Dual coupling of the α -thrombin receptor to signal-transduction pathways involving phosphatidylinositol and phosphatidylcholine metabolism

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Addition of α -thrombin to quiescent IIC9 cells results in the activation of lipid-metabolizing enzymes associated with signal-transduction cascades. These enzymes include phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), phosphatidylcholine (PC)-specific phospholipases C and D and phospholipase A₂ (PLA₂). Whereas the α -thrombin receptor has been shown to couple with PI-PLCs, it is not clear whether this receptor, or a putative second receptor, couples to one or more of the other phospholipases. In this report we determine whether the cloned receptor couples to all or a subset of these enzymes. We show that (i) an α -thrombin-receptor-activating peptide also elicits the above responses and (ii) addition of enterokinase to IIC9 cells,

stably transfected with an α -thrombin receptor (enterokinase-responsive α -thrombin receptor, EKTR) containing an enterokinase cleavage site in place of an α -thrombin cleavage site, stimulates both PI and PC hydrolysis, including PLA₂. Enterokinase also induces mitogenesis in the IIC9s transfected with EKTR. These results indicate that, in addition to initiating a mitogenic signalling cascade, the cloned α -thrombin receptor couples to enzymes involved in generating PC-derived, as well as PI-derived, second-messenger molecules in IIC9s. Additionally, using the cells transfected with EKTR, we further demonstrate that only activated, i.e. cleaved, receptors are desensitized.

INTRODUCTION

The role of lipid metabolism in transducing mitogenic signals is now well documented [1,2]. For many years, agonist-induced breakdown of cellular phosphatidylinositols (PIs), leading to the production of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol [1–3], and the generation of eicosanoids via the activation of phospholipase A₂ (PLA₂), were thought to be the major lipid-mediated signal-transduction cascades. It is now clear that the induced hydrolysis of cellular phosphatidylcholine (PC) via PC-PLC (where PLC is phospholipase C) or PC-PLD (where PLD is phospholipase D) also plays critical roles in a variety of signal-transduction cascades [4], including those involved in mitogenesis. Clearly, to understand the molecular control mechanisms involved in induced lipid metabolism, it is important to identify the receptors involved in initiating the responses. α -Thrombin, a potent mitogen, is known to induce cellular lipid responses in a number of cell types. An α -thrombin cell-surface receptor was cloned from human [5] and hamster [6] cells and has been shown to couple to PI hydrolysis [7,8]. This receptor, also known as PAR1 (protease-activated receptor 1), belongs to the family of receptors containing seven transmembrane domains, which interact with G-proteins [5,6]. α -Thrombin binds to PAR1 and cleaves a region near the N-terminus, generating a 'new' N-terminus that purportedly serves as a tethered agonist by binding to the receptor [5,7]. In support of this notion, many α -thrombin-induced responses are stimulated by the addition of a peptide, referred to as thrombin-receptor-agonist peptide (TRAP), with sequence identical with the first six residues after cleavage [5,8].

Although the activation of PAR1 induces PI hydrolysis, a

second receptor is thought to be involved in the activation of other α -thrombin-induced responses, including PC hydrolysis. TRAP, for example, does not induce all of the responses normally induced by α -thrombin in some systems, suggesting that a second, TRAP-insensitive, receptor is involved in mediating these responses [9,10]. Two other α -thrombin receptors with different tethered peptides, PAR3 and PAR4, have been cloned and found to be expressed in a variety of tissues. The possible presence of PAR1, PAR3 and PAR4 in the same cell suggests that many of the effects of α -thrombin may be a result of activation of more than one receptor. Both PI and PC hydrolysis are stimulated by high concentrations of α -thrombin, except when fibroblasts are first treated with chymotrypsin. In this case, PI hydrolysis is not induced, whereas PC hydrolysis is only modestly blunted [11]. Interestingly, a chymotrypsin cleavage site is present in PAR1 just prior to the N-terminus, so that chymotrypsin treatment would render this receptor inactive, suggesting that the α -thrombin-induced PC hydrolysis in chymotrypsin-treated cells is mediated by another receptor. Consistent with this notion, platelets [12], but not fibroblasts, isolated from *tr*^{-/-} mice, which lack PAR1 [12,13], responded normally to α -thrombin. These data suggest that PAR3 or PAR4 [14] is involved in mediating the stimulation of PC hydrolysis.

The above data, however, do not require that another α -thrombin receptor exists for the stimulation of PC hydrolysis. Two basic models are consistent with the above data (Figure 1). In the first, or the two-receptor model (Figure 1, top panel), PI-PLC and PLA₂ are activated by PAR1, whereas PC-PLC and PC-PLD are activated by a second independent α -thrombin receptor. PAR1 contains multiple potential chymotrypsin-cleavage sites, which would inactivate this receptor by releasing the

Abbreviations used: PC, phosphatidylcholine; PI, phosphatidylinositol; EKTR, enterokinase-responsive α -thrombin receptor; WTR, wild-type receptor; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; TRAP, thrombin-receptor-agonist peptide; PAR, protease-activated receptor; IP₃, inositol 1,4,5-trisphosphate; β ARK2, β -adrenergic receptor kinase 2.

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Figure 1 Two- and single-receptor models

The two-receptor (top panel) and single-receptor (lower panel) models for the stimulation of lipid metabolism by α -thrombin are shown. See the text for details.

tethered peptide into the medium. The other receptor does not contain these sites. α -Thrombin/TRAP activates PC hydrolysis in chymotrypsin-treated cells by interacting with the second receptor. In the second model, or the single-receptor model (Figure 1, bottom panel), PI-PLC, PC-PLC, PLD and PLA₂ are all activated by PAR1 via different G-proteins [15]. In this model, coupling to PC hydrolysis requires activation of fewer receptors than coupling to PI hydrolysis, so that the receptors remaining after chymotrypsin treatment are sufficient for the activation of PC hydrolysis.

The major difference between the two models is that in the single-receptor model activation of PAR1 releases diacylglycerol from both PI and PC whereas in the two-receptor model, PAR1 releases diacylglycerol only from PI. Importantly in this model, PAR1 can also activate PLA₂. The experiments in this report were designed to test, and discriminate between, the above models. We observed that TRAP mimicked all α -thrombin-induced responses, supporting the single-receptor model. We also found that enterokinase stimulates all the responses normally induced by α -thrombin in the cells containing the enterokinase-responsive α -thrombin receptor (EKTR). These data indicate that PAR1 couples to PI and PC hydrolysis, including PLA₂, and therefore support the single-receptor model.

In view of these data, and given the fact that the continual presence of catalytically active α -thrombin is required for sustained responses, desensitization of PAR1 is an important parameter involved in determining the extent and duration of induced responses. Not unlike other cell-surface receptors, desen-

sitization of PAR1 has been shown to undergo identical desensitization [16,17]. Desensitization required the presence of catalytically active α -thrombin and it was not desensitized by activation of other G-protein-coupled receptors. It is not clear if only activated receptors were desensitized. We took advantage of our EKTR-expressing cells to address this question. Addition of enterokinase to these cells results in the desensitization of the EKTR but not the wild-type receptors (WTRs). Similarly, addition of α -thrombin to these cells results in the desensitization of the WTR without affecting the EKTR. These results indicate that only the activated receptors are desensitized.

MATERIALS AND METHODS

Materials

ZAP-cDNA synthesis kit and pBluescript exo/mung DNA sequencing system were purchased from Stratagene (La Jolla, CA, U.S.A.). Sequenase v2.0 was purchased from USB (Cleveland, OH, U.S.A.). The restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.), other modifying enzymes were from New England Biolabs (Boston, MA, U.S.A.). Tissue-culture-media components, lipofectin reagents and geneticin were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Plastic culture dishes were purchased from Falcon Labware (Lincoln Park, NJ, U.S.A.). Highly purified human α -thrombin (approx. 2900 NIH units/mg, 4000 NIH units/ml) and BSA (radioimmunoassay grade, fraction V) were purchased from Sigma (St. Louis, MO, U.S.A.). Highly purified calf intestine enterokinase was purchased from Biozyme (San Diego, CA, U.S.A.). *Escherichia coli* diacylglycerol kinase was obtained from Calbiochem (La Jolla, CA, U.S.A.). AG1X8 resin (200–400 mesh, formate form) was from Bio-Rad (Hercules, CA, U.S.A.). Thin-layer chromatography plates were purchased from EM Diagnostics (Cherry Hill, NJ, U.S.A.) and Analtech (Newark, DE, U.S.A.). CytoScint scintillation-counting fluid was obtained from ICN (Cleveland, OH, U.S.A.). Radioactive materials {[*Me*-³H]choline (84 Ci/mmol); [*Me*-³H]thymidine (6.7 Ci/mmol); [5,6,8,9,11,12,14,15-³H]arachidonic acid (180–240 Ci/mmol); *myo*-[³H]-inositol (10–20 Ci/mmol)} were purchased from Amersham (Arlington, IL, U.S.A.). The peptides were synthesized and HPLC-purified by the Protein Sequencing and Peptide Synthesis Core at the Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A. The oligonucleotides were synthesized by oligonucleotide and peptide synthesis facility in the Department of Biological Chemistry at the Johns Hopkins University School of Medicine.

Receptor cloning, plasmid construction and expression

The IIC9 cDNA library was prepared using ZAP-cDNA synthesis kit (Stratagene). A hamster receptor was cloned from IIC9 cDNA library by screening it with PCR fragments generated from degenerate oligonucleotide primers derived from the cloned α -thrombin receptor; the primers were GCTCTGGAGCGAGAA/GGAA/GAAA/GAAC/TGA and TACGAATTCGAXCCA/GAAT/CTGCCAG/ATC. Only a single species of cDNA was identified. The deduced protein sequence differed from that of Chinese hamster lung fibroblast cell line CCL39 [6] by a single residue insertion (Pro-84). The cDNA differed by two additional nucleotides in the coding region, which resulted in silent changes. The full-length PAR1 α -thrombin receptor cDNA (3 kb) was subcloned into eukaryotic expression vector pRK5 (pRK5-TR). The α -thrombin cleavage site (LFFSRPNV, with cleavage be-

tween S and R) was converted into an enterokinase-sensitive site (LFFSKDDD, cleavage between S and K) by site-directed mutagenesis in pBluescript by the method of Kunkel et al. [18]. The *EcoRI/XbaI* fragment containing the mutation was excised and used to replace a corresponding *EcoRI/XbaI* fragment on pRK5-TR. The resulting plasmid (pRK5-EKTR) was co-transfected with pSV2Neo into IIC9 cells (1:10, w/w) using lipofectin reagent according to the procedure described by the manufacturer (Gibco-BRL). The colonies resistant to 500 μ g/ml G418 were selected, expanded, and then screened by PCR with primers specific to the mutated receptor sequence. Positive clones were assayed for enterokinase-induced arachidonic acid release. Two independent clones (EKTR11.1, EKTR9) with the strongest response were used for all subsequent experiments.

Cells and cell culture

Cells from the IIC9 cell line, a subclone of Chinese hamster embryo fibroblasts [19], were grown, maintained and serum-starved as described previously [20]. Briefly, cultures were grown and maintained in minimal essential medium (Alpha)/Ham's F-12 medium (1:1, v/v) containing 5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (complete media). Sub-confluent cultures were serum-starved by washing three times with Dulbecco's modified Eagle's medium containing 1 mg/ml BSA, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 20 mM Na-Hepes, pH 7.4. The cultures were incubated with this medium supplemented with 5 mg/ml of human transferrin (serum-free medium) for 2 days at 37 °C. Cultures were washed twice, equilibrated in fresh media for 0.5–1 h, and stimulated as indicated.

Arachidonic acid-release assay

The release of arachidonic acid was determined by quantifying the release of [3 H]arachidonic acid and its metabolites from radiolabelled cell cultures as described previously [11].

Diacylglycerol kinase assay

The mass of cellular *sn*-1,2-diacylglycerols was quantified by using *E. coli* diacylglycerol kinase as described by Priess et al. [21] and modified by Wright et al. [20].

Assay for choline and phosphorylcholine

Choline and phosphorylcholine levels were quantified by radiolabelling cultures with [3 H]choline followed by aqueous extraction and separation by thin-layer chromatography as described previously [22].

Assay for inositol phosphates

The levels of inositol phosphates were determined by radiolabelling cultures with *myo*-[3 H]inositol, followed by extraction and separation of the individual inositol phosphates on AG1X8 anion-exchange columns as described previously [11].

Assay for cell growth

The incorporation of [3 H]thymidine into cells was determined by radiolabelling cultures with [3 H]thymidine as described by Raben and co-workers [23]. Cell numbers were obtained by trypsinizing the cells and quantifying cell number in a cell counter (Coulter

Electronics, Hialeah, FL, U.S.A.) as described by Raben et al. [23].

RESULTS

TRAP induces PI and PC hydrolysis, arachidonic acid release and mitogenesis in IIC9 cells

α -Thrombin induces a biphasic increase in cellular diacylglycerol mass in IIC9 cells, an early transient phase from PI hydrolysis and a later prolonged phase from PC hydrolysis [20,24]. Addition of TRAP (SFFLRNP) to quiescent IIC9 fibroblasts results in an increase of diacylglycerols. The magnitude and time courses are similar to those observed with catalytically active α -thrombin (Figure 2). A peak of diglycerides, two-fold above basal levels, is detected at 15 s (Fig 2, inset) followed by a second peak, three-fold above basal, at 5 min. Although the diacylglycerol levels decline, they remain above control for at least 3 h (Figure 2).

As mentioned above, α -thrombin-stimulated diacylglycerols are derived from two phospholipid sources: PIs, which contribute the majority of the diglycerides generated early (15 s); and PCs, which contribute most, if not all, of the diacylglycerols after 5 min [24]. The data in Figure 2 suggest that TRAP stimulates diacylglycerol formation through the same signal-transduction pathways as α -thrombin. We next examined the time course of TRAP-induced PI and PC hydrolysis (Figure 3). Whereas PI hydrolysis lasts only a few minutes (Figure 3, top panel), PC hydrolysis was sustained, remaining elevated for at least 4 h (Figure 3, bottom panel). These data are virtually identical with those seen with α -thrombin (Figure 3), supporting the notion that diacylglycerols generated by TRAP and α -thrombin are derived from the same phospholipid sources.

In addition to stimulating lipid metabolism, α -thrombin is a

Figure 2 TRAP induces an increase in 1,2-diacylglycerol levels

Subconfluent IIC9 cells in culture dishes were serum-starved for 48 h as described in Materials and methods. Main graph: cell cultures were incubated with 1.5 NIH units/ml α -thrombin (Δ), 400 μ M TRAP (SFFLRNP; $\square$) or control serum-free media ($\circ$) for the indicated times. Incubations were terminated by Bligh–Dyer extraction and mass levels of 1,2-diacylglycerol were determined as described previously [20]. Inset: diacylglycerol levels after 15 s, 5 min or 4 h under the conditions described above for controls ($\square$) and TRAP-stimulated ($\blacksquare$) cultures. These data are representative of two independent experiments each performed in duplicate; each point is the average of duplicate determinations, and the error bars represent the range.

Figure 3 TRAP stimulates PI and PC hydrolysis

IIC9 cells were metabolically labelled with [^3H]myo-inositol (top panel) or [^3H]choline (bottom panel) in serum-free media for 24 h as described in Materials and methods. Cultures were then stimulated with 1.5 NIH units/ml α -thrombin (Δ), or 400 μM TRAP (SFFLRNP; $\square$) for the indicated times. Incubations were terminated and [^3H]IP $_3$ or [^3H]choline metabolites were quantified as described previously (see Materials and methods). Data are expressed as the percentage of the [^3H]IP $_3$ or [^3H]choline metabolites present in control cultures ([^3H]IP $_3$ = 180 ± 20 c.p.m./35 mm dish, total ^3H -label in phosphoinositides $\approx 1 \times 10^5$ c.p.m.; [^3H]choline metabolites = 7284 ± 510 c.p.m./35 mm dish) incubated in serum-free medium for the same amount of time. Each point represents the mean $\pm$ S.E.M. of an experiment performed in triplicate and the data are representative of at least two independent experiments, each performed in triplicate.

potent fibroblast mitogen. We therefore examined whether TRAP stimulates cell growth (Figure 4). Addition of TRAP results in a dose-dependent increase in cell number after 2 days. The inactive peptide, FSFLRNP, is ineffective (results not shown).

EKTR couples to PI and PC hydrolysis, arachidonic acid release and mitogenesis in IIC9 cells

The ability of TRAP to induce the above responses does not distinguish between the single-receptor or two-receptor models. To discriminate between these two possibilities, we examined the ability of EKTR to induce PC hydrolysis, arachidonic acid release and mitogenesis in IIC9 cells. IIC9 cells were stably transfected with the modified receptor [25,26], and enterokinase-induced responses were quantified. This allowed us to examine responses induced by this receptor in the absence of contributions

Figure 4 TRAP stimulates cell growth

Serum-starved IIC9 cultures were stimulated with the indicated concentrations of the TRAP (SFFLRNP), then counted 2 days later as described in Materials and methods. Each point represents the mean $\pm$ S.E.M. of an experiment performed in triplicate and the data are representative of two independent experiments.

from the endogenous α -thrombin receptor (WTR), but in the presence of all downstream components required for coupling the receptor to lipid metabolism and mitogenesis.

The modified receptor was first tested for its ability to couple to arachidonic acid release (Figure 5). Enterokinase does not stimulate an increase in the release of arachidonic acid and its metabolites from the parental IIC9 cells expressing only WTR. In contrast with parental IIC9 cells, enterokinase induces a significant release of arachidonic acid and its metabolites into the culture media in cells expressing EKTR (Figure 5). Parental IIC9 cells transfected with control plasmids PKR5-LacZ or pSV2Neo do not respond to enterokinase (results not shown). It is important to note that transfected cells expressing EKTR also respond to α -thrombin (Figure 5). α -Thrombin stimulates virtually identical release of arachidonic acid from IIC9 cells expressing EKTR as parental IIC9 cells (Figure 5, bottom panel). Interestingly, the time course of release and the efficacy of enterokinase are not the same as for α -thrombin. After a 5 min incubation, the amount of arachidonic acid plus metabolites released in response to enterokinase was only one fifth of the release induced by α -thrombin. However, after 30 min of incubation, the increases were the same for both enterokinase and α -thrombin. A comparison of the enterokinase and α -thrombin concentration dependencies of the release of arachidonic acid plus metabolites demonstrated that 2000 units/ml enterokinase is equivalent to a low thrombin concentration or 0.0015 NIH unit/ml α -thrombin (Figure 5, and results not shown).

In addition to the release of arachidonic acid, low concentrations of α -thrombin induce a slow and monophasic increase in diacylglycerol mass in IIC9 cells [11,20]. Interestingly, similar to the arachidonic acid-release response, enterokinase also stimulated a slow increase in diacylglycerols in EKTR-expressing cells (Figure 6). After 5 min, the level of diacylglycerol induced

Figure 5 Enterokinase stimulates arachidonic acid release in cells expressing EKTR

IIC9 cells were metabolically labelled with [3 H]arachidonic acid in serum-free media for 24 h as described in Materials and methods. (Top panel) EKTR-transfected cell line EKTR11.1 cells and (Bottom panel) wild-type IIC9 cells were treated with 2000 units/ml enterokinase, 1.5 NIH units/ml α -thrombin, or control serum-free media for the indicated times. Culture media were collected and counted in a scintillation counter as described in Materials and methods. Each point represents the mean $\pm$ S.E.M. ($n = 3$) and is representative of two independent experiments.

in EKTR-expressing cells was only half that achieved by a high concentration of α -thrombin (1.5 NIH units/ml). This level of diacylglycerol accumulation is the same as that induced by a low concentration (0.0015 NIH unit/ml) of α -thrombin. After 30 min, the enterokinase-induced diacylglycerol level tripled (Figure 6), achieving the same level of diacylglycerol accumulation as that induced by both high and low concentrations of α -thrombin.

Whereas α -thrombin induces a sharp peak of diacylglycerols in EKTR-expressing cells at 15 s, careful analysis of the kinetics of enterokinase-induced diacylglycerol levels shows that enterokinase stimulates only a small 'shoulder' (1.4 ± 0.5 -fold increase in three experiments performed in duplicate, $n = 6$) at this time (Figure 6). Since the first peak of diacylglycerol produced in response to α -thrombin is mostly derived from PI hydrolysis [24], the absence of this peak in EKTR-expressing cells in response to enterokinase indicates that either (i) there is no PI hydrolysis, or (ii) the PI-induced diacylglycerol is very small and largely masked by the diacylglycerols derived from PC hydrolysis. To discriminate between these possibilities, the source of the diacylglycerols induced by enterokinase was investigated by quantifying the water-soluble head groups released in response to enterokinase. The increase in PI hydrolysis observed in response

Figure 6 Enterokinase stimulates an increase in diacylglycerol (DAG) levels in cells expressing EKTR

EKTR11.1 cells were serum starved for 48 h as described in Materials and methods. Cell cultures were incubated with fresh serum-free media ($\square$), 2000 units/ml enterokinase ($\diamond$) or 1.5 NIH units/ml α -thrombin ($\circ$) for the indicated times. Incubations were terminated by Bligh-Dyer extraction and mass levels of 1,2-diacylglycerol were determined as described previously [20]. Each point is the average of duplicate determinations and are representative of two independent experiments. Error bars represent the range of values.

to enterokinase, as evidenced by the increase in IPs (inositol 1-phosphate, inositol 1,4-bisphosphate and IP_3), is similar to that observed in response to a high concentration of α -thrombin (1.5 NIH units/ml) in wild-type cells (Figure 7, top panel). However, in contrast to wild-type cells, PI hydrolysis is observed only in the presence of LiCl (results not shown). PC hydrolysis, on the other hand, is maximally stimulated by enterokinase, virtually identical to α -thrombin-induced PC hydrolysis (Figure 7, bottom panel). These data are consistent with the single-receptor model (Figure 1) and indicate that PC hydrolysis requires fewer activated α -thrombin receptors than PI hydrolysis.

In previous reports, we presented data indicating that induced PC metabolism is a major signal-transduction component in mitogenesis. The above data indicate that induced PC metabolism, in addition to other α -thrombin-induced events, is mediated by PAR1, as previously suggested [27]. To test the role of the cloned receptor in α -thrombin-induced mitogenesis, we tested the ability of enterokinase to induce mitogenesis in cells expressing EKTR. Enterokinase elicits a two-fold increase in [3 H]thymidine incorporation with an EC_{50} of 100 units/ml (Figure 8). A comparable increase in [3 H]thymidine incorporation was observed in cultures treated with 0.0015 NIH unit/ml α -thrombin (results not shown).

Only activated receptors are desensitized in homologous desensitization of the α -thrombin receptor

It has been demonstrated that the α -thrombin receptor undergoes homologous desensitization [17]. Ishii et al. [28] found recently that PAR1 was phosphorylated rapidly upon stimulation, and that β -adrenergic receptor kinase 2 (β ARK2) inhibited α -thrombin stimulation when co-expressed in *Xenopus* oocytes

Figure 7 Enterokinase stimulates both PI and PC hydrolysis in cells expressing EKTR

(Top panel) EKTR11.1 (■) and wild-type IIC9 (□) cells were metabolically labelled with [3 H]myo-inositol in serum-free media for 24 h. Cultures were then first treated with 20 mM LiCl for 1 min, followed by 2000 units/ml enterokinase or 1.5 NIH units/ml α -thrombin for 30 min. (Bottom panel) EKTR11.1 (■) and wild-type IIC9 (□) cells were metabolically labelled with [3 H]choline in serum-free media for 24 h. Cultures were then treated with 2000 units/ml enterokinase or 1.5 NIH units/ml α -thrombin for 30 min. Data are expressed as the percentage of the [3 H]inositol phosphates or [3 H]choline and phosphocholine present in control cultures incubated in serum-free medium for the same amount of time ([3 H]IP = 11880 ± 155 c.p.m./35 mm dish; [3 H]choline metabolites = 5316 ± 516 c.p.m./35 mm.dish). Each point represents the mean $\pm$ S.E.M. of an experiment performed in triplicate, which are representative of two independent experiments each performed in triplicate.

with the α -thrombin receptor. Furthermore, this inhibition was dependent on β ARK2 phosphorylation sites in the receptor's cytoplasmic tail [28]. These data suggest that β ARK2, or a β ARK2-like kinase, inactivated non-activated receptors. The EKTR-expressing cells provide us with an opportunity to determine if only the activated receptors are desensitized or if non-activated, uncleaved, receptors are also affected.

As stated previously, addition of enterokinase to EKTR-expressing cells induces the release of arachidonic acid and its metabolites (Figure 5, top panel). However, enterokinase does

Figure 8 Enterokinase stimulates DNA synthesis in cells expressing EKTR

Serum-starved EKTR cell cultures were serum-starved for 2 days. Cells were then stimulated with the indicated concentrations of enterokinase for 16 h, and pulsed with [3 H]thymidine for 4 h. Cells were harvested and trichloroacetic acid-insoluble material were counted in a scintillation counter. Each point represents the mean $\pm$ S.E.M. of three independent experiments each performed in duplicate.

not stimulate the release of arachidonic acid from wild-type cells (Figure 5, bottom panel). These data, and the data described above, indicate that enterokinase does not activate wild-type receptors. To examine the effects of homologous desensitization, wild-type IIC9 cells exposed to 0.0015 NIH unit/ml α -thrombin for 15 min at 37 °C were washed and incubated with another aliquot of 0.0015 NIH unit/ml α -thrombin in fresh serum-free medium at 37 °C. The second addition of α -thrombin does not induce an increase in arachidonic acid and its metabolites (Figure 9). Similarly, when EKTR-expressing cells are exposed to two incubations of enterokinase, the first addition induces an increase in choline and phosphocholine (Figure 7, bottom panel) and a release of arachidonic acid and its metabolites (Figure 9). However, the second addition is ineffective (Figure 9). These data indicate that the EKTR is desensitized by enterokinase and the α -thrombin receptor is desensitized by α -thrombin. Interestingly, enterokinase induces a release of arachidonic acid and its metabolites from EKTR cells exposed previously to 0.0015 NIH unit/ml α -thrombin for 15 min at 37 °C. Similarly, α -thrombin induces a release of arachidonic acid and its metabolites from EKTR-expressing cells or wild-type cells treated previously with enterokinase (Figure 9). Even 1.5 NIH units/ml α -thrombin does not affect enterokinase-induced arachidonic acid release (Figure 5). Importantly, the desensitization is not due to depletion of radiolabelled arachidonic acid because treatment of EKTR-expressing cells or wild-type cells with fetal calf serum or 1.5 NIH units/ml α -thrombin, which had been exposed to 0.0015 NIH unit/ml α -thrombin or enterokinase, elicits an additional release of [3 H]arachidonic acid and its metabolites. Furthermore, α -thrombin induces the release of arachidonic acid and its metabolites in cells treated previously with α -thrombin (0.0015 NIH unit/ml) for 15 min at 37 °C, washed and incubated in fresh serum-free medium for 15 min at 37 °C (Figure 9).

Figure 9 Only cleaved receptors are desensitized

Arachidonic acid release was measured as for Figure 5. Quiescent EKTR11.1 cells were first treated with 0.3 NIH unit/ml α -thrombin (A), 2000 units/ml enterokinase (EK; B) or 1.5 NIH units/ml α -thrombin (C). Fold increase in arachidonic acid release (relative to quiescent cells, 1200 ± 200 c.p.m./35 mm dish) is indicated as the Control in each panel. Cells were then washed and incubated in serum-free media for 30 min. Samples were then treated again with 2000 units/ml enterokinase, 0.3 NIH unit/ml (Low Th) or 1.5 NIH units/ml (High Th) α -thrombin as indicated. Each bar represents the mean $\pm$ S.E.M. of two independent experiments each performed in duplicate ($n = 4$).

DISCUSSION

α -Thrombin is known to induce a variety of cellular responses, including the hydrolysis of PI and PC [7,29]. An α -thrombin receptor, PAR1, has been cloned from human platelets and hamster fibroblasts. PAR1 has been shown to be a member of a growing class of G-protein-coupled receptors that couple to more than one G-protein [15]. Whereas it is tempting to speculate that PAR1 couples to all of the above-mentioned known responses, there is also considerable evidence that a second α -thrombin receptor exists that is responsible for coupling to PC hydrolysis as well as other α -thrombin-induced responses [9–14]. The present study focuses on determining if PAR1, or a separate α -thrombin receptor, couples to induced PC metabolism.

Whereas at least four PAR receptors have been identified [5,6,14,30,31], the data in this report indicate that the endogenous PAR1-type receptor couples both the α -thrombin-induced PI and PC hydrolysis in IIC9 cells, as well as arachidonic acid release and cell growth. Enterokinase induced all of these

responses in EKTR-expressing cells. In addition, wild-type IIC9 cells respond identically to catalytically active α -thrombin and a TRAP. We cannot completely rule out the possibility that the endogenous PAR1-type receptor requires the presence of a second α -thrombin-responsive receptor for the generation of the responses investigated in this study. This possibility, however, is very unlikely. Although the TRAP used in this study has been shown to activate a trypsin-sensitive PAR (PAR2) [30], this protease does not induce PI or PC metabolism in IIC9 cells (results not shown). Furthermore, if two different receptors are activated by the TRAPs in these cells, they must share the same or very similar peptide-ligand sequences, α -thrombin-binding sites and α -thrombin-recognition and -cleavage sites. Low-stringency hybridization of cloned receptor cDNA of IIC9 mRNA revealed only one species with the size of the cloned receptor (results not shown). PCR using combinations of degenerate receptor sequences also failed to identify a second species (results not shown). This is consistent with the observation that fibroblasts [12] do not respond normally to α -thrombin when the α -thrombin-receptor gene is disrupted in mice [13]. The fact that platelets from these mice respond normally to α -thrombin [12] suggests that another non-PAR1 receptor(s) is involved in modulating platelet, but not fibroblast, responses.

One minor concern regarding the above data involving EKTR-expressing cells is that the 'new' N-terminal ligand tethered to the enterokinase-cleaved EKTR binds to and activates a neighbouring endogenous WTR. This interaction will produce the intracellular responses normally mediated by the WTRs. Whereas recent reports show that, although such intermolecular activation exists among α -thrombin receptors, the intramolecular activation is predominant. The EC_{50} is more than 1000-fold higher for intermolecular activation than for intramolecular activation [32]. It seems very unlikely, therefore, that such intramolecular activation compromises the results presented in this report.

Although the cloned receptor couples to both PI and PC hydrolysis, there are significant differences between PI and PC hydrolysis. First, PC hydrolysis is stimulated at α -thrombin concentrations that are at least 10 times lower than that required for the stimulation of PI hydrolysis [20]. Given the fact that the same receptor couples to both responses, this indicates that fewer receptors are required for activation of PC hydrolysis, suggesting that the α -thrombin receptor couples to PC-specific phospholipase more efficiently. As this receptor couples to G-proteins, it will be interesting to determine if this difference is due, at least in part, to different G-proteins or G-protein subunits coupling to different effectors, or differences in receptor or effector affinities for a specific G-protein or G-protein subunit. Secondly, PC hydrolysis is sustained and requires the continuous presence of catalytically active α -thrombin, whereas PI hydrolysis is transient. Whereas the mechanism for this difference is not yet clear, our data suggest that part of the mechanism may be due to the fact that PC hydrolysis is induced by the hydrolysis of only a few receptors that are present after the initial burst of α -thrombin hydrolysis and/or placed on the cell surface and rapidly cleaved while α -thrombin is present.

There are a number of reports implicating the heterotrimeric G-protein-designated Gq in coupling the α -thrombin receptor to PI hydrolysis. It is also clear that the α -subunit couples to PI-PLC β 1 whereas the $\beta\gamma$ -dimers couple to PI-PLC β 2 [33–36]. In preliminary experiments, this G-protein appears to couple to PI metabolism, via PI-PLC β 1, but not PC metabolism in IIC9 cells (P. Henderson, D. M. Raben and J. J. Baldassare, unpublished work). We are currently examining cells in which specific α -subunits of selected G-proteins have been ablated by antisense RNA to identify the G-protein involved in coupling this receptor

to PC-PLC/D and PLA₂. We are also examining the role of α -subunits and $\beta\gamma$ -dimers in the activation of these enzymes.

In view of our data, another mechanism that is likely to contribute to the differences in PI and PC metabolism is the desensitization of the α -thrombin receptor. There is considerable evidence indicating that this receptor undergoes homologous desensitization, in that desensitization requires the presence of α -thrombin and likely involves phosphorylation via a β ARK2-like enzyme [17,28]. What has not been known is whether unactivated receptors are desensitized or if only those receptors that are activated were desensitized. Taking advantage of our EKTR-expressing cells, our data demonstrate that only the activated receptors were desensitized.

In summary, the data in this report demonstrate that the cloned α -thrombin receptor couples to both PI and PC hydrolysis, the release of arachidonic acid and its metabolites, and mitogenesis. Furthermore, we have shown that whereas cleaved receptors are desensitized, uncleaved receptors remain capable of coupling to the induced responses when cleaved. These results have broad implications for our understanding of how the responses are induced and regulated. Future experiments will be focused on identification of the components involved in inducing and regulating PC metabolism, including the release and metabolism of arachidonic acid.

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