It has recently been reported that protein-tyrosine kinase activity is required for thrombin-induced growth in vascular smooth muscle cells (VSMC). In the present study, we have identified several phosphoproteins that are tyrosine-phosphorylated in response to thrombin in quiescent VSMC. These proteins are insulin-like growth factor-1 receptor β-subunit (IGF-1Rβ), insulin receptor substrate-1 (IRS-1), and phospholipase C-γ1 (PLC-γ1). Thrombin-stimulated phosphorylation of these proteins was rapid; it was maximal at 1 min and reduced thereafter. Thrombin also activated mitogen-activated protein kinases (MAPK) in quiescent VSMC in a biphasic manner with a rapid and larger peak at 10 min (6-fold) followed by a sustained smaller second peak at 2 h (2-fold). Inhibition of protein-tyrosine kinase activity by the use of two structurally different protein-tyrosine kinase inhibitors, genistein and herbimycin A, significantly blocked the thrombin-induced tyrosine phosphorylation of IGF-1Rβ, IRS-1, and PLC-γ1 and decreased thrombin-stimulated DNA synthesis. In contrast, however, inhibition of protein-tyrosine kinase activity had no effect on thrombin activation of MAPK. Collectively, these findings suggest a role for tyrosine phosphorylation of IGF-1Rβ, IRS-1, and PLC-γ1 in thrombin-induced mitogenic signaling events in VSMC. Furthermore, while protein tyrosine phosphorylation is essential for thrombin-induced DNA synthesis, it is not required for thrombin-stimulated MAPK activation. Since thrombin rapidly activated Src in VSMC, Src may be involved in the cross-talk between the G-protein-coupled receptor agonist and a tyrosine kinase receptor such as IGF-1R.

Vascular smooth muscle cell (VSMC) growth (hyperplasia and hypertrophy) is considered to be a significant factor in atherosclerosis and restenosis following balloon angioplasty (1). Several molecules including platelet-derived growth factor (PDGF-A and -B), fibroblast growth factor, and angiotensin II appear to be involved in the induction of paracrine and autocrine growth in VSMC (1). More recently it has been reported that thrombin, a serine protease, in addition to its role in blood coagulation also stimulates growth in certain cell types, notably fibroblasts and VSMC (2, 3). In further support of an important role of thrombin in this vascular lesion formation, it has been documented that hirudin, a specific inhibitor of thrombin, reduces neointimal formation (4, 5).

Thrombin is thought to transmit both its coagulant and mitogenic effects via activation of its receptor, a transmembrane G-protein-coupled receptor, which is identical with or similar to the one we and others recently cloned (6, 7). The observation that hirudin but not the anticoagulant heparin inhibited formation of a neointima suggests that the coagulant and mitogenic effects of thrombin are regulated by differential mechanisms or that there may be more than one type of thrombin receptor (5). In fact, using thrombin receptor-activating peptides and protein-tyrosine kinase inhibitors some investigators have demonstrated that along with activation of its G protein-coupled receptor, thrombin also requires protein-tyrosine kinase activity, possibly of a receptor type for its mitogenic effect (2, 3).

Mitogen-activated protein kinases (MAPK) are implicated in the transmission of growth signals from activated receptor tyrosine kinases and G-protein-coupled receptors (8, 9). MAPK are a family of serine/threonine kinases, which are activated by phosphorylation both at threonine and tyrosine residues. These reactions are catalyzed by a dual specificity enzyme MAPK kinase, also termed MEK (10, 11). MEK itself is activated by at least two types of kinase, Raf-1 (12, 13) and MEK kinase, also known as MEKK (14). Raf-1, which is also activated by phosphorylation, appears to be central in receiving signals from activated upstream kinases such as receptor tyrosine kinases (9, 15), protein kinase C (16), and G-protein-coupled receptors (17, 18). This serine/threonine kinase pathway is thought to be involved in the convergence of growth-initiated early protein-tyrosine kinase signaling events and the subsequent transduction of these signals into the nucleus (19).

Since thrombin is an effector of several important cellular processes and is active in many pathological conditions, it is important to understand precisely the signaling events by which this protease deciphers its diverse biological effects. Therefore, the goal of this study was to investigate signaling events, particularly the role of protein tyrosine phosphorylation and MAPK activation in thrombin-induced growth of VSMC. We report several novel observations. First, thrombin stimulates VSMC DNA synthesis, and tyrosine phosphorylation of several proteins, including IGF-1R, IRS-1, and PLC-γ1, is associated with this phenomena. Second, thrombin activated MAPK in a biphasic manner in quiescent VSMC. Third, inhibition of protein-tyrosine kinase activity blocked the thrombin-induced tyrosine phosphorylation of IGF-1R, IRS-1, and PLC-γ1 and DNA synthesis but not thrombin-induced MAPK.
Thrombin-induced Protein Tyrosine Phosphorylation Events

activation, suggesting that the activity of MAPK is dissociated from early protein-tyrosine kinase events induced by thrombin in VSMC. Fourth, thrombin activated Src rapidly in quiescent VSMC, a result that suggests a possible role for this nonreceptor type protein-tyrosine kinase in the cross-talk between the thrombin-bound G-protein-coupled receptor and a protein-tyrosine kinase receptor such as IGF-1R.

EXPERIMENTAL PROCEDURES

Materials—Apronitin, ATP, bovine myelin basic protein (MBP), EGTA, phenylmethylsulfonyl fluoride, sodium deoxycholate, and sodium orthovanadate were obtained from Sigma. Anti-ERK-1, ERK-2, insulin-like growth-factor-1 receptor β-subunit, IRS-1 rabbit polyclonal antibodies and monoclonal mouse anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal mouse anti-bovine PLC-γ1 antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Genistein and herbimycin A were from Biomole (Plymouth Meeting, PA). [3H]-Hydridine (70 Ci/mm) and [γ-32P]ATP (8000 Ci/mmol) were obtained from DuPont NEN.

Cell Culture—VSMC were isolated from the thoracic aortae of 200–250-g male Sprague-Dawley rats by enzymatic digestion as described earlier (20). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere.

DNA Synthesis—VSMC were plated onto 60-mm dishes, allowed to grow to 70–80% confluence, and then growth-arrested by incubation in DMEM containing 0.1% calf serum for 72 h. Growth-arrested VSMC were exposed to various agonists at the indicated concentrations for 24 h. Cell Kinase pulse-labeled with 1 μCi/ml [3H]thymidine for 2 h just before the end of the incubation period with agonists and harvested by trypsinization followed by centrifugation. The cell pellet was resuspended in cold 10% trichloroacetic acid and vortexed vigorously to lyse the cells. The mixture was allowed to sit on ice for 20 min and then passed through a GF/F glass microfiber filter. The filter was washed once on ice with trichloroacetic acid and 5% TCA and twice with cold 70% ethanol. The mixture was placed in a liquid scintillation vial containing the mixture, and radioactivity was measured in a liquid scintillation counter (Beckman LS 3801).

Western Blot Analysis—Growth-arrested VSMC were incubated at 37 °C in the presence or absence of various agonists for the indicated time periods. Medium was then aspirated, and cells were rinsed with cold phosphate-buffered saline and frozen immediately in liquid nitrogen. Two-hundred fifty microliters of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 1 mM sodium orthovanadate) was added to the frozen monolayers, thawed on ice for 15 min, and scraped into 1.5-ml Eppendorf tubes. The cell lysates were clarified by centrifugation at 12,000 rpm for 30 min at 4 °C. Protein content of the supernatant was determined using Bio-Rad’s Bradford reagent. Cell lysates containing equal amounts of proteins were resolved by electrophoresis on a 0.1% SDS, 10% polyacrylamide gel under reducing conditions. The gels were stained by autoradiography.

RESULTS AND DISCUSSION

Growth-arrested VSMC were treated with various concentrations of thrombin (0.025–0.2 unit/ml) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation into acid-precipitable material as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments performed in triplicate each time.

Src Autophosphorylation—Src activation was determined essentially according to the method described by Gould and Hunter (22). Growth-arrested VSMC were labeled with 300 μCi/ml [32P]orthophosphate for 3 h at 37 °C in phosphate-free DMEM. VSMC were then stimulated with thrombin (0.1 unit/ml) for various time periods in the presence and absence of genistein. Cells were rinsed with cold phosphate-buffered saline and lysed in lysis buffer as described above. Cell lysates were centrifuged by centrifugation at 20,000 rpm for 30 min. The supernatant was harvested by centrifugation at 12,000 rpm for 30 min at 4 °C. Protein content of the supernatant was determined using Bio-Rad’s Bradford reagent. Cell lysates containing equal amounts of proteins were resolved by electrophoresis on a 0.1% SDS, 10% polyacrylamide gel under reducing conditions. The gels were dried and exposed to Kodak X-Omat AR x-ray film with an intensifying screen at –70 °C for 1–2 h.

In-gel Kinase Assay—

Western Blot Analysis—

Src Autophosphorylation—

Thrombin-stimulated VSMC DNA synthesis in a dose-dependent manner. Growth-arrested VSMC were treated with and without the indicated concentrations of thrombin for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation into acid-precipitable material as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments performed in triplicate each time.

Growth-arrested VSMC were treated with various concentrations of thrombin (0.025–0.2 unit/ml) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation into acid-precipitable material as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments performed in triplicate each time.
immunoprecipitated with anti-IGF-IR.

Higher concentrations (50 μM genistein and 10 μM herbimycin A) were found to be toxic to VSMC with a longer incubation period. Therefore, inhibition of DNA synthesis by genistein and herbimycin A suggests that protein-tyrosine kinase activity is required for thrombin-induced growth in VSMC.

Many studies have shown that thrombin stimulates tyrosine phosphorylation of several proteins including Src in various cell types (27–30). To identify the proteins that were potentially tyrosine-phosphorylated by, and involved in, thrombin-induced mitogenic signaling, growth-arrested VSMC were treated with thrombin (0.1 unit/ml) for various time periods, and cell extracts were prepared. Extracts of thrombin-treated and untreated VSMC containing equal amounts of proteins (100 μg) were analyzed by immunoblotting with phosphotyrosine antibodies. We detected three phosphoproteins with apparent molecular masses of 100, 140, and 180 kDa, whose tyrosine phosphorylation in response to thrombin increased rapidly (Fig. 3). Maximum 2–3-fold increases in the phosphotyrosine content of these proteins were observed at 1 min of thrombin treatment, gradually decreasing thereafter. The apparent molecular masses of these three phosphoproteins (100, 140, and 180 kDa) appeared to fall within the range of molecular masses reported for IGF-1R, PLC-γ1, and IRS-1, respectively (31, 32). To establish the identity of these proteins, equal amounts of proteins (500 μg) from thrombin-treated and untreated VSMC were immunoprecipitated with anti-IGF-1R, anti-PLC-γ1, or anti-IRS-1 antibodies and analyzed by Western blotting using phosphotyrosine antibodies. Phosphotyrosine Western blot analysis of anti-IGF-1R immunoprecipitates detected a band with a molecular mass of 100 kDa, whose phosphorylation was increased severalfold in response to thrombin, and this response was sensitive to inhibition by genistein (Fig. 4). Phosphotyrosine Western blot analysis of anti-IRS-1 immunoprecipitates showed a band of 180 kDa in size, and its phosphotyrosine content was increased 2.5-fold by thrombin (Fig. 4). Genistein inhibited the thrombin’s effect on IRS-1 phosphorylation. The identity of the 140-kDa protein as PLC-γ1 was also established using a similar approach (Fig. 4). It has recently been reported that Src phosphorylates IGF-1R in Rat-1 fibroblasts (33). Therefore, to investigate a possible mechanism by which thrombin stimulates IGF-1R phosphorylation, thrombin’s effect of Src activation in growth-arrested VSMC was determined. As shown in Fig. 5, thrombin activated Src rapidly in VSMC with a maximum effect at 1 min (3-fold). In addition, thrombin activation of Src was found to be sensitive to protein-tyrosine kinase inhibition (Fig. 5). Collectively these findings strongly suggest a role for tyrosine phosphorylation of IGF-1R, IRS-1, PLC-γ1, and Src in thrombin-initiated mitogenic signaling events.

It was thought that early protein tyrosine phosphorylation events such as receptor tyrosine kinase phosphorylation transmit mitogenic signals by activation of a “Ras-Raf-MEK-MAPK” pathway via recruitment of adaptor molecules such as GRB2, Shc, and SOS (19, 34–36). Since thrombin requires protein-tyrosine kinase activity for its mitogenic effect and IGF-1R phosphorylation is associated with this, it is paradoxical and intriguing that these tyrosine phosphorylation events may be required for thrombin’s activation of the “Ras-Raf-MEK-MAPK” pathway. Several experiments were performed to test this hypothesis. Growth-arrested VSMC were treated with thrombin (0.1 unit/ml) for various time periods as well as in the presence and absence of protein-tyrosine kinase activity inhibitors, and cell lysates were prepared. MAPK activities in cell lysates were determined by 1) in-gel kinase assay using MBP as a substrate and 2) detection by mobility shift of phosphorylated MAPK on Western blots. Cell lysates containing equal amounts of proteins from control and various treatments were resolved by SDS-PAGE copolymerized with MBP, and the kinase assay was performed by incubating the gel in the kinase buffer containing [γ-32P]ATP, as described under “Experimental Procedures.” Thrombin activated MAPK in a time-dependent biphasic manner with the first and highest peak of activity (6-fold) at 10 min, followed by a second and more sustained lower peak of activity (2-fold) at 2 h (Fig. 6). These results are in agreement with previous reports in hamster fibroblasts (37). Genistein or herbimycin A alone had no effect on MAPK activities in VSMC and did not block the thrombin-stimulated activation of MAPK in these cells (Fig. 6). The lack of the effect of genistein on thrombin-stimulated activation of MAPK is selective, because it blocked the serum-induced activation of MAPK in VSMC (Fig. 7). In fact, Winitz et al. (18) and Hawes et al. (38) also observed that genistein, a protein-tyrosine kinase inhibi-
did not block the muscarinic cholinergic receptor, a G-protein-coupled receptor, mediated activation of MAPK, while it inhibited epidermal growth factor receptor-mediated activation of MAPK.

These findings provide the first demonstration that thrombin, a G-protein-coupled receptor agonist, stimulates tyrosine phosphorylation of IGF-1R, a receptor protein-tyrosine kinase. IGF-1R consists of two α (135 kDa) and two β (95 kDa) subunits linked by disulfide bonds. Ligand binding to the extracellular α-subunit of the receptor produces a conformational change in the β-subunits resulting in autophosphorylation, which is required for its kinase activity. In turn the activated receptor phosphorylates several proteins including its immediate substrate IRS-1 (39). Phosphorylated IRS-1 interacts with downstream signaling molecules such as GRB2 and phosphatidylinositol 3-kinase via Src homology 2 (SH2) domains (40, 41). IGF-1R has a critical role in transmitting its ligand-evoked cellular effects (19, 42), and the importance of IGF-1R phosphorylation in cell growth and transformation has also been demonstrated (39, 43). In fact, Peterson et al. (33) recently reported ligand-independent phosphorylation of IGF-1R in Src-transformed fibroblasts. This work also showed that Src-induced intracellular phosphorylation events are required for IGF-1R phosphorylation in these cells. A similar mechanism may account for thrombin-induced phosphorylation of IGF-1R in VSMC. This suggestion is based on the following observations. 1) Thrombin activated Src in VSMC, and 2) this event is temporally correlated with phosphorylation of IGF-1R by thrombin. As thrombin-induced early protein tyrosine phosphorylation events include phosphorylation of Src, IGF-1R,
IRS-1, and PLC-γ1 and inhibition of protein-tyrosine kinase activity blocks thrombin's phosphorylation of these molecules and DNA synthesis, it is likely that thrombin-induced Src, IGF-1R, IRS-1, and PLC-γ1 phosphorylation are closely associated with thrombin's mitogenic signaling events in VSMC.

Several investigators have established that ligand-induced receptor tyrosine kinase activation is essential for MAPK activation (19, 34–36, 40–42). MAPK are important in regulating transcriptional factors such as c-Jun, c-Myc, and p62TEF (44–46), and they in turn modulate gene transcription. Although our findings demonstrate a biphasic activation of MAPK by thrombin this appears to be dissociated from tyrosine phosphorylation of IGF-1R, IRS-1, PLC-γ1, and Src, since inhibiting phosphorylation of these molecules had no effect on thrombin-induced activation of MAPK. On the other hand, induced tyrosine phosphorylation of IGF-1R, IRS-1, PLC-γ1, and Src appears to be on the pathway that leads to DNA synthesis because inhibiting protein-tyrosine kinase activity blocked phosphorylation of these molecules as well as DNA synthesis. Pages et al. (47) by antisense down-regulation of MAPK and by overexpression of MAPK reported that MAPK are required for fibroblast growth factor and thrombin-induced fibroblastic growth. Our findings and those of Pages et al. (47) imply that the tyrosine phosphorylation events that we observed and the MAPK cascade are independently regulated by thrombin, and both are required for DNA synthesis. If both the MAPK pathway and protein-tyrosine kinase events are required for DNA synthesis, then interference with either pathway will affect growth. Another explanation is that both thrombin-induced IGF-1R, IRS-1, PLC-γ1, and Src, cytoskeletal proteins, and MAPK cascade are independently regulated by thrombin and both are required for DNA synthesis. If both the MAPK pathway and protein-tyrosine kinase events are required for DNA synthesis, then interference with either pathway will affect growth. Another explanation is that both thrombin-induced IGF-1R, IRS-1, PLC-γ1, and Src, cytoskeletal proteins, and MAPK cascade are independently regulated by thrombin and both are required for DNA synthesis. If both the MAPK pathway and protein-tyrosine kinase events are required for DNA synthesis, then interference with either pathway will affect growth. Another explanation is that both thrombin-induced IGF-1R, IRS-1, PLC-γ1, and Src, cytoskeletal proteins, and MAPK cascade are independently regulated by thrombin and both are required for DNA synthesis. If both the MAPK pathway and protein-tyrosine kinase events are required for DNA synthesis, then interference with either pathway will affect growth. Another explanation is that both thrombin-induced IGF-1R, IRS-1, PLC-γ1, and Src, cytoskeletal proteins, and MAPK cascade are independently regulated by thrombin and both are required for DNA synthesis.
Thrombin Stimulates Phosphorylation of Insulin-like Growth Factor-1 Receptor, Insulin Receptor Substrate-1, and Phospholipase C-γ1 in Rat Aortic Smooth Muscle Cells

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