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Blockade of DNA Synthesis Induced by Platelet-Derived Growth Factor by Tranilast, an Inhibitor of Calcium Entry, in Vascular Smooth Muscle Cells

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SUMMARY

The present study was conducted to establish a pharmacological method of controlling growth of vascular smooth muscle cells (VSMC) by blocking calcium entry. In cultured rat VSMC, 1 nM platelet-derived growth factor (PDGF) induced a biphasic elevation of cytoplasmic free calcium concentration, ($[Ca^{2+}]_c$). The second sustained phase of $[Ca^{2+}]_c$ was dependent on extracellular calcium. At lower concentrations, PDGF induced oscillatory changes in $[Ca^{2+}]_c$, and reduction of extracellular calcium attenuated the oscillation. An antiallergic compound, tranilast, abolished the sustained phase of $[Ca^{2+}]_c$ induced by 1 nM PDGF. Tranilast also inhibited the oscillatory changes in $[Ca^{2+}]_c$ induced by 200 pM PDGF. In addition, PDGF-induced calcium influx in the late G_1 phase, as assessed by measuring the initial uptake of ^{45}Ca, was inhibited by tranilast in a concentration-dependent manner. Tranilast also inhibited PDGF-augmented DNA synthesis; the ID_{50} for the inhibition of DNA synthesis was nearly identical to that for calcium influx. Although tranilast blocked PDGF-induced calcium entry, it did not affect PDGF-mediated autophosphorylation of the PDGF receptor, activation of phosphatidylinositol 3-kinase, activation of Ras or mitogen-activated protein kinase. Similarly, PDGF-induced elevation of diacylglycerol was not affected by tranilast. These results suggest that the antiallergic drug tranilast inhibits PDGF-induced DNA synthesis by blocking PDGF-mediated calcium entry. Tranilast may be of use in controlling PDGF-induced DNA synthesis in VSMC.

Arterial injury after PTCA results in proliferation of neointimal VSMC. Clinically significant restenosis takes place in 30 to 50% of patients who have undergone angioplasty (1). Arterial injury causes migration of VSMC into the intimal layer of the arterial wall. Subsequent proliferation of VSMC and synthesis of extracellular matrix lead to the initiation of restenosis. Locally synthesized growth factors and cytokines play a pivotal role in these processes (2). It is therefore possible that blockade of synthesis and/or the action of these factors would be effective in preventing the initiation and progresssion of restenosis. Of the various growth factors and cytokines, PDGF may be particularly important in the genesis of atherogenesis and restenosis (2). PDGF released from platelets during thrombosis induces migration of VSMC into the intima and their subsequent proliferation (3). PDGF is also synthesized in macrophages, endothelial cells, and VSMC (4). Ferns *et al.* (5) reported that administration of anti-PDGF antibody attenuated arterial restenosis after angioplasty. This observation suggests that PDGF plays a critical role in the genesis of restenosis and raises the possibility that blockade of PDGF action would be effective in the prevention of restenosis.

After binding to its receptor, PDGF induces a pleiotropic reaction in target cells (6), including activation of receptor-associated tyrosine kinase, Ras (a G protein), MAP kinase, PI-3-kinase, PL-C-γ and PL-D. Additionally, PDGF induces changes in cell calcium metabolism, including mobilization of calcium from an intracellular pool by a mechanism dependent on inositol-1,4,5-trisphosphate (6). Recent studies indicate that this reaction may not be involved in PDGF-induced DNA synthesis (7, 8). PDGF also augments calcium entry into target cells, but the route of calcium entry and the mechanism by which PDGF activates calcium gating is not fully understood (9). We reported that PDGF induced a sustained elevation of calcium entry in VSMC, which was a

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ABBREVIATIONS: PTCA, percutaneous transarterial coronary angioplasty; VSMC, vascular smooth muscle cells; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; PI-3, phosphatidylinositol-3; PL-C, phospholipase C; PL-D, phospholipase D; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DAG, diacylglycerol; $[Ca^{2+}]_c$, cytoplasmic free calcium concentration.

prerequisite for PDGF-induced DNA synthesis (10). When calcium entry was inhibited by either the reduction of extracellular calcium or the addition of nickel chloride, an inorganic blocker of the calcium channel, PDGF-induced DNA synthesis was completely blocked (10). This raises the possibility that blockade of PDGF-induced calcium entry may be useful in inhibiting the proliferation of VSMC.

Tranilast is an antiallergic drug and blocks calcium entry in mast cells (11). Because this compound is shown to inhibit growth and migration of VSMC (12), we investigated whether tranilast inhibits growth of VSMC by blocking calcium entry. The results indicate that tranilast blocks calcium entry induced by PDGF. Given that tranilast is effective in preventing restenosis after PTCA in humans (13), blockade of calcium entry may provide a pharmacological method to prevent restenosis after PTCA.

Experimental Procedures

Culture of VSMC. Rat VSMC were obtained from explants of aortic media of male Wistar rats that weighed approximately 200 g (14). For measurement of DNA synthesis, cells between the fourth and seventh subcultures were seeded at a density of 2×10^4 cells/cm^2 and cultured for 5 days in DMEM containing 10% fetal calf serum (GIBCO, Grand Island, NY). To obtain quiescent cells, confluent cells were washed with serum-free DMEM and cultured for 3 days in serum-free DMEM (15).

Measurement of DNA synthesis. DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material. Quiescent VSMC were incubated for 24 hr with various agents and 1 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured by the method of McNiel *et al.* (15). In some experiments, nuclear labeling was measured using bromodeoxyuridine (Amersham Japan, Tokyo, Japan), as described previously (16).

Measurement of $[Ca^{2+}]_c$. The $[Ca^{2+}]_c$ in a single VSMC was monitored by measuring fluo-3 fluorescence (17). Cells cultured on a coverglass were incubated with 30 μM fluo-3/acetoxymethyl ester (Dojin Laboratories, Kumamoto, Japan) in the presence of Pluronic F-125 (Molecular Probes, Eugene, OR) for 60 min at room temperature, and each coverglass was then placed on a flow-through chamber mounted on the stage of a TMD microscope (Nikon, Tokyo, Japan). The perfusion medium contained 135 mM NaCl, 4.5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM HEPES/NaOH, pH 7.4. Fluo-3 fluorescence was measured using CAM-250 (Nihon Bunko, Tokyo, Japan) (18). The fluorescence excited at 506 nm was measured at 526 nm. $[Ca^{2+}]_c$ was calibrated as described by Minta *et al.* (19). When extracellular calcium was reduced to 1 μM, the calcium-EGTA buffer was employed (19).

Measurement of unidirectional calcium influx rate. Calcium influx rate was determined by measuring the initial uptake of ^{45}Ca (10). Briefly, cells were cultured in 35-mm dishes and were serum-fasted as described above. The cells were incubated for the indicated time (see Fig. 4) in DMEM containing PDGF. The cells were washed quickly with modified Hanks' solution (pH 7.8, adjusted with 20 mM HEPES/NaOH). The cells were incubated with the same solution containing 2 μCi/ml [^{45}Ca]$CaCl_2$ and PDGF for either 15 or 75 sec in the presence or absence of tranilast. Cells were washed quickly three times with ice-cold solution containing 120 mM NaCl, 5 mM $CaCl_2$, 20 mM HEPES/NaOH, pH 7.8, solubilized by adding 0.1% SDS and the radioactivity associated with the cells was counted. Calcium influx rate was calculated by measuring the amount of calcium taken up in 60 sec. (19).

Analysis of Ras-bound guanine nucleotides. Cells were grown in 60 mm-dishes in serum-free medium as described above. The cells were labeled with ^{32}P by incubating them for 3 hr with phosphate-free DMEM that contained 100 μCi of [^{32}P]orthophosphate and were then incubated with PDGF. Determination of the GTP/GDP ratio of $p21^{ras}$ was executed as described by Burgering *et al.* (20). Briefly, the cells were washed rapidly 3 times with ice-cold Tris-buffered saline and lysed in the lysis buffer (1× = 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-114, 1 mg/ml bovine serum albumin, 10 μg of aprotinin, 10 μg of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 μm GTP, 100 μm GDP, 1 mM ATP, 1 mM ATP, 1 mM sodium pyroposphate, and 1 mM sodium orthovanadate) (20). Nuclei were removed by centrifugation at 15,000 rpm for 20 min at 4°. For separation of Triton-X-114 and the aqueous phase, a 300-ml cushion of 6% (w/v) sucrose, 100 mM Tris·HCl, pH 7.4, 150 mM NaCl and 0.06% Triton X-114 was placed at the bottom of a 1.5-ml Eppendorf microfuge tube. The clear sample (supernatant) was then overlaid on this sucrose cushion, the tube was incubated for 3 min at 37°, and was then centrifuged (2,500 rpm) for 3 min at room temperature. The aqueous phase was transferred to another new tube, which was used for the detection of PDGF receptor autophosphorylation. The detergent phase was used for the determination of GTP/GDP ratio of $p21^{ras}$ (20).

Analysis of PDGF receptor autophosphorylation. After phase separation as described above, the aqueous phase was incubated for 1 hr with an antiphosphotyrosine polyclonal antibody coupled to protein G-sepharose. The immunoprecipitates were collected and washed 4 times with the washing buffer (1× = 50 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS, 1 mg/ml bovine serum albumin, 1 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) as described above. The sample buffer (1× = 125 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol blue, and 5% β-mercaptoethanol) was then added and heated at 90° for 5 min. Proteins were analyzed by SDS-PAGE and autoradiograms were obtained.

Measurement of PI-3-kinase activity. Serum-fasted cells grown in 60-mm dishes were incubated for the indicated period (Fig. 7) with PDGF in the presence and absence of tranilast. The cells were rinsed with an ice-cold buffer that consisted of 20 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, and 0.1 mM Na_3VO_4 and then lysed in 300 μl of this buffer supplemented with 10% glycerol, 1% Nonidet P-40 (Sigma Chemical, St. Louis, MO), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A. The cell lysates were centrifuged at 15,000 rpm for 20 min and the supernatant was immunoprecipitated with an appropriate amount of polyclonal antiphosphotyrosine antibody coupled to protein G-Sepharose. PI-3-kinase activity in the immunoprecipitate was measured as described by Sato *et al.* (21).

Measurement of MAP kinase activity. Cells were incubated for the indicated time (see Fig. 8A) with PDGF and were homogenized in buffer containing 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin. Cellular debris was precipitated by centrifuging at 25,000 × *g* for 20 min. MAP kinase activity was measured using the p42/p44 MAP kinase assay system (Amersham Japan).

Measurement of diacylglycerol. Cells cultured in 35-mm dishes were incubated for the indicated time (see Fig. 8A) with PDGF in the presence and absence of tranilast. The reaction was stopped by adding ice-cold phosphate-buffered saline (1× = 127 mM NaCl, 2.6 mM KCl, 1 mM KH_2PO_4, and 8 mM Na_2HPO_4) and cells were scraped off using a rubber policeman. The cellular lipids were extracted by adding chloroform/methanol (1:2, v/v) containing 0.5% HCl. DAG was measured by the method of Preiss *et al.* (22).

Materials. PDGF-BB was purchased from Pepro Tech (Los Angeles, CA). Tranilast [*N*-(3′,4′-dimethoxy cinnamoyl) anthranilic acid (*N*-5′)] was provided by Kissei Pharmaceuticals (Nagano, Japan). Nifedipine, nicardipine, verapamil, diltiazem, indomethacin, meclofenamate, and quinacrine were obtained from Sigma. Tetramethrin was provided from Sumitomo Chemical Inc. (Osaka, Japan) and BW755c was from Wellcome Research Laboratories (Kent, UK). Other chemicals were of reagent grade.

Results

Effects of tranilast on PDGF-induced calcium entry. The effects of various compounds on PDGF-induced elevation of $[Ca^{2+}]_c$ in VSMC were tested to identify an inhibitor of PDGF-induced calcium entry. The compounds that were tested are listed in Table 1. Among the compounds tested, an antiallergic agent, tranilast, attenuated PDGF-induced calcium entry in VSMC (Fig. 1). As shown in Fig. 2A, 1 nM PDGF elicited a rise in $[Ca^{2+}]_c$ in a single VSMC. In the presence of 1.25 mM extracellular calcium, the elevation of $[Ca^{2+}]_c$ was biphasic; the initial peak of $[Ca^{2+}]_c$ was followed by a sustained elevation of $[Ca^{2+}]_c$. The second phase of the rise in $[Ca^{2+}]_c$ was caused largely by calcium entry, because reduction of the extracellular calcium concentration to 1 μM abolished the sustained phase of $[Ca^{2+}]_c$ (Fig. 2A). PDGF induced a transient elevation of $[Ca^{2+}]_c$ in a low calcium-containing medium. Elevation of the extracellular calcium concentration to 1.25 mM restored the sustained phase of $[Ca^{2+}]_c$ (Fig. 2B). Lower concentrations of PDGF (200 pM) induced oscillatory changes in $[Ca^{2+}]_c$ (Fig. 2C). Reduction of extracellular calcium to 1 μM gradually decreased the peak values of $[Ca^{2+}]_c$; the oscillation disappeared within 3 min. In the presence of 100 μM tranilast, 1 nM PDGF induced monophasic change in $[Ca^{2+}]_c$ (Fig. 3A). Although the peak value was nearly identical to that observed in the absence of tranilast, the second sustained phase of $[Ca^{2+}]_c$ was not observed. The sustained elevation of $[Ca^{2+}]_c$ was observed after removal of tranilast, although the elevation of $[Ca^{2+}]_c$ was not prompt. The reversal of the inhibitory effect of tranilast occurred rather slowly. The inhibitory effect of tranilast on the second phase was detected at 25 μM and was maximal at 100 μM. The addition of 100 μM tranilast after the occurrence of the second phase of PDGF-induced elevation of $[Ca^{2+}]_c$ abolished the sustained phase of $[Ca^{2+}]_c$ (Fig. 3B). Oscillation of $[Ca^{2+}]_c$ induced by 200 pM PDGF was also attenuated by the addition of 100 μM tranilast (Fig. 3C).

We have shown previously that PDGF induces a sustained elevation of calcium influx and that the calcium influx rate reaches its maximal level 90 min after the addition of PDGF (10). To analyze quantitatively the effect of tranilast on PDGF-induced calcium entry, we measured the unidirectional calcium influx rate in the late G_1 phase, 9 hr after the addition of PDGF (10). Fig. 4 demonstrates the effect of tranilast on PDGF-induced calcium influx. At concentrations higher than 60 μM, tranilast inhibited PDGF-induced calcium entry into VSMC. At a concentration of 100 μM, tranilast almost completely blocked the effect of PDGF.

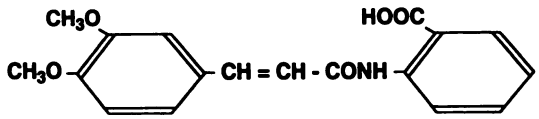

Fig. 1. Structure of tranilast

Effect of tranilast on PDGF-mediated DNA synthesis. In the subsequent experiments, we investigated the effect of tranilast on PDGF-induced DNA synthesis in VSMC. As shown in Fig. 5, tranilast inhibited DNA synthesis induced by PDGF. The inhibitory effect of tranilast was concentration-dependent; it was detectable at 30 μM, whereas at 100 μM, DNA synthesis was almost completely blocked. The ID_{50} for the inhibitory action of tranilast was approximately 45 μM. When VSMC were cultured in a sparse condition, 100 μM tranilast completely blocked the increase in cell number induced by PDGF (Fig. 6). The effect of tranilast was reversible because PDGF increased cell number after the removal of tranilast (data not shown).

Effect of tranilast on signaling system activated by PDGF. To determine the specificity of the action of tranilast, we examined the effects of tranilast on the intracellular signaling system activated by PDGF. As shown in Fig. 7A, PDGF induced the phosphorylation of a 170-kDa protein that was immunoprecipitated by antiphosphotyrosine antibody, and was presumed to be the PDGF receptor. Tranilast did not affect the phosphorylation induced by PDGF. Phosphatidylinositol 3-kinase is activated by PDGF (6) and is thought to play a critical role in PDGF-induced DNA synthesis. Tranilast did not affect the activation of PI-3-kinase, which was elicited by PDGF (Fig. 7B). Another important signaling molecule involved in the action of PDGF is Ras, a low-molecular weight G protein that is activated by the PDGF receptor via the Grb2-Sos pathway (23). In some types of cells (e.g., neurons), it has been shown that the activity of Ras is affected by calcium entry (24). We therefore examined whether tranilast would affect PDGF-induced activation of Ras. In VSMC, tranilast did not affect PDGF-induced activation of Ras (data not shown). MAP kinase is a serine-threonine kinase that is activated by mitogens, including PDGF (25), and has been implicated in the mitogenic action of these growth factors. The MAP kinase pathway is located downstream of Ras and is also activated by other mechanisms, including protein kinase C (25). We therefore examined the effect of tranilast on MAP kinase. Fig. 8A depicts the time course of MAP kinase activity in PDGF-stimulated cells. The activity of MAP kinase was markedly augmented within 5 min of stimulation, and the activity remained elevated for at least 120 min. Tranilast did not affect the elevation of MAP kinase activity induced by PDGF. PL-D is activated by the PDGF receptor via the activation of PL-C (26) and has been shown to be involved in the mitogenic action of PDGF (27). We therefore examined the effect of tranilast on PDGF-induced elevation of DAG, a product of activated PL-C and PL-D. As shown in Fig. 8B, tranilast did not affect the increase in DAG content induced by PDGF.

TABLE 1

Compounds tested

Various compounds were tested as to whether or not they inhibited PDGF-induced calcium entry by measuring calcium influx rate as shown in Fig. 4. Compounds listed (except tranilast) had little effect at the concentrations indicated.

Compound	Concentration (M)
Nifedipine	5×10^{-5}
Nitrendipine	5×10^{-5}
Nicardipine	5×10^{-5}
Verapamil	1×10^{-4}
Diltiazem	1×10^{-4}
SKF96365	5×10^{-5}
Tetramethrin	5×10^{-5}
Tranilast	1×10^{-4}
Clotrimazole	1×10^{-5}
Miconazole	1×10^{-5}
Indomethacin	1×10^{-4}
Meclofenamate	1×10^{-4}
Quinacrine	1×10^{-4}
BW755c	1×10^{-4}

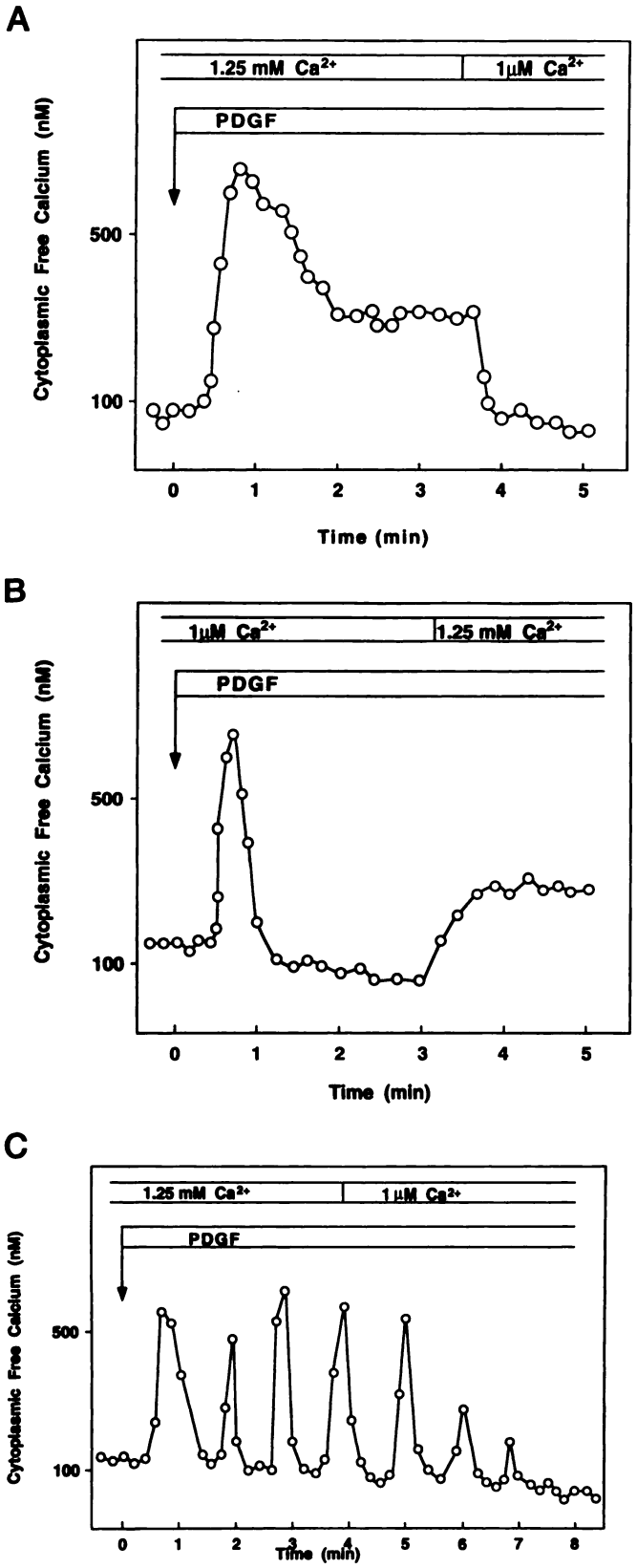

Fig. 2. Effect of changes in extracellular calcium on PDGF-induced rise in $[Ca^{2+}]_c$. Fluo-3-loaded VSMC were stimulated by either 1 nM (A, B) or 200 pM PDGF (C) in the presence of 1.25 mM or 1 μM extracellular calcium. $[Ca^{2+}]_c$ in a single cell was monitored as described in Experimental Procedures.

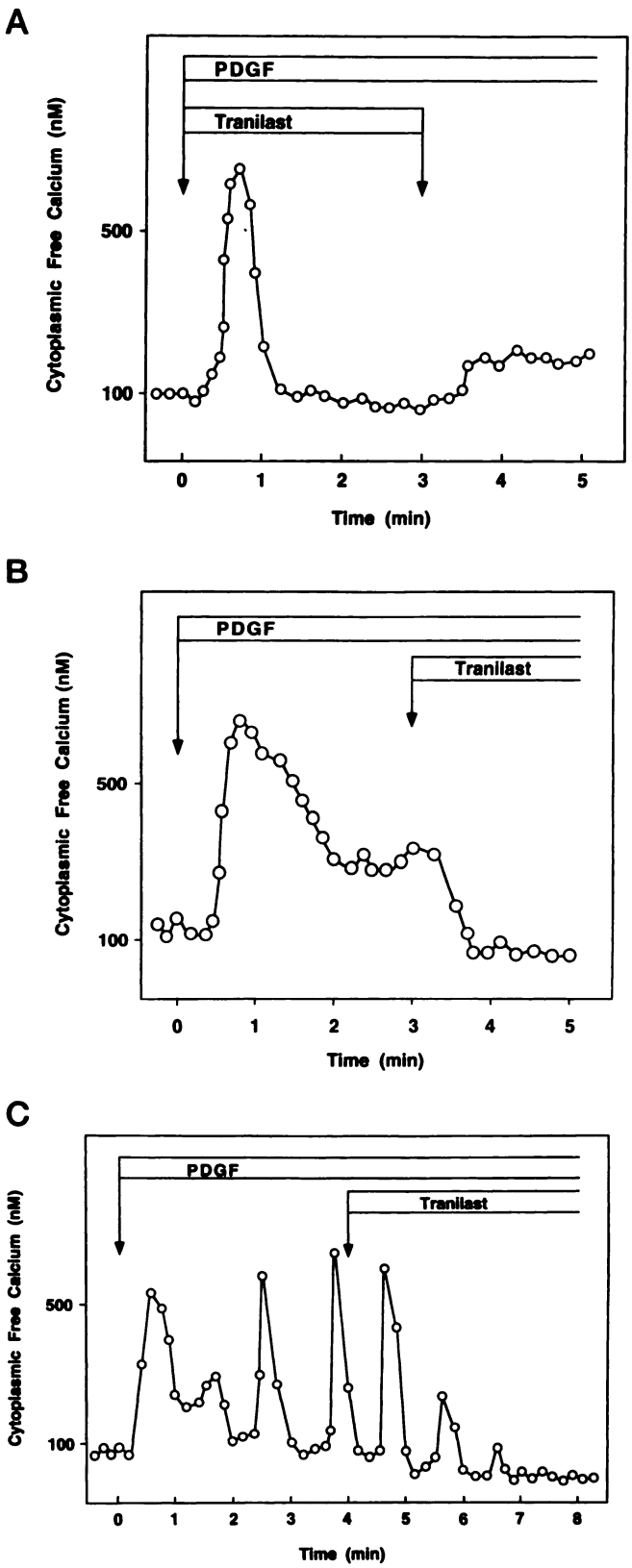

Fig. 3. Effect of tranilast on PDGF-induced rise in $[Ca^{2+}]_c$. Fluo-3-loaded VSMC were stimulated by either 1 nM (A, B) or 200 pM PDGF (C) in the presence or absence of 100 μM tranilast. $[Ca^{2+}]_c$ in a single cell was monitored.

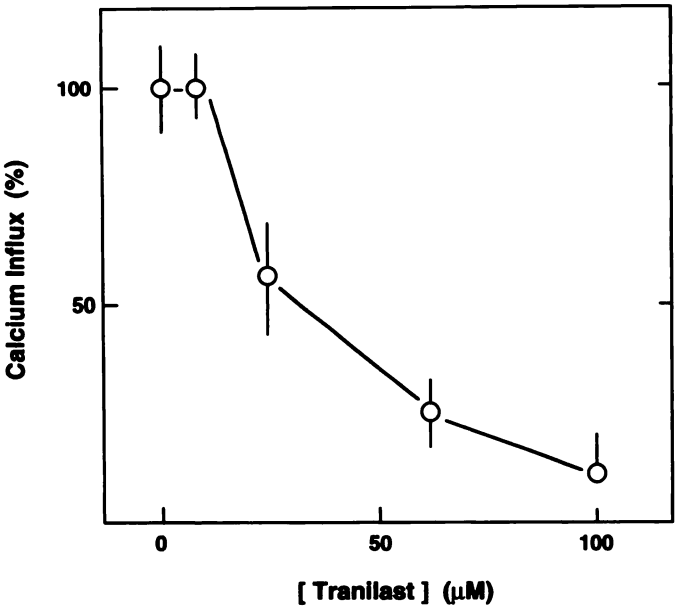

Fig. 4. Effect of tranilast on calcium influx induced by PDGF. VSMC were incubated for 9 hr with 1 nM PDGF. Then ^{45}Ca was added in the presence of various concentrations of tranilast. Calcium influx rate was determined by measuring the uptake of ^{45}Ca in 60 sec. Values are the means ± standard error for four experiments. Basal and PDGF-stimulated calcium influx rates were 934 ± 88 and 2234 ± 208 pmol/min/mg of protein, respectively.

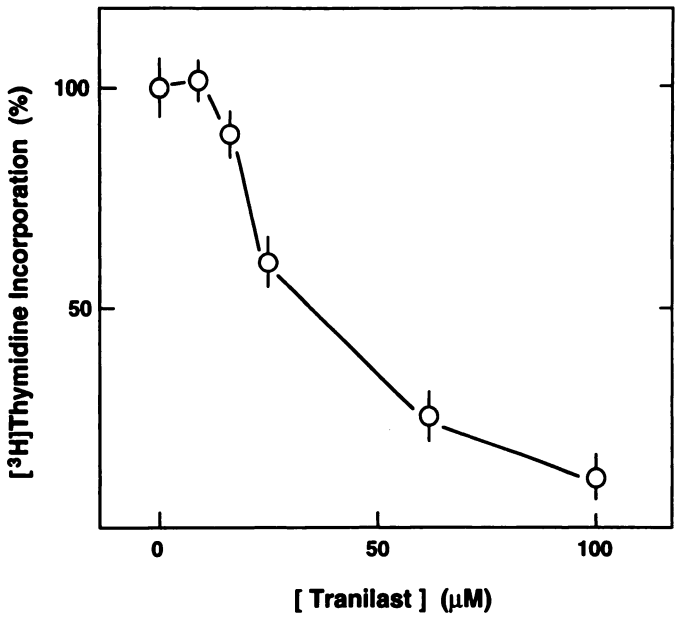

Fig. 5. Effect of tranilast on DNA synthesis induced by PDGF. VSMC were incubated for 24 hr with 1 nM PDGF in the presence of various concentrations of tranilast. [^{3}H]Thymidine incorporation was measured. Values are the means ± standard error for four experiments. [^{3}H]Thymidine incorporations in PDGF-treated and untreated cells were 98,432 ± 6,811 and 8,643 ± 496 cpm/well, respectively.

Discussion

PDGF stimulates calcium entry in VSMC in a sustained manner (10). Although the route of calcium entry is not clear, involvement of voltage-dependent calcium channels is unlikely, because dihydropyridines are ineffective (10). Recently, Matsunaga *et al.* (28) reported the presence of PDGF-activated, voltage-independent calcium-permeable cation channel in cultured mesangial cells. The PDGF-sensitive cation channel was tightly regulated by the PDGF receptor and the unitary conductance was extremely small. Given that mesangial cells resemble VSMC in many respects, it is possible that PDGF activates a similar type of calcium-permeable cation channel in VSMC. The PDGF-mediated calcium entry may be at least partly attributable to the activation of such a calcium-permeable channel.

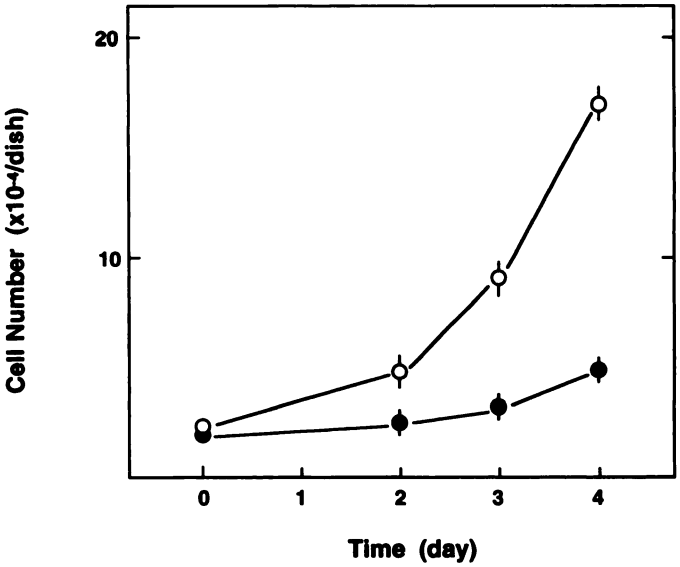

Fig. 6. Effect of tranilast on PDGF-induced proliferation of VSMC. VSMC were cultured for various periods with 1 nM PDGF in the presence (●) or absence (○) of 100 μM tranilast. Cell numbers were then counted. Values are the means ± standard error for four experiments.

As shown in Fig. 3, tranilast inhibited the sustained elevation in $[Ca^{2+}]_c$ induced by PDGF. This effect was the result of an attenuation in calcium entry, because it was qualitatively similar to that induced by the reduction of the extracellular calcium or the addition of $NiCl_2$, an organic blocker of calcium entry (data not shown). In agreement with this notion, PDGF-induced calcium influx was blocked by tranilast (Fig. 4). In particular, tranilast blocks calcium entry in the late G_1 phase. Tranilast was originally developed as an antiallergic drug (29). It suppresses histamine release from mast cells through inhibition of calcium entry (11). Based on the structure of the IgE receptor FcεRI, which has recently been determined, the β subunit acts as a calcium-permeable cation channel (30). Presumably, tranilast may inhibit the activity of this cation channel in mast cells. In B lymphocytes, the structure of the cell-surface antigen CD20 is characteristic of an ion channel and, in fact, acts as a calcium-permeable cation channel (31). Interestingly, the primary structure of CD20 has a high degree of homology with the β subunit of FcεRI, and it is thought that CD20 and FcεRI belong to a superfamily of calcium-permeable cation channels. We have found that tranilast also inhibits calcium entry via CD20.[1] Therefore, tranilast blocks calcium entry through cation channels belonging to the FcεRI superfamily. We showed recently that expression of CD20 accelerated G_1 progression in BALB/c3T3 cells (32). The results suggest that CD20 can function as a calcium-permeable channel (32). It is

[1] M. Kanzaki and I. Kojima, unpublished observations.

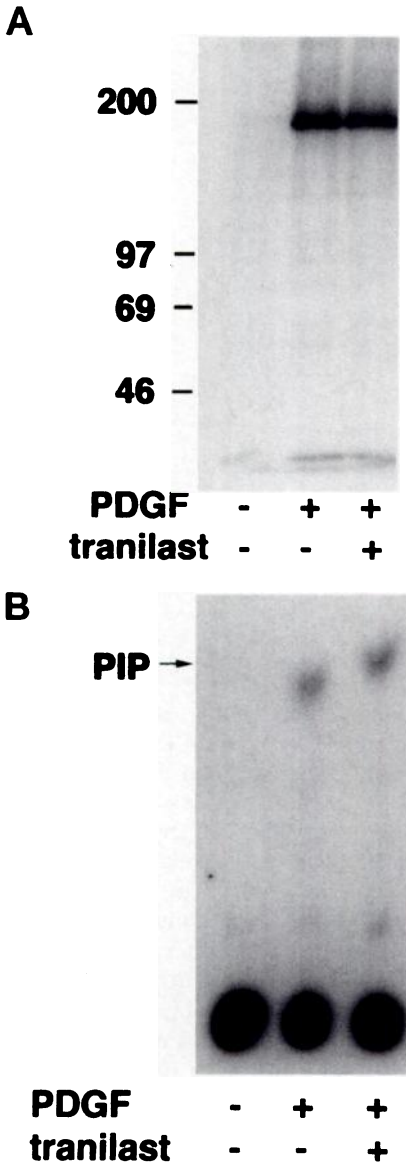

Fig. 7. Effect of tranilast on PDGF-induced phosphorylation of the PDGF receptor and activation of PI-3-kinase. A, VSMC labeled with ^{32}P were incubated for 5 min with 1 nM PDGF in the presense and absence of 100 μM tranilast. Phosphotyrosine-containing proteins were immunoprecipitated with antiphosphotyrosine antibody and were separated by SDS-PAGE. B, VSMC were incubated for 5 min with 1 nM PDGF in the presence and absence of 100 μM tranilast. PI-3-kinase activity was measured by measuring incorporation of ^{32}P into PI as described in Experimental Procedures. *PIP*, PI-3-phosphate.

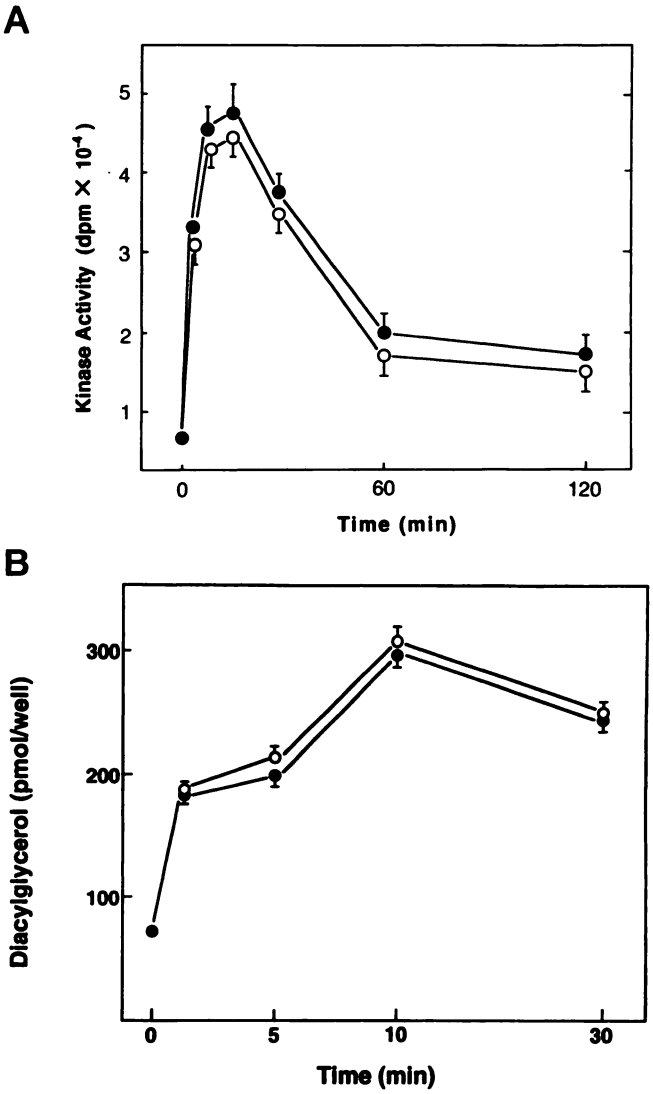

Fig. 8. Effect of tranilast on PDGF-induced activation of MAP kinase and production of DAG. A, VSMC were incubated for indicated time with 1 nM PDGF in the presence (●) and absence (○) of 100 μM tranilast. MAP kinase activity was then measured. Values are the means ± standard error for four experiments. B, VSMC were incubated for indicated time with 1 nM PDGF in the presence (●) and absence (○) of 100 μM tranilast. DAG mass was measured as described in Experimental Procedures. Values are the means ± standard error for three experiments.

therefore conceivable that tranilast also affects the calcium entry induced by PDGF.

The dose-response relationships for the action of tranilast on PDGF-induced calcium entry and DNA synthesis are fairly similar. Given that blockade of calcium entry attenuates PDGF-induced DNA synthesis (10), these results suggest that tranilast attenuates DNA synthesis by inhibiting calcium entry. Consistent with this observation, tranilast did not affect the PDGF-induced intracellular signaling, including receptor autophosphorylation, activation of the Ras-MAP kinase pathway, and activation of PI-3-kinase. With regard to the phospholipases, although tranilast inhibited calcium entry, it did not affect PDGF-induced calcium release from the intracellular pool, which is mediated by inositol-1,4,5-trisphosphate (33). It is likely that tranilast does not inhibit the activity of PL-C-γ. The lack of effect of tranilast on PDGF-induced elevation of DAG mass suggests that the compound does not inhibit PL-D. Taken together, these results indicate that tranilast does not affect many of the early signaling events induced by the PDGF receptor except the stimulation of calcium entry. At present, the mechanism by which inhibitors of calcium entry block the G_1 progression is not totally clear. It is kown that deprivation of calcium in eukaryotic cells results in growth arrest in the late G_1 phase (34). In fibroblasts, reduction of extracellular calcium attenuates the activation of cyclin-dependent kinases and subsequent phosphorylation of the RB protein (35). In PDGF-treated VSMC, the protooncogene c-*myb* is expressed in the late G_1 phase. The protooncogene product of c-Myb may lead

to an elevation of $[Ca^{2+}]_c$. Interestingly, the elevation in $[Ca^{2+}]_c$ is dependent on calcium entry via a dihydropyridine-insensitive pathway (36). Collectively, the evidence suggests that the blockade of calcium entry by tranilast may halt the cell cycle in the late G_1 phase by interfering with the machinery necessary for its entrance into the S phase. In this regard, a recent study by Benzaquen *et al.* (37) showed that clotrimazole inhibited cell proliferation by blocking calcium fluxes across the plasma membrane. An inhibitor of calcium fluxes may be a powerful tool for manipulating uncontrollable cell proliferation.

The present results correspond with a recent report by Tanaka *et al.* (12), which showed that tranilast inhibits migration and proliferation of VSMC in culture. They suggested that the compound may be useful in the prevention of restenosis. However, their report did not address the mechanism of the inhibitory action of tranilast. Our results extend the earlier observations by showing that tranilast inhibits proliferation of VSMC by blocking calcium entry. More importantly, when a standard clinical dose of tranilast (300 mg/day) is administered in humans, the plasma concentration of tranilast exceeds 100 μM (12). Therefore, tranilast may be able to inhibit DNA synthesis of VSMC *in vivo*. In fact, the effectiveness of tranilast in preventing restenosis after PTCA was shown recently in a double-blind study performed in humans (13). The results suggest that tranilast not only blocks growth of cultured VSMC but is also effective *in vivo*. Thus, pharmacological control of PDGF-induced DNA synthesis by inhibition of calcium entry may now be possible using tranilast. Tranilast or its analogues provide a potential therapeutic approach for preventing restenosis after PTCA.

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