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# Thrombin and Trypsin Act at the Same Site to Stimulate Phosphoinositide Hydrolysis and Calcium Mobilization

LINDA G. JONES, PATRICK M. MCDONOUGH, and JOAN HELLER BROWN
*Department of Pharmacology, University of California at San Diego, La Jolla, California 92093*
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## SUMMARY

Thrombin stimulates polyphosphoinositide hydrolysis in embryonic chick heart cells and in 1321N1 astrocytoma cells and increases intracellular $Ca^{2+}$ in the 1321N1 cells. The serine protease trypsin mimics these actions in a dose-dependent fashion, whereas the proteolytically inactive thrombin derivatives diisopropyl fluorophosphate-thrombin (DIP-thrombin) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone-thrombin (PPACK-thrombin) are ineffective in this regard. The phosphoinositide responses to thrombin or trypsin and the muscarinic agonist carbachol are additive, but no additivity is observed between the responses to thrombin and trypsin. Unlike the response to carbachol, the phosphoinositide and $Ca^{2+}$ responses to thrombin and trypsin desensitize, with no recovery of the calcium response even when $Ca^{2+}$ stores are replenished. Cross-desensitization of phospholipase C activation and calcium mobilization between these proteases is also observed. In addition, PPACK-thrombin, which elicits no response itself, effectively inhibits trypsin-stimulated phosphoinositide hydrolysis. It is proposed that thrombin and trypsin act through the same receptor. Proteolysis appears to be important in the mechanism by which these agonists elicit phosphoinositide hydrolysis, calcium mobilization, and, perhaps, subsequent receptor desensitization.

While best characterized for its role in blood clotting, thrombin has also been shown to promote a number of physiological responses, in various tissues, that are typical of those stimulated by hormones. These responses include PI turnover (1–3), calcium mobilization (4, 5), inhibition of adenylate cyclase (6), arachidonic acid release (7), secretion (8–10), stimulation of the $Na^+/H^+$ antiport (11, 12), and mitogenesis (13, 14). The actions of thrombin have been studied primarily in the platelet, but other cell types such as fibroblasts (11–15), endothelial cells (16, 17), and neuroblastoma cells (18) have also been shown to be responsive to thrombin.

Although many of these physiological responses to thrombin have been well characterized, the mechanism of action of thrombin is still controversial (19–21). In addition, the properties of the thrombin receptor(s) remain enigmatic. Thrombin has been shown to bind to a number of different cell surface proteins (for review, see Refs. 19 and 20), and there is evidence suggesting that the thrombin receptor differs according to cell type or species (13, 16, 22). Receptor subtypes (23) or receptors of various affinities (24) on the same cell type have been described as well.

The serine protease trypsin has been found to mimic several of the actions of thrombin in platelets (25, 26). These effects of trypsin are of interest because they give support to a role for proteolysis in platelet activation. However, trypsin may also cause effects not elicited by thrombin. For example, trypsin has been shown to promote tyrosine kinase activity of the insulin receptor (27) and to stimulate adenylate cyclase (28, 29). In this report, we compare the effects of thrombin and trypsin on PI hydrolysis and calcium mobilization in CHC and 1321N1 astrocytoma cells. We demonstrate that both agents promote $PIP_2$ hydrolysis and stimulate the release of intracellular calcium. In contrast to the effects of muscarinic stimulation on PI hydrolysis (30) and intracellular $Ca^{2+}$ release (31), the effects of thrombin and trypsin desensitize. We present data that suggest that actions of thrombin and trypsin on PI hydrolysis and $Ca^{2+}$ mobilization are mediated through a common receptor and that proteolysis may be involved in the activation and subsequent desensitization of this receptor.

## Experimental Procedures

**Cell preparation.** Cells from 13-day-old chick embryo hearts were isolated by enzymatic dissociation and cultured overnight in serum-

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**ABBREVIATIONS:** PI, phosphoinositides; CHC, embryonic chick heart cells; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; InsP, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; fura-2, {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzo-furan-5-oxy]-2-(2′-amino-5′-methylphenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid}; fura-2/AM, the cell-permeant pentaacetoxymethyl, ester of fura-2; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DIP, diisopropyl fluorophosphate, and PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (thrombin inactivators); PMSF, phenylmethylsulfonyl fluoride; G protein; guanine nucleotide-binding protein; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

supplemented DMEM, as previously described (32). Cultures of 1321N1 astrocytoma cells were cultured in 35-mm cell culture dishes and maintained as described earlier (33). The PI in the CHC were labeled for measurement of [$^3$H]InsP formation by including 1–3 $\mu$Ci/ml [$^3$H] inositol in the incubation medium. This concentration was sometimes increased to 15 $\mu$Ci/ml when [$^3$H]InsP$_3$ and [$^3$H]InsP$_2$ were also quantified. Astrocytoma cells were labeled with 5 $\mu$Ci/ml [$^3$H]inositol for all experiments.

**Assay conditions.** Following overnight incubation at 37° in a 90% air/10% $CO_2$ atmosphere, cell monolayers were washed with a physiological salt buffer that contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES or with DMEM buffered with 20 mM HEPES. The culture plates were then incubated on a tray in a water bath at 37°, for 10 min. HEPES-buffered salt solution or DMEM was replaced with 0.75–1.0 ml of the same medium containing 10 mM LiCl and the appropriate drugs. For assays requiring permeabilized cells, the cells were permeabilized with saponin and assayed in an intracellular buffer containing 10 mM LiCl, as we have described previously (32). After the drug incubation, the reactions were terminated by the addition of trichloroacetic acid (10% final). Samples were neutralized by ether extraction and the inositol phosphates were separated by anion exchange column chromatography (32).

**Preparation of PPACK-thrombin.** Thrombin (450 nM) was treated with PPACK (1 $\mu$M) in a 50 mM PIPES-buffered salt solution at 25° for 30 min (34). The unreacted PPACK was then removed by overnight dialysis in cold distilled water. Thrombin alone, i.e., without exposure to PPACK, was treated in a similar fashion. Aliquots of the samples were lyophilized, stored at −20°, and resuspended in the assay buffer before use.

**Intracellular calcium measurements.** Intracellular calcium concentrations were monitored using the fluorescent calcium indicator fura-2. Cell suspensions of the astrocytoma cells were prepared by replacing the culture medium with a HEPES-buffered physiological salt solution and releasing the cells by gentle agitation. Cell suspensions prepared in this manner were 90–95% viable (as determined by trypan blue exclusion) and consisted mostly of single cells. Cells were incubated with fura-2/AM for 20–30 min, centrifuged, and resuspended in buffer that was free of fura-2/AM, immediately before the fluorescence measurements. Fluorescence was monitored at 340 and 380 nm excitation, 510 nm emission, as described previously (35).

**Materials.** Fertilized white leghorn chicken eggs were from McIntyre Poultry and Eggs (San Diego, CA). Male Sprague-Dawley rats (300–350 g) were obtained from Charles River Laboratories (Wilmington, MA), and ventricular heart cells were prepared as previously described (36). Human erythroleukemic cells and BC3H-1 cells were kindly provided by Drs. Harvey Motulsky and Palmer Taylor, respectively (University of California at San Diego, La Jolla, CA). Carbamylcholine (carbachol) chloride, thrombin (T6759 from human plasma, purity >98%, 3000 NIH units/mg of protein), carboxypeptidase A (type I-DFP), carboxypeptidase B (type I-DFP), $\alpha$-chymotrypsin (type VII), papain (type IV), aprotinin, hirudin, and soybean trypsin inhibitor (type II-S) were from Sigma, (St. Louis, MO). Trypsin (TRL3 from bovine pancreas, purity >95%, 210 units/mg of protein) was from Worthington, Millipore Corp. (Bedford, MA). PMSF and PPACK were from Calbiochem (La Jolla, CA). DIP-thrombin was a gift of Dr. Elliot Richelson (University of Michigan, Ann Arbor, MI). GTP$\gamma$S was from Boehringer Mannheim (Indianapolis, IN). [$^3$H]Inositol was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA).

## Results

**Thrombin-induced PI hydrolysis is mimicked by the serine protease trypsin.** Thrombin promotes the accumulation of InsP in embryonic CHC and in 1321N1 cells in a dose-dependent manner, with half-maximal effects at 0.07 units/ml (0.6 nM) and 0.3 units/ml (2.7 nM), respectively, in the two cell types (Fig. 1A). In both the CHC and 1321N1 cell another serine protease, trypsin, mimicks the effect of thrombin on the stimulation of IP formation, with a half-maximal effect at 10–20 nM and maximal effect by 100 nM (Fig. 1B). Papain (2.7 $\mu$M), another protease that also displays specificity for arginyl or lysyl bonds, also promotes PI hydrolysis but is only 25% as effective as trypsin (data not shown). Specificity of proteolytic action is implied by the finding that the proteases with a different specificity than trypsin ($\alpha$-chymotrypsin, carboxypeptidase A, and carboxypeptidase B) are ineffective in eliciting PI hydrolysis (data not shown). Further evidence that proteolysis might be required for the activation of PI hydrolysis is the observation that the proteolytically inactive thrombin derivatives DIP-thrombin and PPACK-thrombin are ineffective

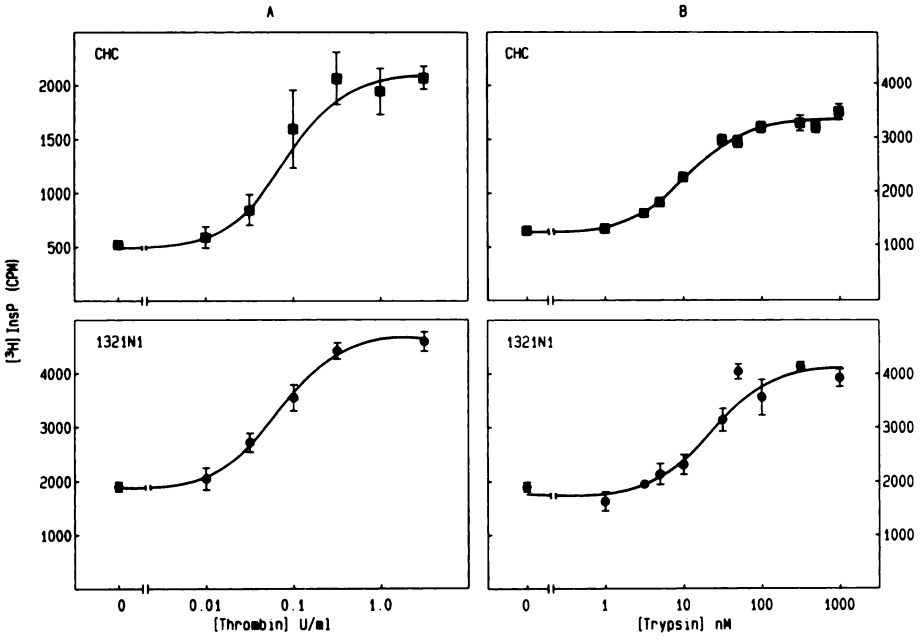


**Fig. 1.** Dose response of [$^3$H]InsP accumulation to thrombin (A) and trypsin (B) in CHC and 1321N1 astrocytoma cells. These *curves* represent accumulation of [$^3$H]InsP during 5 min except for the *top left* panel, which represents accumulation over 30 min. Values are means ± standard errors of three to six determinations.

TABLE 1

**Comparative effects of protease inhibitors on thrombin- and trypsin-stimulated PI hydrolysis in CHC**

Cells were incubated 20–30 min in the presence of thrombin or trypsin with or without inhibitors, which were added simultaneously with the proteases and at the following concentrations: PMSF, 100 μM; soybean trypsin inhibitor (STI), 100 nM; and aprotinin (APRO), 0.425 units/ml. These data are from representative experiments; with two to four determinations in each.

| | InsP | | | |
|---|---|---|---|---|
| | None | PMSF | STI | APRO |
| | % of control | | | |
| Thrombin (1 unit/ml) | 1047 ± 48 | 263 ± 35 | 974 ± 27 | 1065 ± 28 |
| DIP-Thrombin[a] | 123 ± 7 | | | |
| PPACK-Thrombin[b] | 85 ± 2 | | | |
| Trypsin (100 nM) | 1355 ± 239 | 225 ± 82 | 102 ± 2 | 106 ± 15 |

[a] Molar equivalent to 0.5 units/ml thrombin.
[b] Molar equivalent to 7 units/ml thrombin.

in eliciting the PI response (Table 1). Table 1 also shows the results obtained when inhibitors of thrombin and trypsin were used. The serine protease inhibitor PMSF blocks the effects of both thrombin and trypsin. Two other protease inhibitors, aprotinin and soybean trypsin inhibitor, also block the stimulation of InsP formation by trypsin. Thrombin is not a substrate for these agents and its action is, therefore, not inhibited.

**Reversal of thrombin- and trypsin-induced PI hydrolysis.** Because proteolysis appears to be involved in thrombin-stimulated PI hydrolysis, we wondered whether an initial proteolytic event might lead to sustained activation of phospholipase C. Fig. 2A shows a time course for the accumulation of InsP in CHC stimulated by a maximal dose of thrombin. At the times indicated by the arrows, cells were washed to remove thrombin and were incubated for an additional 15 min in the presence of hirudin, which antagonizes the action of any remaining thrombin by binding to it. There was no additional accumulation of InsP during this 15-min incubation. Similar results were obtained with trypsin treatment (Fig. 2B) and with thrombin and trypsin treatment in the 1321N1 cell (data not shown). Thus, phospholipase C is not irreversibly activated by the proteolytic actions of thrombin and trypsin. Rather, the continued presence of thrombin or trypsin seems to be required to maintain PI hydrolysis, as would be the case with most receptor-active hormones.

**Activation of a polyphosphatidylinositol-specific phospholipase C by thrombin and trypsin.** Thrombin (0.5 units/ml, 4.5 nM) and trypsin (0.5 units/ml, 100 nM) stimulate not only the production of InsP but also that of $InsP_2$ and $InsP_3$ in both CHC (Table 2) and astrocytoma cells (Table 3). These data provide evidence that thrombin and trypsin promote the hydrolysis of $PIP_2$ by a polyphosphoinositide-specific phospholipase C. This response is similar to that which we have previously observed to carbachol in CHC (32) and 1321N1 cells (30). The significant increase in the accumulation of $InsP_3$ at early times suggests that the hydrolysis of $PIP_2$ is the immediate receptor-linked response to thrombin and trypsin.

**Possible involvement of a G protein.** Most receptors that couple to phospholipase C and mediate $PIP_2$ hydrolysis do so through a G protein. To investigate whether a G protein couples thrombin stimulation to PI hydrolysis, we used a permeabilized CHC preparation (32) in which the intracellular guanine nucleotide concentration could be regulated. We previously reported that addition of GTPγS, the nonhydrolyzable GTP analog, greatly increased the accumulation of [$^3$H]InsP, [$^3$H]$InsP_2$, and [$^3$H]$InsP_3$ in the permeabilized CHC (32) and in membranes from 1321N1 cells (37). Thrombin alone promotes

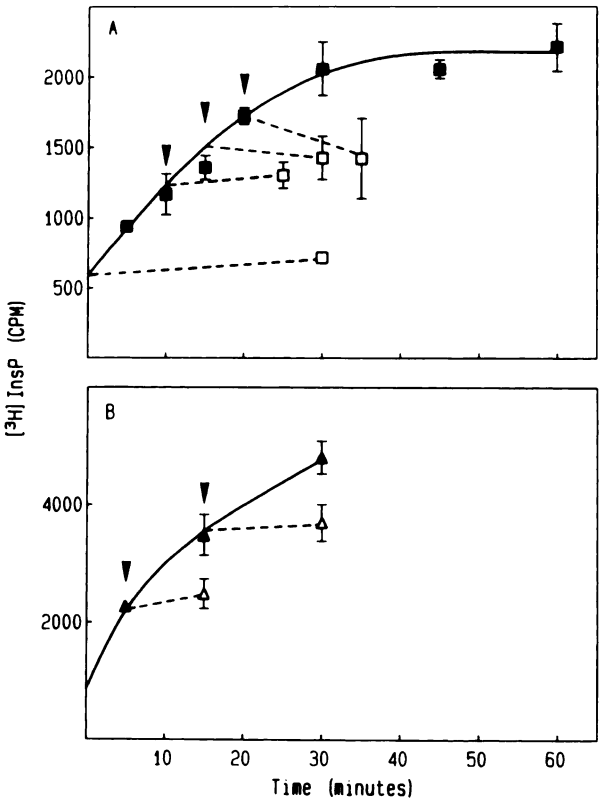


**Fig. 2.** Time course of accumulation of [$^3$H]InsP in CHC in response to thrombin and trypsin. The concentration of thrombin used in A was 0.5 units/ml (4.5 nM) and of trypsin used in B was 100 nM. At the times indicated by the *arrows*, some of the cells were washed and incubated for the remaining period of time (*dashed line*) in the absence of thrombin or trypsin, with (A) or without (B) the thrombin inactivator hirudin (1 unit/ml). The *closed symbols* on the *solid line* represent the values obtained when samples were incubated continuously with thrombin or trypsin. The *y*-intercepts represent the control values; three or four determinations were performed.

a small accumulation of $InsP_2$ and $InsP_3$ in the permeabilized CHC; the response to hormone in the absence of added guanine nucleotides may reflect remaining endogenous GTP. Nevertheless, addition of GTPγS alone has a marked stimulatory effect, and GTPγS potentiates the effect of thrombin (Table 4). Overnight treatment of intact cells with 100 ng/ml pertussis toxin did not block the stimulation of PI hydrolysis by thrombin or trypsin (data not shown), indicating that thrombin and trypsin do not act through a G protein that is a pertussis toxin substrate.

**Lack of additivity of responses to thrombin and tryp-**

TABLE 2

**Thrombin and trypsin increase inositol polyphosphate formation in CHC**

Cells were incubated for the times indicated with either carbachol, thrombin, or trypsin. The values shown are pooled from four separate experiments, (three to six determinations) and represent percentage of the control value at a given time.

| | Time | Inositol phosphate formation | | |
|---|---|---|---|---|
| | | Carbachol (100 μM) | Thrombin (0.5 units/ml) | Trypsin (100 nM) |
| | *min* | | *% of control* | |
| InsP | 0.15 | 98 ± 9 | 80 ± 6 | 96 ± 6 |
| | 1.0 | 107 ± 4 | 90 ± 6 | 132 ± 10 |
| | 5.0 | 219 ± 7 | 158 ± 15 | 209 ± 6 |
| | 30.0 | 684 ± 14 | 521 ± 29 | 703 ± 84 |
| $InsP_2$ | 0.15 | 108 ± 8 | 99 ± 5 | 200 ± 27 |
| | 1.0 | 215 ± 13 | 146 ± 8 | 399 ± 87 |
| | 5.0 | 558 ± 14 | 430 ± 30 | 424 ± 22 |
| | 30.0 | 758 ± 95 | 607 ± 18 | 975 ± 112 |
| $InsP_3$ | 0.15 | 161 ± 10 | 113 ± 12 | 298 ± 69 |
| | 1.0 | 294 ± 12 | 156 ± 9 | 436 ± 115 |
| | 5.0 | 342 ± 23 | 250 ± 13 | 213 ± 11 |
| | 30.0 | 292 ± 46 | 220 ± 30 | 381 ± 66 |

TABLE 3

**Thrombin and trypsin increase inositol polyphosphate formation in 1321N1 astrocytoma cells**

Cells were incubated for the times shown in the presence of thrombin or trypsin. Values are from three separate experiments (3 to 10 determinations) and represent percentage of the control value at a given time.

| | Time | Inositol phosphate formation | |
|---|---|---|---|
| | | Thrombin (0.5 units/ml) | Trypsin (100 nM) |
| | *min* | *% of control* | |
| InsP | 1.0 | 127 ± 4 | 105 ± 13 |
| | 5.0 | 237 ± 48 | 194 ± 24 |
| $InsP_2$ | 1.0 | 319 ± 10 | 182 ± 30 |
| | 5.0 | 370 ± 74 | 284 ± 42 |
| $InsP_3$ | 1.0 | 327 ± 34 | 193 ± 23 |
| | 5.0 | 240 ± 27 | 178 ± 13 |

TABLE 4

**Accumulation of [$^3$H]$InsP_{1-4}$ in permeabilized CHC in response to thrombin and GTPγS**

Cells were permeabilized with saponin, washed, and incubated for 30 min with 1 unit/ml thrombin, 10 μM GTPγS, or a combination. In this experiment, the effects of the combination of thrombin and GTPγS were significantly ($p = 0.02$) greater than the additive effects of the agents used alone. These data are representative of three separate experiments (three determinations in each).

| | Inositol phosphate accumulation | |
|---|---|---|
| | *cpm* | *cpm over basal* |
| Basal | 165 ± 4 | |
| Thrombin | 345 ± 34 | 180 |
| GTPγS | 464 ± 7 | 299 |
| Thrombin + GTPγS | 778 ± 31 | 613 |

**sin.** To assess the independence in mechanism of action of thrombin, trypsin, and carbachol, we asked whether additivity could be observed in the PT responses to maximal doses of these agonists. The effect of either thrombin or trypsin on $InsP_3$ accumulation is additive with that of carbachol in 1321N1 cells (Fig. 3, A and B) and in CHC (data not shown). However, the effects of thrombin and trypsin are not additive with each other in 1321N1 cells (Fig. 3C) or CHC (data not shown). These data suggest that thrombin and trypsin stimulate $PIP_2$ hydrolysis by a common mechanism independent of that used by carbachol.

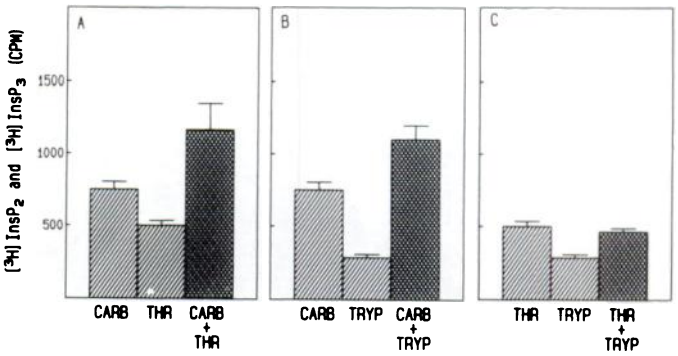


**Fig. 3.** Accumulation of [$^3$H]inositol polyphosphates in 1321N1 astrocytoma cells in response to single and combined hormone treatment. Cells were incubated for 5 min in the presence of 100 μM carbachol (*CARB*), 0.5 units/ml thrombin (*THR*) 100 nM trypsin (*TRYP*), or combinations of these drugs. Fractions containing [$^3$H]$InsP_2$ and [$^3$H]$InsP_3$ were combined. The basal value (128 cpm) has been subtracted from each *bar*, which represents the mean ± standard error from four determinations. Similar results were obtained in three other experiments (two of which were in CHC).

**Thrombin and trypsin appear to act through a common receptor.** In order to test the hypothesis that thrombin and trypsin act through a common receptor, we measured PI hydrolysis in response to these agents in other cell types. As observed in Fig. 4, PI hydrolysis is stimulated by thrombin in a human erythroleukemic cell line, and trypsin is also effective in these cells. However, neither protease effectively stimulates accumulation of the inositol phosphates in the adult rat ventricular cardiomyocyte or the BC3H-1 smooth muscle-like cell (adrenergic clone), when compared with the response seen with norepinephrine in these cells.

**Competition for receptor activation by PPACK-thrombin and trypsin.** To provide more conclusive evidence that thrombin and trypsin were competing for the same site, we prepared an inactive derivative of thrombin and tested it as a competitive inhibitor of trypsin-stimulated $InsP_3$ production. Treatment of 1321N1 cells with a 100-fold excess of PPACK-thrombin completely blocked the stimulation of $InsP_3$ formation by thrombin; the response to trypsin was also inhibited by approximately 80% under these conditions (Fig. 5). Similarly, treatment of CHC with an excess of PPACK-thrombin blocked the stimulation of $InsP_3$ formation by thrombin and trypsin by approximately 70 to 80% (data not shown). PPACK-thrombin alone did not elicit a PI response, whereas thrombin that had been dialyzed but not reacted with PPACK was still nearly 75% as effective as thrombin in this regard.

**Release of intracellular calcium and cross-desensitization of responses to thrombin and trypsin.** The hydrolysis of $PIP_2$ by thrombin and trypsin suggests that these agents may use $InsP_3$ as a second messenger. We examined changes in intracellular calcium in response to these agents using the fluorescent calcium indicator fura-2. As discussed elsewhere (38), we have been unable to measure a significant increase in intracellular calcium in response to muscarinic receptor stimulation in the CHC. Thrombin and trypsin were, likewise, ineffective in raising intracellular calcium by more than 20 nM in the CHC. However, thrombin and trypsin are effective in promoting a rapid dose-dependent increase in intracellular calcium in the 1321N1 cell. Maximal increases in intracellular calcium (to values >1 μM) are observed in response to 0.3 units/ml (2.7 nM) thrombin and 100–1000 nM trypsin (Fig. 6). These

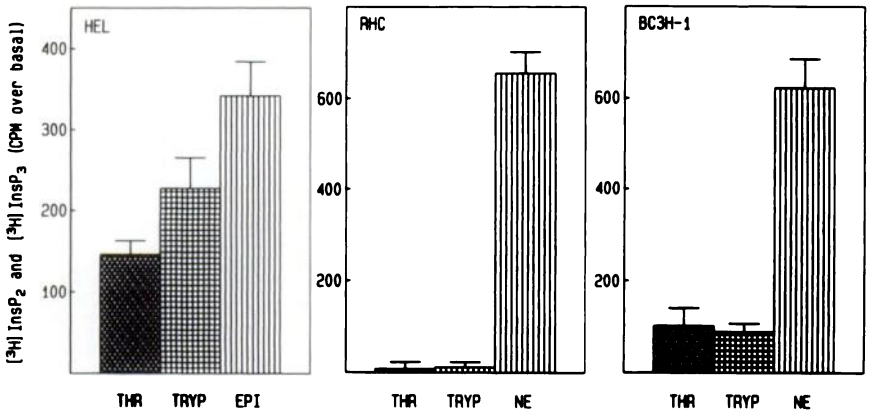


**Fig. 4.** Accumulation of inositol polyphosphates in human erythroleukemic cells (*HEL*), rat ventricular cardiac myocytes (*RHC*), and smooth muscle-like BC3H-1 cells (the adrenergic clone). Cells were incubated for 5 min in the presence of thrombin (*THR*) at 0.1 units/ml in HEL cells and 0.5 units/ml in RHC and BC3H-1 cells; trypsin (*TRYP*) at 20 nM in HEL cells and 100 nM in RHC and BC3H-1 cells; epinephrine (*EPI*) at 10 μM; or norepinephrine (*NE*) at 30 μM in RHC and 10 μM in BC3H-1 cells. The fractions containing [$^3$H]InsP$_2$ and [$^3$H]InsP$_3$ were combined. These data represent the mean ± standard error of four determinations from single experiments except for RHC, which is representative of three experiments.

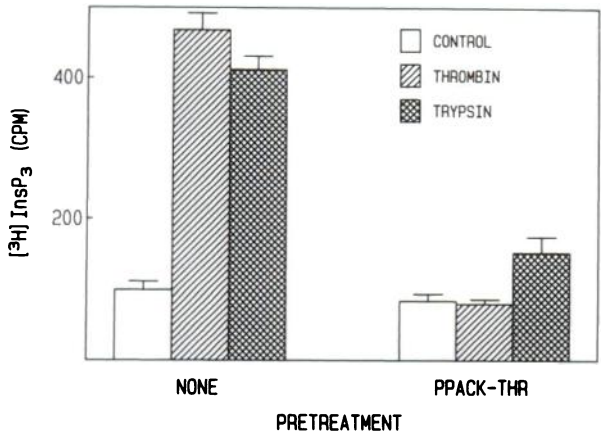


**Fig. 5.** Accumulation of [$^3$H]InsP$_3$ in 1321N1 astrocytoma cells with and without prior exposure to PPACK-thrombin. Cells were incubated for 5 min in the presence of 0.21 units/ml thrombin, 60 nM trypsin, or no drug, in the presence (*right*) or absence (*left*) of 21 units/ml PPACK-thrombin (*PPACK-THR*). Cells were exposed to PPACK-thrombin for 10 min in the absence of LiCl before the addition of drugs and LiCl. This is a representative experiment of three, two of which were performed in CHC. Values shown represent the means ± standard errors from three determinations.

calcium responses are only slightly attenuated in the absence of extracellular calcium (data not shown). In addition, the dose response for raising intracellular calcium correlates well with that observed for $InsP_3$ production, with half-maximal effects elicited by 0.04 units/ml (0.4 nM) thrombin and 25 nM trypsin.

Fig. 6 shows that treatment of the cells with carbachol, thrombin, or trypsin inhibits calcium mobilization to an immediate rechallenge by any of these agents. Parallel studies (data not shown) demonstrated that $InsP_3$ formation in response to thrombin or trypsin is not blocked by pretreatment with carbachol (and vice versa). Therefore, the loss of the calcium response does not reflect failure of the second hormone treatment to elevate $InsP_3$. Rather, it suggests that carbachol, thrombin, and trypsin elicit $Ca^{2+}$ release from a common and limited intracellular calcium pool, which is depleted by the first challenge with a maximal dose of hormone. A similar conclusion concerning the effects of carbachol and histamine was reached in a previous detailed study in our laboratory (31).

The most complete loss of the calcium response to a second hormone challenge is seen in cells that are challenged with thrombin following trypsin stimulation (and trypsin following thrombin stimulation) (Fig. 6, B and C). This suggests that for these two agonists there is an additional site of desensitization

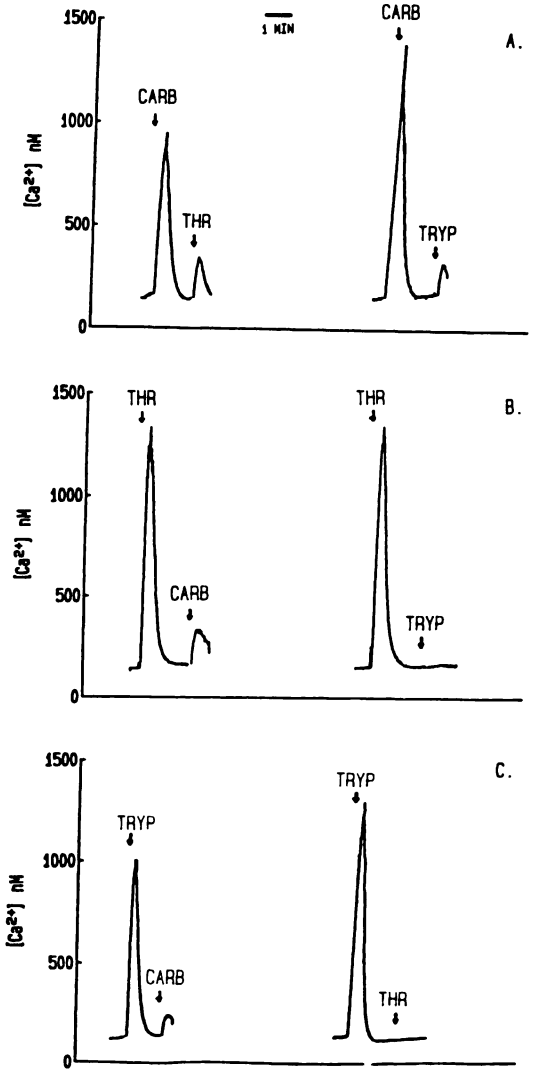


**Fig. 6.** Agonist-stimulated release of intracellular calcium in 1321N1 astrocytoma cells. These are representative *tracings* of calcium spikes in six separate samples that have been challenged with two drugs, one administered within three min of the other. The concentrations of drugs used were 300 μM carbachol (*CARB*), 0.5 units/ml thrombin (*THR*), and 1 μM trypsin (*TRYP*). *Tracings* are representative of those obtained in three or four separate experiments.

proximal to calcium release. This is supported by the observation that pretreatment with trypsin completely densensitizes the subsequent PI response to rechallenge by either agonist (data not shown). Thus, in contrast to that observed with carbachol, the responses to thrombin and trypsin demonstrate cross-desensitization at an early step in the activation pathway.

We also compared the time courses of calcium pool recovery following treatment of 1321N1 cells with carbachol, thrombin, or trypsin. After brief exposure (4 min) to these agents, the cells were washed and divided into aliquots, which were then allowed increasing time for refilling of the calcium pool before a second drug challenge. As seen in Fig. 7A, the calcium signal stimulated by carbachol is >80% recovered by 10 min after wash-out of drug. However, after an initial stimulation by thrombin (Fig. 7B), the calcium spike to subsequent challenges

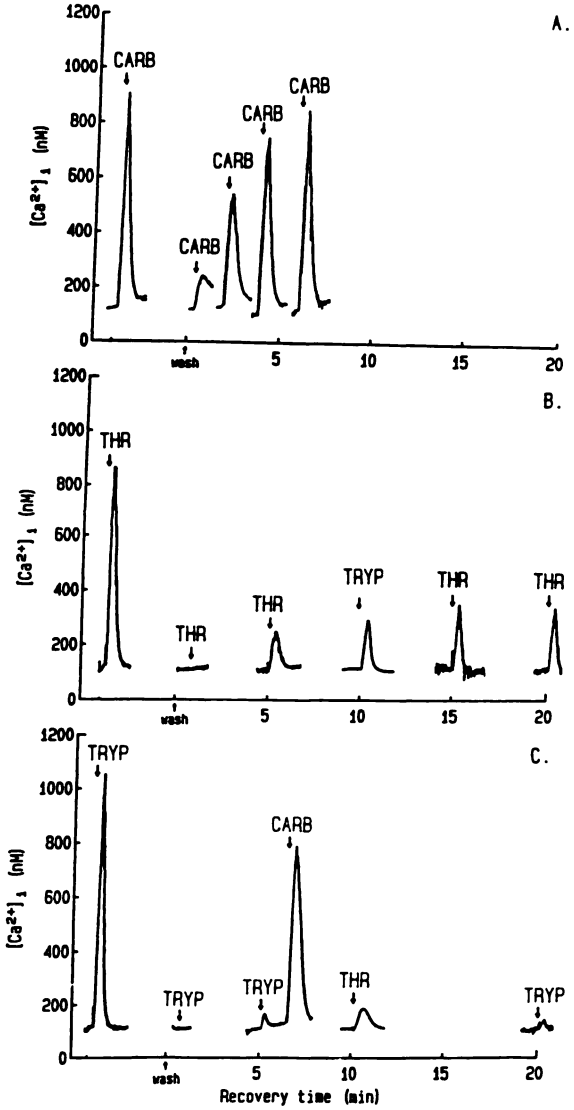


**Fig. 7.** Recovery of the agonist-stimulated $Ca^{2+}$ response in 1321N1 astrocytoma cells. Each of the *tracings* following the initial spike represent separate samples that were washed after the initial 4-min challenge with 300 μM carbachol (*CARB*) (A), 0.5 units/ml thrombin (*THR*) (B), or 1 μM trypsin (*TRYP*) (C) and were allowed increasing time in a physiologic salt buffer with 1.8 mM calcium for calcium pool replenishment. At the recovery time shown, a given sample was rechallenged by the drug indicated. *Tracings* are representative of those obtained in three or four separate experiments.

by thrombin recovers only very slowly (25% after 20 min). The calcium response to trypsin (Fig. 7C) shows even less recovery (<10%) over 20 min following wash-out of the initial trypsin challenge. Thrombin also failed to elicit a substantial $Ca^{2+}$ response after 10 min of recovery time following an initial trypsin challenge (Fig. 7C) and vice versa (Fig. 7B). Proof that the calcium pool had been replenished is the observation that carbachol is able to stimulate a calcium response in cells that had been previously calcium depleted by either thrombin (data not shown) or trypsin (Fig. 7C). These data also demonstrate cross-desensitization to the effects of thrombin and trypsin and further suggest that these two agents act at the same receptor.

## Discussion

Both thrombin and trypsin stimulate polyphosphoinositide hydrolysis in CHC and 1321N1 astrocytoma cells, in a dose-dependent fashion. The hydrolysis of $PIP_2$ (and a consequent increase in the production of $InsP_3$) appears to be the initial response to thrombin or trypsin stimulation. It is likely that thrombin uses $InsP_3$ as a second messenger in the 1321N1 cells, because an increase in cytosolic calcium is also observed in response to these agents, with a dose dependency similar to that observed for $InsP_3$ accumulation.

The finding that the continued presence of thrombin or trypsin is required to sustain PI hydrolysis is consistent with these agents acting like hormones at a specific cell surface receptor. Similar results showing a requirement for the continuous presence of thrombin to maintain accumulation of inositol phosphates has also been described in the platelet (39).

As for many other receptor-mediated events, the literature contains ample evidence that a G protein mediates at least some of the responses to thrombin (6, 11, 40–43). In a permeabilized cell preparation, we show that the stimulation of $InsP_2$ and $InsP_3$ accumulation by thrombin is modestly potentiated by GTPγS and we suggest the involvement of a G protein in the transduction of the PI signal generated by thrombin in these cells. The failure of pertussis toxin to block the PI response to thrombin in 1321N1 cells and CHC, at a concentration that inhibits adenylate cyclase in these cells (44), suggest that this coupling protein is neither $G_i$ nor $G_o$.

Our findings suggest that thrombin and trypsin elicit the PI response in CHC and 1321N1 cells through a receptor distinct from that activated by carbachol or other hormones we have studied. The effects of thrombin and trypsin are additive with that of carbachol in the CHC and in the 1321N1 cell (implying independent pathways) but not with each other in either cell type. Furthermore, unlike the response to carbachol, the PI response to thrombin and trypsin appears to desensitize at the receptor level. This is supported by the observation that the calcium response to thrombin or trypsin shows little recovery following brief exposure to either protease, although calcium stores are replenished. This is in contrast to what is seen with carbachol or histamine (31).

Cross-desensitization to the effects of thrombin and trypsin on PI hydrolysis and calcium mobilization was also observed and provides evidence that the actions of these agents are mediated through the same receptor. The similarity of the responses to thrombin and trypsin, the lack of additivity observed between the agents, and the observation that stimulation of the PI response by trypsin occurs only in cells that are also responsive to thrombin provide further evidence that the pro-

teases act at the same site. The strongest evidence for this hypothesis is the demonstration that the active site-blocked thrombin derivative PPACK-thrombin is an effective inhibitor of trypsin-induced phospholipase C activation. This suggests that PPACK-thrombin binds and prevents access of trypsin to a common site. That this is a competitive inhibition is indicated by the observation that, at a higher concentration of PPACK-thrombin (or at a lower dose of trypsin), PPACK-thrombin more effectively inhibits the $InsP_3$ response to trypsin. From these data, we conclude that thrombin and trypsin are acting at the same receptor and that the desensitization induced by these proteases is, therefore, of a homologous nature.

Jakobs and Aktories (45) recently published evidence that thrombin and trypsin were acting at the same site (presumably a surface receptor) to stimulate high affinity GTPase activities in platelet membranes, which might, in part, be associated with a G protein that regulates phospholipase C. These data support our conclusion that thrombin and trypsin act at a common receptor to activate a G protein-coupled polyphosphoinositide-specific phospholipase C.

The mechanism by which thrombin elicits its biochemical responses in cells and platelets remains controversial because it displays properties of both an enzyme and a hormone. Yet, the hypothesis that thrombin and trypsin act through a receptor does not preclude proteolysis as the mechanism whereby the receptor is activated or desensitized. Models of action that take into account both the enzymatic and the hormone-like characteristics of thrombin have been proposed (46). Cleavage of the thrombin receptor by thrombin (8, 13) and of the insulin receptor by trypsin (27) has been suggested as the means by which these agents cause receptor activation. Thus, there is a precedent in the literature for such a proteolytic event for at least some of the responses to thrombin and trypsin. In our experiments, appropriate protease inhibitors blocked the PI response to thrombin and trypsin. Furthermore, the inactive thrombin derivatives DIP-thrombin and PPACK-thrombin were ineffective in promoting PI hydrolysis on their own, yet PPACK-thrombin effectively competed with thrombin and trypsin for phospholipase C activation, suggesting its binding was unimpaired. However, one could consider that a change in the active site makes these thrombin derivatives conformationally unfit to serve as agonists, independent of their proteolytic activity. On the other hand, the most straightforward explanation of receptor activation by thrombin or trypsin involves proteolysis. Thus, these data support (although do not prove) a proteolytic mechanism of action by thrombin and trypsin.

The desensitization of the PI response and the lack of recovery of the calcium response to thrombin and trypsin (following brief exposure to these agents) also suggest a change in the receptor so that it is no longer functional. Postreceptor sites of desensitization seem unlikely because trypsin or thrombin pretreatments do not affect the PI and calcium responses to carbachol. Whereas proteolysis of the receptor seems a likely possibility, other mechanisms of receptor desensitization such as receptor phosphorylation and internalization must also be considered, because they can not be ruled out by our data.

Paris *et al.* (21) reported that, in fibroblasts, the proteolytic activity of thrombin seems to be required for both the activation of phospholipase C and subsequent receptor desensitization, because PPACK-thrombin was ineffective. They concluded that the thrombin-induced desensitization of PI hydrolysis occurs at the level of the receptor, because the G protein coupling to phospholipase C remained intact and because the rate of desensitization depended on thrombin concentration (i.e., receptor occupancy). They have also shown that thrombin-induced desensitization is independent of protein kinase C activation and cannot be accounted for by receptor internalization. They suggested that the mechanism for desensitization may be thrombin-induced cleavage of its own receptor but also suggested the possibility of receptor phosphorylation by a kinase other than protein kinase C. Interestingly, it has also been reported (47) that elevation of cAMP levels decreases the responses to thrombin and the level of the receptor. The complexity of desensitization of the PI response to thrombin has also been described in platelets (39, 48) and endothelial cells (17).

Although the mechanism by which thrombin (and trypsin) initiate cellular responses remains unresolved, we have provided at least circumstantial evidence for the involvement of proteolysis. More importantly, we have demonstrated that thrombin and trypsin act at a common receptor to elicit PI hydrolysis and calcium mobilization. We were somewhat surprised by our initial findings that thrombin and trypsin were able to stimulate these responses in CHC and 1321N1 cells. Conceivably, these agents would promote a biochemical cascade of events (e.g., PI hydrolysis, calcium mobilization, protein kinase C activation, and sequelae such as secretion or mitogenesis) in other cells not *a priori* assumed to be responsive to these agents. This possibility should be considered when trypsin is used to prepare primary cell cultures, to passage cultured cells, or to remove cells from plates for subsequent study, especially because the concentration of trypsin found to be maximally effective in our studies is 100- to 1000-fold less than that usually used for these manipulations.

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**Send reprint requests to:** Linda G. Jones, Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093.