcAMP), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), 4-phorbol-12-myristate-13-acetate (PMA), collagenase type I, staurosporine, and EDTA were also obtained from Sigma. Cell permeant 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetomethylester (BAPTA-AM), and fura-2 acetoxymethylester (fura-2/AM) were obtained from Molecular Probes (Eugene, OR). Pertussis toxin (PTX) was obtained from List Laboratories (Cambell, CA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, and cell culture grade trypsin-EDTA were purchased from Gibco (Grand Island, NY). Fetal calf serum was obtained from HyClone Labs (Logan, UT). Penicillin and streptomycin were supplied by Fisher (Pittsburgh, PA). Fungizone was obtained from the local pharmacy. Disposable, cell culture grade labware was supplied by Corning (Corning, NY).

2.2. Endothelial cell culture

Microvascular endothelial cells from the rat adrenal medulla (RAMEC) were isolated with a modification of the method of Mizrachi et al. [33]. Briefly, after removal of the adrenal glands from 5–6 animals, the medullae were excised under a stereo microscope, triturated, and digested with collagenase type I. Pure cultures of endothelial cells were obtained following differential plating and, after establishing colonies of primary isolates, cloning by limited dilution. RAMEC were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone, and passaged with trypsin every 4 days. The endothelial nature of all cultures was routinely confirmed by their hystiotypic, contact-inhibited cobblestone morphology, and by the ability of the cells to endocytose di-indocarbocyamine labelled, acetylated low-density lipoprotein and to express von Willebrand factor as previously described [29]. RAMEC from passages 5 to 20, which were used in our experiments, maintain expression of all EC-specific characteristics mentioned above.

2.3. Measurement of cAMP

Cells were plated in 12-well plates at a density of 15 000 cells per cm^2. One day after reaching confluence (3–4 days after plating), the cells were washed with serum-free medium twice and then stimulated with the various compounds for 5 min in serum-free medium. Physiological activation of the adenylyl cyclase cascade was obtained with the β-adrenergic receptor agonist isoproterenol. In preliminary experiments, we established that maximal stimulation of cAMP accumulation was obtained with 1 μM isoproterenol (data not shown). Therefore, this concentration of the agonist was used in all subsequent experiments. The diterpene, forskolin, which binds to, and activates adenylyl cyclase with an EC_{50} ranging between 5–40 μM [34,35], was also used, at concentrations of 10 or 100 μM. Further, a lipid-soluble analogue of cAMP, dbcAMP, which is taken up by cells, was used to obtain high intracellular cAMP levels without activation of the upstream signaling cascade. Finally, the phosphodiesterase inhibitor IBMX was used to inhibit cAMP degradation by phosphodiesterases. This is often necessary as the intracellular concentration of cAMP in many cell types, including EC, despite constant production by basally active adenylyl cyclase, is usually kept very low due to continuous degradation of cAMP by phosphodiesterases [35].

At the end of an experiment, cAMP was extracted, and measured by a competitive radioligand binding assay, as previously described [35]. The sensitivity of the cAMP assay was 0.25 pmol cAMP per assay tube. The protein content of the wells was determined with a commercially available colorimetric assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.4. Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured as described elsewhere [36]. Briefly, cells were seeded on glass cover slips at 10 000 cells per cm^2 and used 2–3 days after plating. The cells were incubated for 30 min at 37°C with 2 μM fura-2/AM dissolved in modified Krebs' buffer of the following composition (in mM): 140.0 NaCl, 1.5 $CaCl_2$, 5.9 KCl, 1.2 $MgCl_2$, 11.5 HEPES-NaOH, and 10.0 glucose, pH 7.4. The cells were placed on the stage of a Zeiss inverted microscope (equipped with a 40 × fluorite objective) and superfused with Krebs buffer at a rate of 1.5 ml/min. Individual cells were excited alternatively at 360/390 nm via a rotating filter wheel (speed of 2–3 s^{-1}), and fluorescence was measured at 510 nm. Autofluorescence was measured on cell-free parts of the coverslips and automatically subtracted from the Ca^{2+} signals. Apparent free Ca^{2+} concentrations were calculated from the ratio of the background-corrected fluorescence signals. The effective dissociation constant of Fura-2 was obtained from in vitro calibrations [36].

2.5. Statistical analysis

Each experiment included triplicate wells for each condition tested and was repeated at least 3 times, unless otherwise specified. The results for each experimental group are expressed as mean ± S.E.M. The significance of variability amongst various groups was determined by one-way analysis of variance (ANOVA test). Calculations of IC_{50} were done with the Origin software package (MicroCal Software, Northampton, MA).

3. Results

3.1. Thrombin modulation of basal and agonist-stimulated cAMP accumulation

Incubation of RAMEC for 5 min with isoproterenol, forskolin, IBMX, or dbcAMP, resulted in significantly elevated cAMP levels as compared to unstimulated cells (Table 1). When applied to the cells for 1–30 min, and at concentrations of up to 1 U/ml, α-thrombin did not significantly affect intracellular cAMP levels (Table 1 and data not shown). When applied immediately before addition of the cAMP-elevating compounds, 0.1 U/ml of α-thrombin significantly inhibited cAMP accumulation induced by isoproterenol, forskolin, or IBMX, but it did not reduce high intracellular cAMP levels obtained with dbcAMP (Table 1). Thus, the inhibitory effect of thrombin does not result from its direct effect on the formed cAMP, but occurs at a step of the adenylyl cyclase cascade prior to cAMP formation.

The inhibitory effect of thrombin on both basal (in the presence of IBMX) as well as isoproterenol- and forskolin-induced cAMP levels was further studied. Significant inhibition of the effect of 1 μM of isoproterenol was found with 0.001 U/ml of α-thrombin; this inhibition showed an IC_{50} of approx. 0.0015 U/ml, and reached a maximum level of 85% inhibi-

Table 1
Effect of various cAMP-generating compounds on intracellular cAMP levels in RAMEC in the absence and presence of α-thrombin

Stimulus	No thrombin (pmol cAMP/mg protein)	+Thrombin (pmol cAMP/mg protein)	Inhibition (%) [a]
None ($n = 17$)	18.6 ± 2	17.7 ± 2.4	4.8 ± 1.6
Isoproterenol 1 μM ($n = 17$)	919 ± 238	161 ± 61	84.9 ± 2.2 ***
Forskolin 10 μM ($n = 6$)	245 ± 82	114 ± 11	57.5 ± ± 10.3 **
Forskolin 100 μM ($n = 16$)	7474 ± 907	3202 ± 369	57.3 ± 4.2 **
IBMX 1 mM ($n = 9$)	184 ± 35	105 ± 24	47.6 ± 4.0 **
DbcAMP 50 μM ($n = 4$)	95 ± 115	92 ± 54	1.4 ± 3.5
DbcAMP 500 μM ($n = 4$)	1027 ± 209	1008 ± 608	2.5 ± 8.6

Thrombin was used at 0.1 U/ml. Values are mean ± S.E.M. of the number of experiments indicated.
[a] Values in this column denote percent inhibition of agonist-stimulated cAMP accumulation in the presence of thrombin, extracted from the raw data presented in the other 2 columns. Basal cAMP levels have been deducted in each case before calculation of the inhibitory effect of thrombin. Two or three asterisks indicate $P < 0.01$ and $P < 0.001$ level of statistical significance, respectively.

tion at 0.1 U/ml of α-thrombin (Fig. 1). In the cases of forskolin- or IBMX-induced cAMP levels, the threshold concentrations of thrombin required to elicit detectable inhibition were right-shifted by approx. one order of magnitude, while its maximal inhibitory effect on both agents was approx. 50–60% and was reached also with 0.1 U/ml (Table 1, Fig. 1). The inhibitory effect of higher thrombin concentrations (up to 1 U/ml) was unchanged in the case of isoproterenol, but it was slightly reduced in the cases of IBMX and forskolin (Fig. 1 and data not shown).

The time requirements for thrombin inhibition were determined by exposing the cells to α-thrombin for 0–15 min, while isoproterenol was added only during the last 5 min of the incubation period. The inhibitory effect was maximal when α-thrombin was applied concomitantly with, or up to 2 min before, addition of the agonist, and gradually declined if thrombin was applied for longer or shorter periods (Fig. 2). This rapid fall of thrombin's effect is consistent with the previously reported desensitization of the thrombin receptor shortly after activation [11].

To ensure that the reduction of intracellular cAMP levels in thrombin-treated cells was not due to increased extrusion of cAMP from the cell, we measured both the intracellular and medium content of cAMP in cells stimulated with either isoproterenol or forskolin in the presence and absence of thrombin. These experiments showed that the thrombin-induced decrease in agonist-stimulated intracellular cAMP accumulation is not due to increased extrusion of cAMP in the media (data not shown).

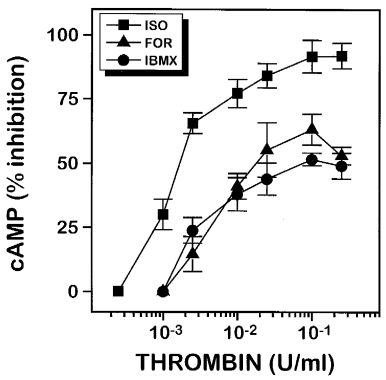

Fig. 1. Effect of thrombin on isoproterenol-, forskolin-, and IBMX-stimulated cAMP accumulation in RAMEC. The cells were stimulated for 5 min with either 1 μM isoproterenol (ISO, squares), 100 μM forskolin (FOR, triangles), or 1 mM IBMX (circles) in the presence of increasing amounts of α-thrombin (0.00025–0.25 U/ml). Results are expressed as percent inhibition of the agonist-stimulated cAMP levels in the presence of thrombin and represent the mean ± S.E.M. from 3 independent experiments, each performed in triplicate wells.

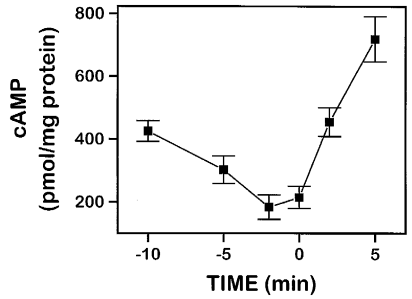

Fig. 2. Time-dependence of the effect of thrombin on isoproterenol-induced cAMP accumulation. The cells were stimulated for 5 min with 1 μM isoproterenol. Thrombin (0.025 U/ml) was added to the cells, 10, 5, or 2 min before, simultaneously with, or 2 min after, isoproterenol (These time points correspond with graph points (−10, −5, −2, 0, and 2 min, respectively). Results are expressed as pmol cAMP/mg protein and represent the mean ± S.E.M. of triplicate determinations from one representative experiment out of two that gave similar results.

3.2. Thrombin receptor involvement

Thrombin activates a G protein-coupled cell surface receptor by proteolytic cleavage and exposure of a new NH_2-terminus, which in turn binds to and activates the receptor [9]. A synthetic thrombin receptor activating peptide (TRAP) with sequence mimicking this terminus activates the receptor in the absence of thrombin and is able to reproduce many of the cellular actions of this protease [9,10]. A concentration of 10 μM of TRAP inhibited isoproterenol-induced cAMP accumulation to a similar extent as 0.025 U/ml of α-thrombin (Table 2). The reduced efficiency of the peptide (by more than 3 orders of magnitude) in comparison to thrombin is probably due to the ability of one thrombin molecule to cleave and thus activate many receptor molecules, while each molecule of TRAP can only bind to and activate only one receptor molecule [9]. Further, γ-thrombin, which lacks the fibrinogen binding site of native α-thrombin but retains full proteolytic activity [31], inhibited isoproterenol-stimulated cAMP accumulation almost as efficiently as α-thrombin (Table 2).

Table 2
Inhibitory effect of TRAP, active and inactive thrombin analogs, and other proteases on isoproterenol-stimulated cAMP levels in RAMEC

Stimulus	% Inhibition	(*n*)
Set A:		
α-thrombin (0.025 U/ml)	72.0 ± 3.6 **	(4)
TRAP (10 μM)	54.8 ± 5.4 **	(4)
γ-thrombin (0.025 U/ml)	65.2 ± 7.0 **	(4)
PPACK-α-thrombin (25 nM)	2.4 ± 4.3 n.s.	(3)
DIP-α-thrombin (25 nM)	7.8 ± 6.7 n.s.	(2)
Set B:		
α-thrombin (0.025 U/ml)	77.8 ± 6.3 **	(3)
trypsin (0.1 U/ml)	32.3 ± 5.4 *	(3)
trypsin (1 U/ml)	53.9 ± 7.5 *	(3)
plasmin (0.2 U/ml)	35.0 ± 6.8 *	(3)
elastase (0.2 U/ml)	14.3 ± 9.3 n.s.	(2)

Values are mean $\pm$ S.E.M. of the number of experiments indicated in the parenthesis. 0.025 U/ml of α-thrombin correspond to a concentration of approx. 0.25 nM. Results are expressed as percent inhibition of agonist-stimulated cAMP accumulation in the presence of the indicated compounds. In each case, basal cAMP levels were deducted from values in the stimulated cells before calculation of the inhibitory effect of each compound.
One or two asterisks indicate $P < 0.05$ and $P < 0.01$ level of statistical significance, respectively.
n.s. not significant.

By contrast, the proteolytically inactive thrombin analogs PPACK-α-thrombin and DIP-α-thrombin [32] had no effect on agonist-induced cAMP accumulation, even when used at concentrations 100 times higher than α-thrombin (Table 2). Finally, the inhibitor of thrombin activity, hirudin, reduced the inhibitory action of thrombin, but had no effect on the inhibitory action of TRAP (Fig. 3). These data suggest that the inhibitory action of thrombin on agonist-induced cAMP accumulation is mediated by a proteolytically-activated thrombin receptor.

3.3. Inhibition by other proteases

It has been shown that activation of the thrombin receptor following proteolytic cleavage is not a unique property of thrombin but can also be achieved with other serine proteases possessing similar cleavage site specificity, such as trypsin and plasmin [9,37]. Both trypsin, a lysine/arginine-specific protease, and plasmin, which has a similar cleavage site specificity as trypsin but with higher specificity for lysine, significantly inhibited isoproterenol-stimulated cAMP accumulation. However, their effect was smaller than that of thrombin even when used at several fold higher proteolytic activities than thrombin (Table 2). By contrast, elastase, which hydrolyses amides and esters, was without effect (Table 2). These data provide further evidence that proteolytic activation of the thrombin receptor by other serine proteases is sufficient for inhibition of agonist-induced cAMP accumulation, although alternative explanations are possible (see Discussion).

3.4. Pertussis toxin sensitivity

Adenylyl cyclase is under the dual control of stimulatory and inhibitory G-proteins [12]. Inhibitory G_i-proteins that couple extracellular receptors to adenylyl cyclase can be inactivated by pertussis toxin (PTX)-catalyzed ADP ribosylation [19]. To test whether such proteins are involved in thrombin's inhibitory effect, we preincubated RAMEC monolayers with various concentrations of PTX (0.1–500 ng/ml) for 4 h before agonist application. A concentration of 10 ng/ml of PTX effectively abrogated the inhibitory effect of thrombin on isoproterenol-stimulated cAMP accumulation (Fig. 4a). It should be noted that PTX, even at a higher concentration (100 ng/ml), did not affect either basal (17.2 ± 1.7 and

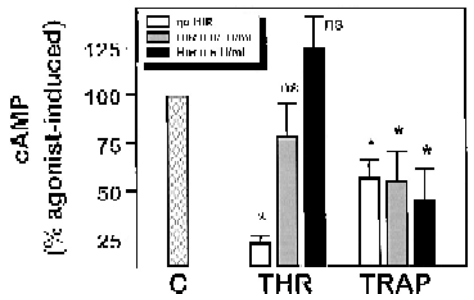

Fig. 3. Effect of hirudin on the inhibitory action of thrombin or TRAP on isoproterenol-induced cAMP accumulation. RAMEC were preincubated for 10 min with either 0.05 or 0.5 U/ml hirudin (HIR). Thereafter, 0.025 U/ml thrombin (THR) or 2.5 μM TRAP were added, followed by 1 μM isoproterenol. The experiment was terminated 5 min after addition of isoproterenol. Results are expressed as percent of agonist-stimulated cAMP levels (depicted at column C) and represent the mean $\pm$ S.E.M. of triplicate determinations from one representative experiment out of two that gave similar results. Asterisk (*) denotes values significantly ($P < 0.02$) lower than in column C.

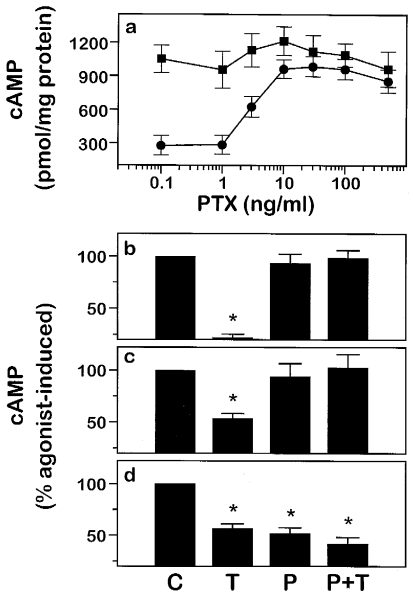

Fig. 4. Effect of PTX on the inhibitory action of thrombin on agonist-induced cAMP accumulation in RAMEC. (a) Following preincubation for 4 h with PTX (0.1–500 ng/ml), the cells were stimulated for 5 min with 1 μM isoproterenol, in the absence (squares) and presence (circles) of 0.1 U/ml thrombin. Results are expressed as pmol/mg protein and represent the mean $\pm$ S.E.M. of triplicate determinations from one representative experiment out of three that gave similar results. (b–d) Following preincubation for 4 h with 100 ng/ml PTX (P), the cells were stimulated for 5 min with 1 μM isoproterenol (b), 1 mM IBMX (c), 100 μM forskolin, or (d), in the presence and absence of 0.1 U/ml thrombin (T). Results are expressed as percent of agonist-induced cAMP levels in each case (column C), and represent the mean $\pm$ S.E.M. of 3 experiments each done in triplicate wells. Asterisk (*) denotes values significantly ($P < 0.01$) lower than in column C.

18.4 ± 1.5 ng/ml protein in unstimulated and PTX-treated cells, $n = 3$) or isoproterenol-stimulated cAMP levels (Fig. 4b). Similarly, PTX did not alter the IBMX-stimulatory effect on cAMP levels, but it abolished the inhibitory action of thrombin (Fig. 4c). These results suggest that thrombin is coupled to adenylyl cyclase through a PTX-sensitive G_i-protein.

In forskolin-stimulated cells, PTX (100 ng/ml for 4 h), not only did not abrogate the inhibitory effect of thrombin on cAMP accumulation, but it had a significant inhibitory effect of its own, similar to the effect of thrombin (Fig. 4d). In the presence of PTX, thrombin was unable to significantly reduce cAMP levels (Fig. 4d). Similar results were obtained with higher concentrations of PTX (up to 1 μg/ml). Thus, the presumed direct activation of adenylyl cyclase by forskolin [34] may involve a PTX-sensitive step.

3.5. Protein kinase C activators and inhibitors

Previous experiments suggested a cross-talk between PKC and adenylyl cyclase activity [28]. The ability of thrombin to activate PKC in several EC types is well documented [6,10] and is also implied from our results (see below, Section 3.7). To assess the possible involvement of PKC in thrombin-induced inhibition of agonist-stimulated cAMP accumulation, we used the PKC inhibitors H7 and staurosporine, at concentrations considered rather specific for PKC inhibition (H7, 10 μM; staurosporine, 100 nM) [38]. In 2 independent experiments, each containing 3 wells for each condition tested, preincubation for 30 min with either inhibitor had no effect on either basal, isoproterenol-, or forskolin-stimulated cAMP levels, in the presence or in the absence of α-thrombin (data not shown). It should be noted that both inihibitors were $> 90\%$ effective in inhibiting PKC activity, as determined by using a commercial, non-radioactive PKC assay (MESACUP from Medi-

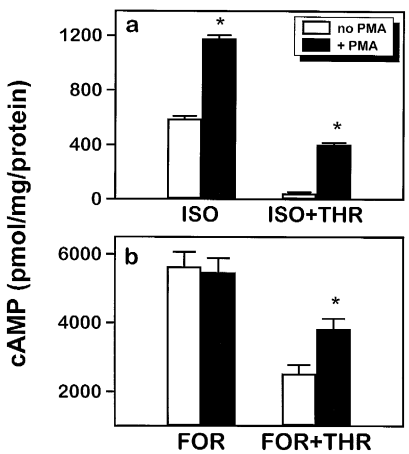

Fig. 5. Effect of PMA on agonist-stimulated cAMP accumulation. The cells were stimulated for 5 min with either (a) 1 μM isoproterenol (ISO), or (b) 100 μM forskolin (FOR), in the presence and absence of PMA (100 nM for 10 min), or thrombin (THR, 0.1 U/ml for 5 min), or both. Results are expressed as pmol/mg protein and represent the mean $\pm$ S.E.M. of triplicate determinations from one representative experiment out of three that gave similar results. Asterisk (*) indicates values significantly ($P < 0.01$) higher in the presence than in the absence of PMA.

cal and Biological Laboratories, Watertown, MA) according to the manufacturers' instruction (data not shown). Thus, the involvement of PKC in thrombin-induced reduction of cAMP levels in RAMEC. is unlikely.

PKC activation with PMA (1 μM for 5 min or 100 nM for 10 min) did not affect basal cAMP levels (20 ± 4.5 and 24.3 ± 6.4 pmol/mg protein in unstimulated and PMA-treated cells, respectively, $n = 3$), but it significantly potentiated isoproterenol-stimulated cAMP levels when applied concomitantly with the agonist (Fig. 5a). By contrast, forskolin-induced cAM2P production was unaffected by PMA (Fig. 5b). Interestingly, PMA partially reversed the inhibitory effect of thrombin on cAMP levels induced by either of the above agonists. For example, in the case of isoproterenol-induced cAMP accumulation, the inhibitory effect of thrombin was $93 \pm 5.6\%$ in the absence of PMA, but only $64.6 \pm 5\%$ in its presence ($n = 3$, and $P < 0.05$, in both cases). In the case of forskolin-induced cAMP accumulation, the PMA-induced partial reversal of thrombin's effect on forskoln-induced cAMP is apparent from the data shown in Fig. 5b. Collectively, our results with PMA and PKC inhibitors suggest that, while thrombin-activated PKC does not affect cAMP homeostasis, PMA-activated PKC affects both the isoproterenol-stimulated cAMP elevation and the thrombin-induced inhibition of this elevation. This apparent dichotomy may reflect differences in the nature of PKC activation by PMA and physiological agonists such as thrombin [39].

3.6. Intracellular Ca^{2+} chelation

The regulation of adenylyl cyclase activity by intracellular Ca^{2+} has been well documented [17]. To investigate the possibility that thrombin-induced Ca^{2+} elevation may be involved in the inhibitory effect of this agonist on cAMP accumulation, we used the cell-permeant Ca^{2+} chelator BAPTA-AM. Incubation of RAMEC with 25 μM BAPTA-AM for 60 min did not affect basal cAMP levels (19.2 ± 5 and 16.4 ± 4 pmol/mg protein in unstimulated and BAPTA-AM-treated cells, respectively, $n = 3$), but it potently reduced isoproterenol-stimulated cAMP levels both in the absence and presence of α-thrombin (Fig. 6a). The inhibitory effect of thrombin, however, was not

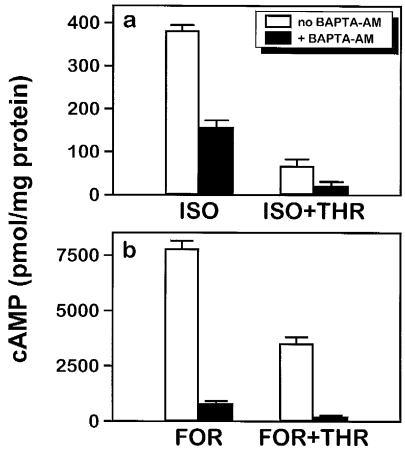

Fig. 6. Effect of BAPTA-AM on agonist-stimulated cAMP accumulation. The cells were stimulated for 5 min with either (a) 1 μM isoproterenol (ISO), or (b) 100 μM forskolin (FOR), in the presence and absence of BAPTA-AM (25 μM for 60 min) or thrombin (THR, 0.1 U/ml for 5 min) or both. Results are expressed as pmol/mg protein and represent the mean $\pm$ S.E.M. of triplicate determinations from one representative experiment out of three that gave similar results.

affected by intracellular Ca^{2+} chelation (75.9 ± 3.6 and $73.5 \pm 5\%$ inhibition in the absence and presence of BAPTA-AM, respectively, $n = 3$). Similar results were obtained in forskolin-stimulated cells, although the inhibitory effect of BAPTA-AM was much more potent in this case (Fig. 6b). These results suggest that, while intracellular Ca^{2+} homeostasis is pivotal for the generation of cAMP by extracellular stimuli, thrombin-induced elevation of intracellular Ca^{2+} is inconsequential for the inhibitory effect of this protease on agonist-stimulated cAMP accumulation.

3.7. Thrombin stimulation of intracellular Ca^{2+}

Several studies have shown that thrombin activates phospholipase C and evokes subsequent Ca^{2+} release from intracellular stores in various types of EC (reviewed in [10]). However, no such studies have been reported in RAMEC. We found that monolayers of RAMEC, cultured under identical conditions as in the cAMP measurement studies, responded to thrombin (0.1–1 U/ml) with a fast, transient increase in intracellular Ca^{2+} levels which peaked 15–30 s after addition of the agonist and returned to almost basal levels within 5 min (data not shown). The nature of the thrombin response is characteristic for inositol

trisphosphate-induced Ca^{2+} release from intracellular stores. This result confirms that thrombin activates the phospholipase C cascade also in RAMEC.

4. Discussion

The major finding of this study is that thrombin inhibits cAMP accumulation in cultured microvascular EC. This action of thrombin (i) requires the proteolytic cleavage and activation of the thrombin receptor, and (ii) is sensitive to the bacterial toxin PTX. Together, these findings suggest that the thrombin receptor in this cell type is negatively coupled to adenylyl cyclase through a PTX-sensitive G_i-protein. To the best of our knowledge, our study is the first to demonstrate inhibitory coupling of adenylyl cyclase to the thrombin receptor in endothelial cells. Previous studies found either no effect or increased cAMP levels in response to thrombin [3,24–27]. The reasons for the differences between our findings and the previous studies may reside in the types of EC used. Most of the above studies were done in HUVEC or other large vessel EC, while we used cells of microvascular origin. Our preliminary studies with diverse EC types confirm the previously reported stimulatory effect of thrombin on adenylyl cyclase activity in HUVEC, but also suggest that the effect of thrombin on intracellular cAMP levels in different EC types can be inhibitory, stimulatory, or none, depending on the tissue and species origin of the cells (Manolopoulos, V.G. and Lelkes, P.I., manuscript in preparation). This EC type-specific action of thrombin on intracellular cAMP homeostasis further extends our previous findings on the heterogeneity of the adenylyl cyclase signaling system in various types of EC [35].

In an earlier study in fibroblasts, Magnaldo et al. [21] found a biphasic effect of thrombin on stimulated adenylyl cyclase consisting of a PTX-sensitive inhibitory effect at concentrations up to 0.1 nM (roughly equivalent to 0.01 U/ml), followed by a PKC-mediated potentiating effect at higher concentrations (0.1–10 nM). A similar mechanism may account for the partial reduction of the inhibitory effect that we found with higher thrombin concentrations (0.25–1 U/ml) when forskolin or IBMX were used to activate adenylyl cyclase (Fig. 1 and data not shown). It should be noted, however, that no such reversal was seen in the case of isoproterenol-activated adenylyl cyclase even at a thrombin dose (1 U/ml) equal to the highest dose used in the above mentioned study (data not shown).

The inhibitory effect of thrombin on adenylyl cyclase fulfilled several established criteria [9] required for ascribing a cellular action of thrombin to proteolytic activation of the G-protein-coupled thrombin receptor (Table 2, Fig. 3): (i) TRAP, a synthetic peptide resembling the exposed NH_2-terminus of the thrombin receptor mimicked the inhibitory action of thrombin. (ii) Hirudin, an established thrombin inhibitor, abolished the inhibitory action of thrombin but not that of TRAP. (iii) γ-Thrombin, which lacks the fibrinogen binding site of α-thrombin but retains full proteolytic activity, was able to reproduce the inhibitory action of thrombin. (iv) PPACK-α-thrombin and DIP-α-thrombin, two thrombin analogs lacking proteolytic activity, did not affect agonist-stimulated cAMP levels. Thus, these results leave little doubt that the effect of thrombin on adenylyl cyclase activity is mediated through proteolytic activation of the thrombin receptor.

The efficacy of γ-thrombin is somewhat surprising, as the current model of thrombin receptor activation predicts that the anion-binding site of α-thrombin (which is absent in γ-thrombin) may be important in positioning the protease on the thrombin receptor for appropriate cleavage of the receptor amino-terminus [9]. However, this has not been proven to be critical in every case and there are examples in the literature showing that γ-thrombin has almost the same efficacy as α-thrombin [40–42]. Also, in another study in RAMEC, we found that α- and γ-thrombins show similar efficacy in stimulating extracellular matrix protein deposition [43]. Moreover, the possibility has not been ruled out that thrombin may proteolytically activate distinct receptors in different species/cell types, each of them with different thrombin binding requirements for full activation.

In agreement with previous reports [9,37], our results that trypsin and plasmin also reduce agonist-stimulated cAMP levels (Table 2) suggest that proteolytic cleavage of the thrombin receptor after an arginine residue by proteases other than thrombin may be sufficient for its activation. However, the extent of proteolysis of the known thrombin receptor

by these proteases was not determined, and it is thus possible that other protease-activated receptors may be involved in the observed effects of trypsin and plasmin. For example, a protease-activated receptor (PAR-2) distinct from the thrombin receptor was recently cloned in the mouse and subsequently found also in human EC [44,45]. This receptor is sensitive to trypsin but not to thrombin, thus it is unlikely that the action of thrombin observed in our study is mediated through PAR-2 activation. Nevertheless, the possibility cannot be excluded that the inhibitory effects of trypsin and/or plasmin on adenylyl cyclase may be mediated through proteolytic activation of PAR-2 or other protease activated-receptors likely to be discovered in the future [46].

Pertussis toxin ADP-ribosylates and thus inactivates the alpha subunit of the inhibitory G_i proteins coupled to adenylyl cyclase [12,19]. The presence of G_i proteins that are ADP-ribosylated by PTX has been demonstrated in virtually all cell types [13,47], including several types of endothelial cells [25,27, 41,48–50]. Thus, although no direct detection of ADP-ribosylation substrates in response to PTX was performed in this study, the ability of PTX to negate the inhibitory effect of thrombin on cAMP accumulation in RAMEC strongly suggests that a G_i protein directly couples the thrombin receptor to adenylyl cyclase in this cell type. Such a coupling has been previously reported in platelets, fibroblasts, and megakaryocytes, but not in EC [19–22]. Interestingly, in intact platelets, PKC activation with phorbol ester reversed G_i-dependent, hormone-sensitive inhibition of adenylyl cyclase, without affecting the G_i-independent portion of the inhibition [51]. In our study, PMA had a similar effect (Fig. 5), further supporting the idea that the inhibitory effect of thrombin on adenylyl cyclase activity in RAMEC is mediated by G_i.

Forskolin-stimulated cAMP levels were inhibited by PTX to a similar extent as by thrombin. This is a surprising result, as PTX is expected to potentiate (through removal of the G_i inhibitory component) rather than inhibit agonist-induced cAMP accumulation, and it suggests that the presumed direct activation of adenylyl cyclase by forskolin might also comprise a PTX-sensitive G_i protein. One possible explanation may be provided by a recent study which showed that certain adenylyl cyclase isoforms have independent sites for interaction with $G_{s\alpha}$ (site 1, stimulatory) and $G_{i\alpha}$ (site 2, inhibitory), and that high concentrations of $G_{i\alpha}$ can interact with site 1 and activate the enzyme [15]. Forskolin may somehow produce high levels of $G_{i\alpha}$ which in turn may activate adenylyl cyclase. Further studies are needed to clarify this point.

Cross-talk between signal transduction pathways plays an important role in the ability of single cells to integrate multiple signal inputs into a unitary cellular response. Extensive interactions between the phospholipase C and adenylyl cyclase pathways have been reported in several cell types including EC [17]. In line with these reports, our results with PMA and BAPTA-AM (Figs. 5 and 6) show that both facets of the phospholipase C signaling, i.e. PKC activation and elevation of intracellular Ca^{2+}, effectively modulate agonist-stimulated adenylyl cyclase activity in RAMEC. However, the lack of effect of PKC inhibitors and BAPTA-AM argue against a role of phospholipase C signaling in mediating the inhibitory action of thrombin on adenylyl cyclase in this cell type.

Adenylyl cyclase is expressed in at least 8 distinct isoforms with different tissue distributions [13,17]. All isoforms are responsive to forskolin and $G_{s\alpha}$, but they are differentially regulated by other components of the cellular signaling machinery, such as PKC, Ca^{2+}, $G_{i\alpha}$-proteins, and $G_{\beta\gamma}$-protein subunits [14–17]. Some clues as to the identity of the isoforms involved in the effects of isoproterenol and thrombin in RAMEC emerge from our results. Firstly, the fact that thrombin activation of a G_i protein almost completely inhibits the isoproterenol stimulatory effect suggests that the adenylyl cyclase isoform(s) activated by isoproterenol are the ones sensitive to $G_{i\alpha}$. Thus, isoforms I and II can be excluded, as inhibition of isoform I by $G_{i\alpha}$ is largely absent when the $G_{s\alpha}$-stimulated activity is examined, while activation of G_i results in activation rather than inhibition of isoform II [14]. In any case, isoform I is an unlikely candidate in EC, as its expression has been shown by several studies to be limited to the brain [13,47]. Further, we found that PMA has no significant effect on basal or forskolin-induced cAMP, but it strongly enhances isoproterenol-induced cAMP levels (Fig. 6). Similar results were recently reported in HT4 neural cells, which express only adenylyl cyclase isoforms I and VI [16].

In a previous study, we characterized the expression profile of adenylyl cyclase isoforms in RAMEC: isoforms III and VI are predominant, followed by small amounts of isoform V and only trace amounts of isoforms II and IV [30]. Based on these data, we hypothesize that isoform VI — and perhaps also V — may be the principal adenylyl cyclase isoforms mediating the effects of isoproterenol and thrombin on cAMP homeostasis in RAMEC. Additional support for this hypothesis is provided by Taussig et al. [15] who showed that $G_{s\alpha}$- and forskolin-activated isoforms V and VI are strongly inhibited by $G_{i\alpha}$. However, it should be noted that in a cell containing many of the known isoforms of adenylyl cyclase, all of which are regulated in a highly complex manner, it is unlikely that a single isoform is solely responsible for the observed effects on cAMP levels. Studies with isoform-specific neutralizing antibodies or antisense oligonucleotides are needed to verify this notion.

Both thrombin and cAMP regulate several functions in EC, and often they do so in an opposing fashion. For example, in HUVEC, thrombin induces tissue factor synthesis and cell surface expression, effects that can be inhibited by elevation of cAMP [4]. In the same EC type, thrombin-stimulated platelet activating factor production is inhibited by agents that increase cAMP [6]. Also, thrombin is a potent stimulator of EC monolayer permeability, while elevated cAMP not only reduces the permeability of quiescent EC monolayers, but also abolishes the stimulatory action of thrombin [5,8]. Moreover, in a chick chorioallantoic membrane angiogenesis model, thrombin stimulates angiogenesis [52], while cAMP-elevating agents potently inhibit it [53]. We hypothesize that, through its ability to inhibit adenylyl cyclase, thrombin may provide an endogenous system to antagonize excessive signal transduction by agonists (e.g. adrenaline) that exert their physiological actions through activation of the adenylyl cyclase pathway. Such a control system may be of special relevance in EC at the adrenal medulla, which represent the first cell layer in the vasculature to be exposed to circulating catecholamines produced by chromaffin cells.

In conclusion, we have shown that thrombin inhibits basal and agonist-stimulated cAMP accumulation in RAMEC; this inhibitory action requires proteolytic activation of the thrombin receptor and is sensitive to PTX. These findings suggest that the thrombin receptor in this cell type is negatively coupled to adenylyl cyclase through a G_i-protein.

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