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EFFECTS OF THROMBIN RECEPTOR ACTIVATING PEPTIDE ON PHOSPHOINOSITIDE HYDROLYSIS AND PROTEIN KINASE C ACTIVATION IN CULTURED RAT AORTIC SMOOTH MUSCLE CELLS: EVIDENCE FOR "TETHERED-LIGAND" ACTIVATION OF SMOOTH MUSCLE CELL THROMBIN RECEPTORS

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Abstract - Phosphoinositide hydrolysis and protein kinase C (PKC) activation were examined in response to treatment of rat aortic smooth muscle cells with α-thrombin and a seven amino acid thrombin receptor activating peptide (TRAP-7; SFLLRNP). α-Thrombin and TRAP-7 stimulated total inositol phosphate (IP) accumulation and phosphorylation of a specific endogenous substrate for activated PKC. Acetylated TRAP-7 and "reverse" TRAP (FSLLRNPNDKYEPF) were ineffective in stimulating signal transduction. The active site inhibitor, MD805 (argatroban), and the anion-binding exosite inhibitor, BMS 180,742, reduced the IP response to α-thrombin in a concentration-dependent manner. In contrast, the TRAP-7-induced IP response was not affected by either inhibitor. These data are consistent with the tethered-ligand hypothesis for thrombin receptor activation in rat aortic smooth muscle cells.

α-Thrombin is a serine protease which plays a major role in hemostasis. It exerts diverse effects on multiple cell types including vasoconstriction of vascular smooth muscle [1], induction of platelet-derived growth factor expression in endothelial cells [2], proliferation and chemotaxis of inflammatory cells [3], and mitogenesis of neonatal rat vascular smooth muscle cells [4]. α-Thrombin stimulates several intracellular signaling events associated with the growth response such as phosphoinositide hydrolysis, calcium transients and protein kinase C (PKC) activation [5,6]. These events are thought to be mediated by a novel receptor-ligand interaction termed the "tethered-ligand" hypothesis [7]. Two domains of thrombin interact with the thrombin receptor. The active site of thrombin interacts with the cleavage recognition sequence of the receptor while the anion binding exosite interacts with the receptor at the hirudin C-terminal-like domain. Thrombin cleaves the receptor exposing a new amino terminus, SFLLRNPNDKYEPF, for thrombin receptor activating peptide (TRAP), which acts as a tethered-ligand. This new terminus is capable of activating the thrombin receptor directly, i.e. in the absence of thrombin-induced proteolytic cleavage. The tethered-ligand hypothesis has been investigated in platelets where thrombin and TRAP stimulate aggregation [8], inhibit adenylate cyclase [9], and activate phosphoinositide turnover [10]. However, despite the cloning of the rat vascular smooth muscle receptor [11], the role of TRAP in smooth muscle cell thrombin receptor activation is less clear. Thus, it was of interest to examine the effects of α-thrombin and TRAP on phosphoinositide hydrolysis and PKC activation in order to elucidate the mechanisms by which these proteins act in smooth muscle cells.

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MATERIALS AND METHODS

Cell culture. Primary RASM cells were obtained from male Sprague-Dawley rats and were provided by Dr. Marschall Runge of Emory University at passage 2. These cells were serially passaged in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated calf serum (HICS), 2 mM glutamine, and 1% penicillin-streptomycin (Gibco, Grand Island, NY), in a 95% air, 5% CO_2 humidified atmosphere at 37° [6]. To measure the production of inositol phosphate (IP), 1-2 x 10^5 cells were seeded in growth medium for 24 hr. The medium was replaced with DMEM containing 0.4% HICS and 4 μCi/mL [^{3}H]*myo*-inositol (93 Ci/mmol, Amersham, Arlington Heights, IL). After incubation at 37° for 36-72 hr, cells were washed three times with DMEM containing 0.1% bovine serum albumin and used in phosphoinositide turnover experiments. All experiments were conducted in RASM cells from passages 8 to 16.

Phosphoinositide hydrolysis. RASM cells were incubated in medium with 10 mM lithium chloride for 15 min at 37°. Stimuli were added to the medium and the incubation was continued for 5 min at 37°. Inhibitors were added 5 min prior to the stimulus. Reactions were terminated by aspiration, addition of 2.5 mL of boiling 2 mM EDTA and microwaving for 20 sec. Detached radioactivity was placed on a 0.5-mL Dowex AG1X8 (Bio-Rad, Richmond, CA) column for the separation of IPs [12] and 1 mL of eluent was counted in the presence of 15 mL of Ultima Gold scintillation fluid (Packard, Downers Grove, IL) in a Packard Tri-Carb 4640 scintillation counter.

Estimation of PKC activation by analysis of endogenous p80 protein ("MARCKS") phosphorylation. Phosphorylation of endogenous RASM cell proteins was performed essentially as described [13,14]. Briefly, confluent RASM cells were labeled for 4 hr with 0.25 mCi/mL [^{32}P]orthophosphate (9000 Ci/mmol, NEN, Boston, MA). Cells were challenged with 12-myrisate, 13-acetate (PMA), α-thrombin, TRAP-7, or acetylated TRAP for 30 min. Labeled proteins were extracted with 10% (w/v) trichloroacetic acid on ice for 30 min, and then solubilized in 0.3 mL of lysis buffer consisting of 9.5 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol, and 2% ampholines (pH range, 3.5 to 10, Bio-Rad). Two-dimensional polyacrylamide gel electrophoresis was performed as described [14,15]. Radioactivity in individual protein spots was determined by phosphorimage analysis (Molecular Dynamics Phosphorimager Model 400E, Sunnyvale, CA). Phosphorylation of p80, a specific protein PKC substrate, MARCKS [16], was normalized by a ratio method in which the radioactive incorporation of the p80 spot was compared to reference phosphoproteins in the same gel [14].

RESULTS

Both α-thrombin and TRAP-7 caused concentration-dependent increases in total IP accumulation (Fig. 1). The EC_{50} values for α-thrombin and TRAP-7 were 0.1 ± 0.04 nM and 20 ± 2.9 μM, respectively (n=3). Maximum IP accumulation was attained at 1-10 nM α-thrombin and at 100 μM to 1 mM TRAP-7. In all cases, the maximal response to TRAP-7 was slightly greater than that to α-thrombin; however, the difference was not statistically significant and α-thrombin and TRAP-7 in combination at maximal concentrations did not produce a significant additive IP accumulatory effect (data not shown). Total IP accumulation was also examined in response to acetylated TRAP (Ac TRAP) and "reverse" TRAP in which the first two amino acids are reversed (FSLLRNPNDKYEPF). These peptides did not stimulate IP accumulation at concentrations up to 10 μM (Fig. 2). Similarly, the IP response to γ-thrombin was weaker than that to α-thrombin or TRAP-7 (data not shown).

The effects of the active site inhibitor, MD805 (4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; see ref. 17), and the anion-binding exosite inhibitor, BMS 180,742 (succinyl-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-cyclohexylalanine-Gln; see ref. 8), on α–thrombin- and TRAP-7-induced phosphoinositide metabolism were examined. MD805 (Fig. 3A) and BMS 180,742 (Fig. 3B) inhibited the accumulation in total IP induced by α-thrombin (10 nM) in a concentration-dependent manner. Neither MD805 nor BMS 180,742 affected TRAP-7-induced stimulation of phosphoinositide hydrolysis (Fig. 3C, 3D).

We also examined the ability of α-thrombin and TRAP-7 to activate cellular PKC, a ubiquitous cytosolic serine, threonine protein kinase implicated in signal transduction and cell growth. In these assays, increased phosphorylation of the p80-kDa acidic MARCKS protein [13,16] was used as a marker for PKC activation. Addition of phorbol PMA (200 ng/mL), a potent direct-acting PKC agonist, to serum-starved RASM cells induced a 6-fold increase in p80 phosphorylation (Fig. 4). In parallel cultures, we found that addition of α-thrombin (10 nM) or TRAP-7 (100 μM) each induced a 3-fold increase in the phosphorylation of p80, as measured 30 min after agonist addition, while acetylated TRAP (100 μM) failed to induce a significant increase in p80 phosphorylation (Fig. 4).

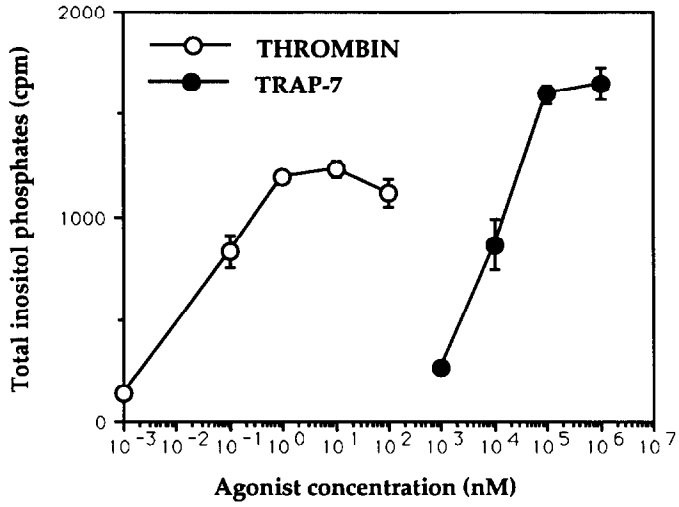

Fig. 1. Total inositol phosphate response to α-thrombin and TRAP-7. Data points are means ± SEM (n = 3). The figure is representative of three similar experiments.

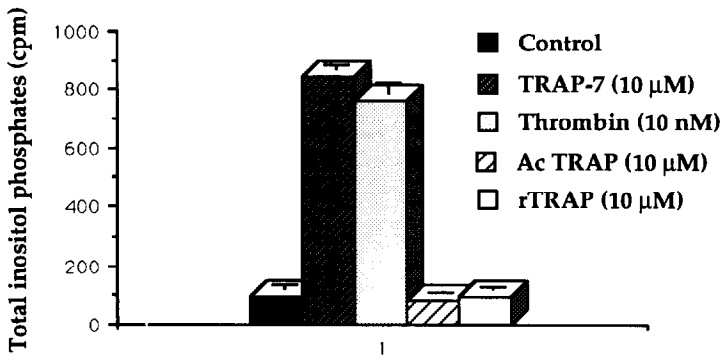

Fig. 2. Effects of α-thrombin, TRAP-7, acetylated TRAP-7 (Ac TRAP) and reverse TRAP (rTRAP) on phosphoinositide hydrolysis. Data are means ± SEM (n = 3) from three similar experiments.

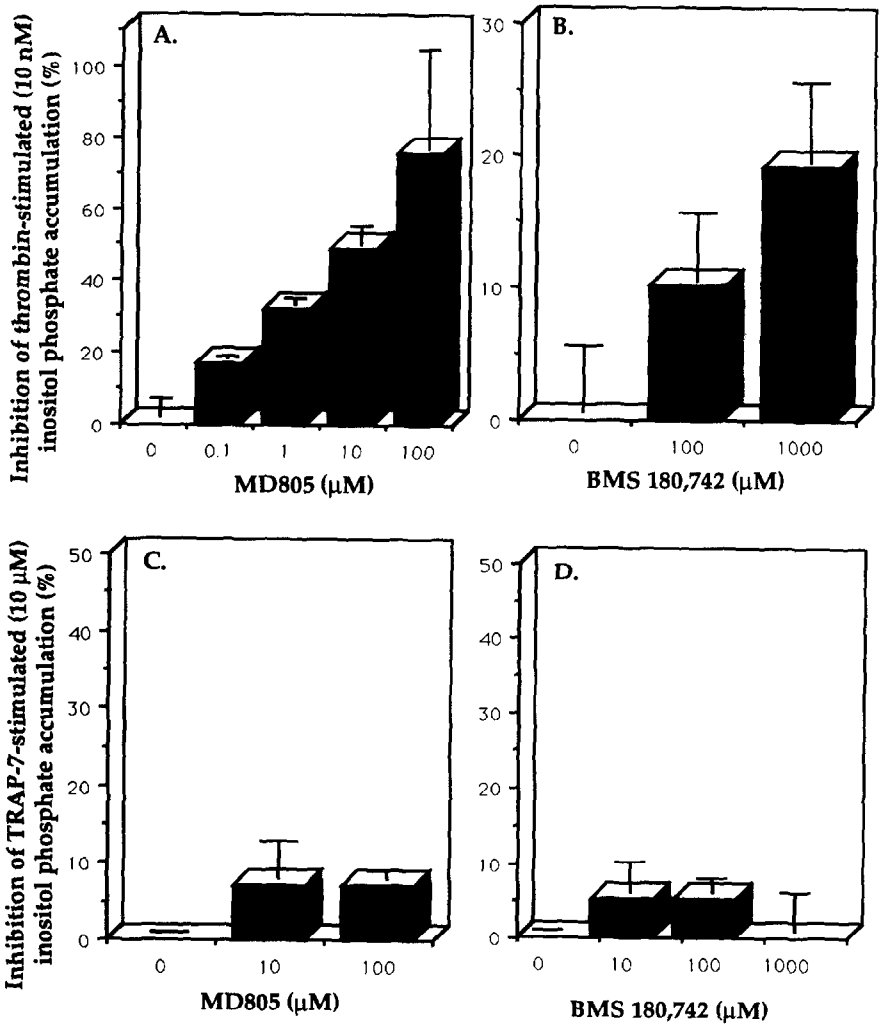

Fig. 3. Effects of MD805 (A and C) and BMS 180,742 (B and D) on α-thrombin (10 nM; panels A and B) or TRAP-7 (10 μM; panels C and D) stimulated inositol phosphate accumulation in rat aortic smooth muscle cells. Values are means ± SEM (n=3) and represent three similar experiments.

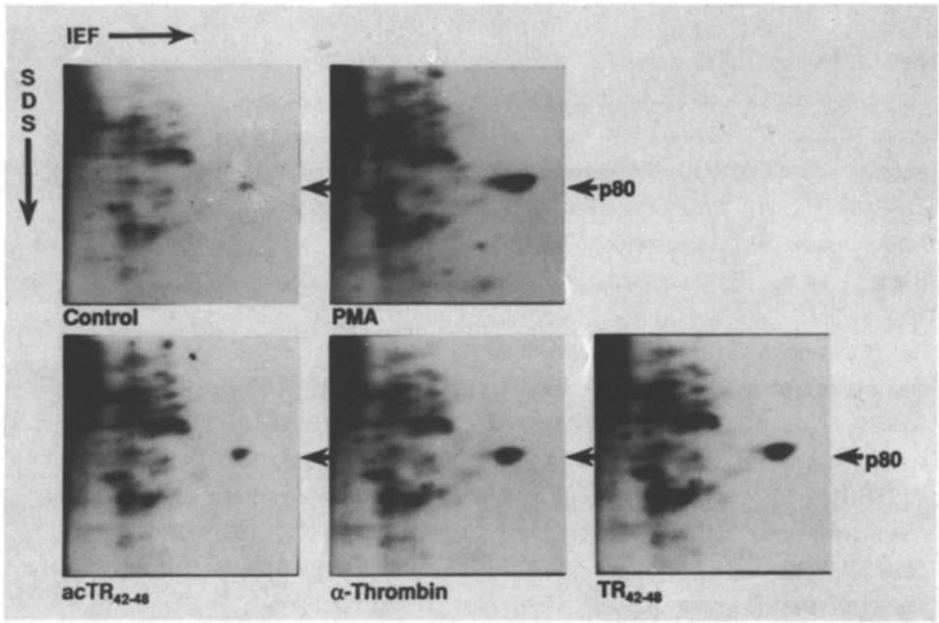

Fig. 4. Effects of α-thrombin and TRAP-7 (TR_{42-48}) on the phosphorylation of a specific protein kinase C substrate. [^{32}P]Orthophosphate-labeled cells were either left unstimulated (control) or were treated with PMA (200 ng/mL), α-thrombin (10 nM), TRAP-7 (TR_{42-48}, 100 μM) or acetylated TRAP-7 ($acTR_{42-48}$, 100 μM) for 30 min as indicated. The position of the specific 80 kDa protein kinase C phosphoprotein substrate is indicated by arrows. Results are representative of experiments performed in duplicate.

Discussion

The data presented here demonstrate that α-thrombin and human TRAP-7 (SFLLRNP *vs* rat TRAP-7 of SFFLRNP) stimulate phosphoinositide hydrolysis and endogenous PKC activity in cultured rat aortic smooth muscle cells. PKC is likely activated by membrane diacylglycerols released as a consequence of PLC catabolism of membrane phospholipids. α-Thrombin has been shown previously to stimulate PLC activity in cultured rat aortic smooth muscle cells [5,6]. Taken together, these data lend further support to the hypothesis that activation of membrane-spanning domain receptors in RASM cells stimulates intracellular signaling pathways that may be involved in cell proliferation.

The EC_{50} values for α-thrombin and TRAP-7 activation of phosphoinositide hydrolysis compare favorably with those for agonist-induced platelet aggregation responses [8]. Thus, the potency of α-thrombin and TRAP-7 in RASM cells is consistent with that in human platelets. The inhibition of α-thrombin-stimulated (10 nM) IP accumulation by MD805 (IC_{50} = 10 μM) and BMS 180,742 (IC_{50} > 1 mM) demonstrates that both thrombin catalytic activity and anion binding exosite function are required for receptor activation. Several investigations have demonstrated that SFLLR-containing peptides can directly stimulate thrombin receptors [7,8]. The selectivity of the present TRAP-7 effect is shown by the lack of stimulation of phosphoinositide hydrolysis by acetylated TRAP-7 and reverse TRAP. These data suggest that a free amino terminus on the first serine residue is required for activation of PLC. Consistent with the tethered-ligand hypothesis of thrombin receptor activation, inhibitors of the exosite and active site of thrombin had no effect on TRAP-7 stimulation of phosphoinositide hydrolysis. Structure-activity relationships associated with TRAP peptides continue to be a topic of great interest as non-peptidic small molecules are sought to bind specifically to the receptor [18-20].

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