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Stimulation of aortic smooth muscle cell mitogenesis by serotonin

(platelet-derived growth factor/atherogenesis)

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ABSTRACT **Bovine aortic smooth muscle cells *in vitro* responded to 1 nM to 10 μM serotonin with increased incorporation of [^{3}H]thymidine into DNA. The mitogenic effect of serotonin was half-maximal at 80 nM and maximal above 1 μM. At a concentration of 1 μM, serotonin stimulated smooth muscle cell mitogenesis to the same extent as human platelet-derived growth factor (PDGF) at 12 ng/ml. Tryptamine was ≈1/10th as potent as serotonin as a mitogen for smooth muscle cells. Other indoles that are structurally related to serotonin (D- and L-tryptophan, 5-hydroxy-L-tryptophan, *N*-acetyl-5-hydroxytryptamine, melatonin, 5-hydroxyindoleacetic acid, and 5-hydroxytryptophol) and quipazine were inactive. The stimulatory effect of serotonin on smooth muscle cell DNA synthesis required prolonged (20–24 hr) exposure to the agonist and was attenuated in the presence of serotonin D receptor antagonists. When smooth muscle cells were incubated with submaximal concentrations of serotonin and PDGF, synergistic rather than additive mitogenic responses were observed. These data indicate that serotonin has a significant mitogenic effect on smooth muscle cells *in vitro*, which appears to be mediated by specific plasma membrane receptors.**

Interactions between blood cells and the arterial wall have been proposed as early events in atherogenesis (1–3). The possibility that cell-derived growth factors are involved in the initiation and progression of the myointimal lesions, which are characteristic of atherosclerosis, has received particular attention. Recent work has focused on platelet-derived growth factor (PDGF), a potent mitogen for vascular smooth muscle cells *in vitro* (4, 5) that is thought to be released from the α granules of platelets adhering to exposed subendothelial connective tissue or foam cells (2, 3). Prior to the onset of DNA synthesis, PDGF elicits a variety of responses in aortic smooth muscle cells, including increased prostacyclin biosynthesis (6). Coughlin *et al.* (6) have reported that the prostacyclin response to PDGF is potentiated in the presence of serotonin, which is also released during platelet degranulation. This observation suggested that serotonin might influence the magnitude of the smooth muscle cell mitogenic response to PDGF. The present investigation was undertaken to evaluate this hypothesis.

EXPERIMENTAL PROCEDURES

Cell Cultures. Bovine aortic smooth muscle cells were propagated from medial explants essentially as described by Ross and Karlya (7). The cultures showed focal areas of overgrowth and prominent bundles of myofilaments, both of which are characteristic of vascular smooth muscle cells *in vitro*. All incubations were carried out at 37°C under a humidified atmosphere of 95% air/5% CO_2.

[^{3}H]Thymidine Incorporation. Bovine aortic smooth muscle cells from frozen stock (passages 3–12) were suspended in Dulbecco's modified Eagle's medium (DMEM) (pH 7.4) supplemented with 10% calf serum/6 mM glutamine/penicillin (100 units/ml)/streptomycin (100 μg/ml) for seeding into 16-mm 96-well microtiter plates at a density of ≈50,000 cells per well. After 48 hr, the growth medium was replaced with serum-free quiescing medium consisting of DMEM and Ham's F12 (1:1; pH 7.4) supplemented with ovalbumin (1 mg/ml)/ascorbic acid (25 μg/ml)/selenium (25 ng/ml)/dialyzed Cohn fraction IV (10 μg/ml)/epidermal growth factor (10 ng/ml)/transferrin 10 μg/ml) 50 μM hydrocortisone/6 mM glutamine/penicillin (100 units/ml)/streptomycin (100 μg/ml) (8). Smooth muscle cells were incubated in the quiescing medium for 96 hr. The medium was changed once during this period. Compounds to be tested were added to smooth muscle cells in quiescing medium 1 hr before serotonin and/or PDGF. The cultures were exposed to serotonin and/or PDGF for 20 hr before [^{3}H]thymidine (2.0 μCi/ml; 1 Ci = 37 GBq) was included in the quiescing medium. Four hours after the addition of [^{3}H]thymidine, experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco's phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid, and ethanol/ether (2:1). Phase-contrast microscopy was used to inspect the microwells for evidence of cell detachment or changes in cell morphology. Acid-insoluble [^{3}H]thymidine was extracted into 0.5 M NaOH and quantified by using a liquid scintillation counter.

Materials. Serotonin, tryptamine, L-tryptophan, D-tryptophan, *N*-acetyl-5-hydroxytryptamine, 5-hydroxy-L-tryptophan, melatonin, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, propranolol, atropine, cimetidine, 1-methyl-3-isobutylxanthine, ovalbumin, and Cohn fraction IV were obtained from Sigma; quipazine (2-[1-piperazinyl]quinoline maleate) was from Miles; phentolamine (Regitine) was from CIBA–Geigy; forskolin was from Calbiochem–Behring; human PDGF [300,000–500,000 units/mg, where a unit is defined as the amount of PDGF needed to stimulate the incorporation of [^{3}H]thymidine into 3T3 fibroblast DNA to the same extent as 5% calf serum (9)] was from Cellular Products (Buffalo, NY) and Larry D. Witte (Columbia University College of Physicians and Surgeons); selenium, epidermal growth factor, transferrin, and hydrocortisone were from Collaborative Research (Lexington, MA); [*methyl*-^{3}H]thymidine (2.0 Ci/mmol) was from New England Nuclear; BW501C (*N*-[2-(3-chlorophenoxy)-propyl]-2-phenylaminoethanimidamide) and 32-550 (9,10-dihydro-2-β-isopropyl-5′α-*sec*-butyl-6′-desoxo-ergopeptin) were from Brian P. Richardson (Sandoz, Ltd., Basel, Switzerland). Serotonin was dissolved in DMEM (pH 7.4) con-

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Abbreviation: PDGF, platelet-derived growth factor.
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taining 0.6 mM ascorbic acid, 1 mM pargyline, 6 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml immediately before the beginning of an experiment. DMEM containing 2 mg of bovine serum albumin per ml was the diluent for PDGF, BW501C, propranolol, phentolamine, cimetidine, and atropine. 1-Methyl-3-isobutylxanthine and 32-550 were solubilized in dimethyl sulfoxide. Forskolin was dissolved in absolute ethanol. The final concentration of dimethyl sulfoxide or ethanol in any assay system did not exceed 0.1%.

RESULTS

In agreement with Williams *et al.* (5), we observed that bovine aortic smooth muscle cells *in vivo* responded to human PDGF with concentration-dependent increases in [^{3}H]thymidine incorporation into DNA (Fig. 1). The ED_{50} for the mitogenic response to PDGF was ≈16 ng/ml. Stimulation of [^{3}H]thymidine incorporation was maximal at PDGF concentrations above 28 ng/ml. Exposure of smooth muscle cells to serotonin (1 nM to 10 μM) was similarly associated with enhanced DNA synthesis (Fig. 2). The ED_{50} for serotonin was ≈80 nM. The mitogenic effect of serotonin was maximal above 1 μM. At this concentration, serotonin increased smooth muscle cell [^{3}H]thymidine incorporation to the same extent as 12 ng of PDGF per ml.

The ability of serotonin to stimulate smooth muscle cell mitogenesis was structurally specific. Although tryptamine elicited a concentration-dependent mitogenic response in smooth muscle cells (ED_{50}, 0.8 μM), it was only ≈1/10th as potent as serotonin in this regard (Fig. 2). At 10 μM, quipazine (10) caused an increase in smooth muscle cell [^{3}H]thymidine incorporation that was no greater than that seen in response to 20 nM serotonin. Data presented in Table 1 additionally indicate that the stimulatory effect of serotonin on smooth muscle cell DNA synthesis was not mimicked by a number of other indoles that were examined.

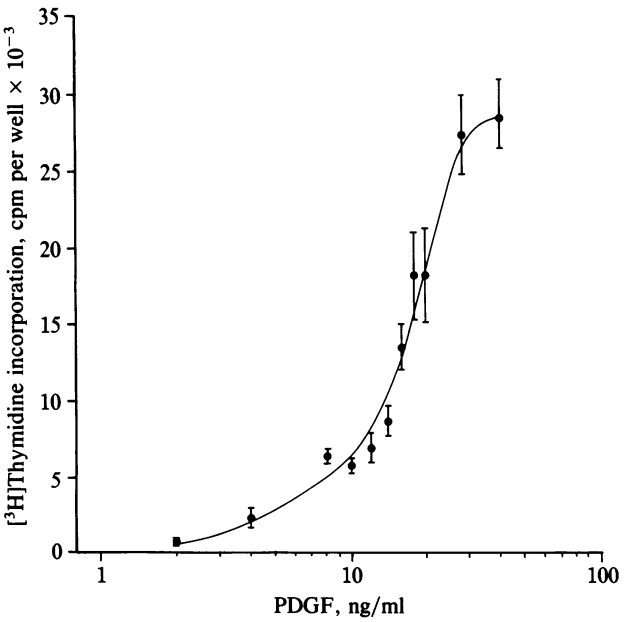

FIG. 1. Concentration-dependent stimulation of smooth muscle cell mitogenesis in response to PDGF. [^{3}H]Thymidine incorporation into DNA was determined in cultured bovine aortic smooth muscle cells exposed to various concentrations of human PDGF. Values are means ± SEM for determinations on three different cultures. Control [^{3}H]thymidine incorporation: 2045 ± 330 (n = 3) cpm per well. Data are representative of results from three separate experiments.

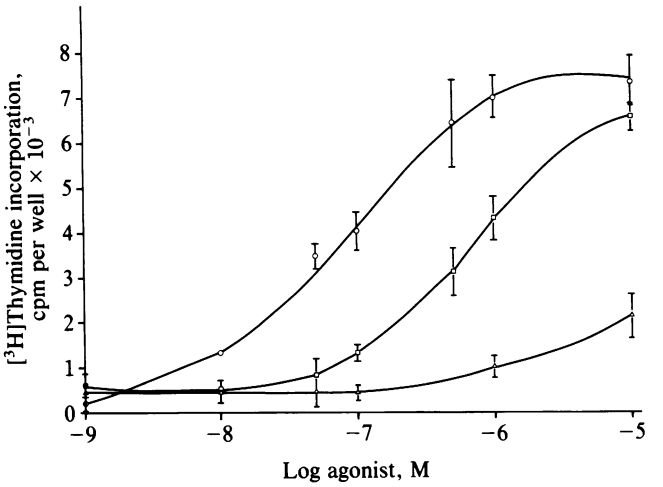

FIG. 2. Concentration-dependent stimulation of smooth muscle cell mitogenesis in response to serotonin or serotonin agonists. [^{3}H]Thymidine incorporation into DNA was determined in cultured bovine aortic smooth muscle cells exposed to various concentrations of serotonin (○), tryptamine (□), or quipazine (△). Values are means ± SEM for determinations on three different cultures. Control [^{3}H]thymidine incorporation: 2641 ± 113 (n = 6) cpm per well. Data are representative of results from three separate experiments.

Serotonin-induced increases in smooth muscle cell DNA synthesis required prolonged exposure to the agonist. In the experiment summarized in Fig. 3, [^{3}H]thymidine incorporation in smooth muscle cultures exposed to 0.1 μM serotonin for 3, 6, 9, or 12 hr was comparable to the control value. Enhanced mitogenesis was observed when serotonin was included in smooth muscle cell incubation medium for 20 hr. Under these conditions, however, the mitogenic response to serotonin was attenuated relative to that seen when the indole was present for 24 hr.

Serotonin appeared to stimulate smooth muscle cell mitogenesis by interacting with specific plasma membrane receptors. The stimulatory effect of 0.3 μM serotonin on [^{3}H]thymidine incorporation was markedly attenuated in the presence of either BW501C (0.01–10 μM) or the ergot derivative 32-550 (0.1–10 μM) (Fig. 4). BW501C has been reported to selectively antagonize radioligand binding to type 2 serotonin D receptors (11). 32-550 has been found to inhibit vascular responses mediated by serotonin D receptors (B. P. Richardson, personal communication). The mitogenic response to 10 ng/ml PDGF was maintained and, in some

Table 1. Incorporation of [^{3}H]thymidine in smooth muscle cells in response to various indoles

Addition to incubation medium	[^{3}H]Thymidine incorporation, cpm per well
None	2,641 ± 113 (6)
Serotonin (10 μM)	10,006 ± 558 (3)
L-Tryptophan (10 μM)	2,428 ± 302 (3)
D-Tryptophan (10 μM)	2,492 ± 326 (3)
N-acetyl-5-hydroxytryptamine (10 μM)	2,802 ± 309 (3)
5-Hydroxy-L-tryptophan (10 μM)	3,557 ± 248 (3)
Melatonin (10 μM)	2,421 ± 30 (3)
5-Hydroxyindoleacetic acid (10 μM)	2,723 ± 423 (3)
5-Hydroxytryptophol (10 μM)	3,518 ± 549 (3)

[^{3}H]Thymidine incorporation into smooth muscle cell DNA was estimated as described. Values are means ± SEM for determinations on the number of different cultures indicated in parentheses.

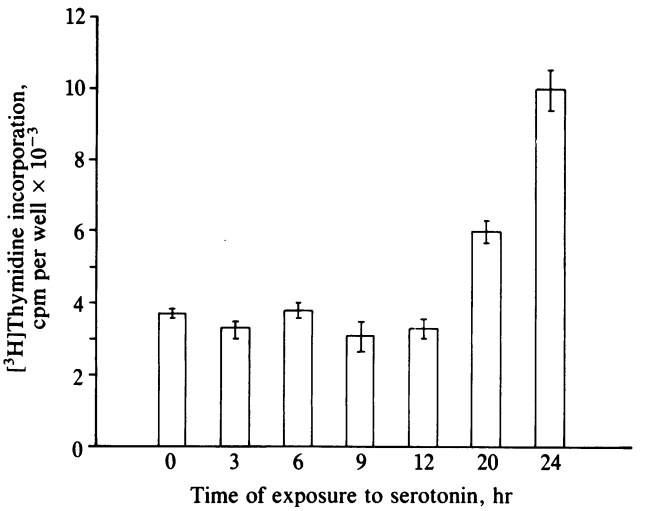

FIG. 3. Mitogenic response in bovine aortic smooth muscle cells as a function of time of exposure to serotonin. Smooth muscle cells were incubated with 0.1 μM serotonin for 3, 6, 9, 12, and 20 hr. The medium containing serotonin was subsequently replaced with fresh medium that did not contain the indole. [^{3}H]Thymidine was added 20 hr after the cultures had first been exposed to serotonin and the incubations were continued for 4 hr. Control cells—i.e., those represented by the zero time point—were incubated with the serotonin vehicle for 24 hr. Smooth muscle cultures exposed to serotonin for 24 hr did not undergo a medium change. Values are the means ± SEM for determinations on four or five different cultures.

cases, slightly increased in smooth muscle cells exposed to concentrations of BW501C or 32-550 below 10 μM.

In contrast to the response to serotonin D receptor antagonists, serotonin M receptor antagonists did not significantly affect the mitogenic response to the indole even at concentrations as high as 100 μM (data not shown). As indicated in Table 2, 1-methyl-3-isobutylxanthine and forskolin decreased serotonin-stimulated [^{3}H]thymidine incorporation into DNA. Since 1-methyl-3-isobutylxanthine and forskolin would be expected to potentiate cyclic AMP-mediated responses (12, 13), this observation was consistent with the activation of type 2 serotonin D receptors that are not coupled to adenylate cyclase (14). Neither propranolol (a β-adrenergic receptor antagonist), atropine (a muscarinic cholinergic receptor antagonist), nor cimetidine (an H_2 receptor antagonist) blocked the stimulatory effect of serotonin. Phentolamine, an α-adrenergic receptor antagonist with anti-serotonergic activity, suppressed the mitogenic response to the indole.

The interaction between serotonin and PDGF on smooth muscle cell mitogenesis is shown in Fig. 5. Simultaneous exposure of cultured smooth muscle cells to submaximal concentrations of serotonin and PDGF resulted in synergistic mitogenic responses. For example, in the presence of 8 ng of PDGF per ml and 0.1 nM serotonin, [^{3}H]thymidine incorporation (12,673 ± 731 cpm per well) was nearly 3 times greater than the sum of the individual responses to the growth factor (2110 ± 234 cpm per well) and the indole (2244 ± 418 cpm per well). A positive interaction between serotonin and PDGF was observed at each combination of the growth factor and the indole that was tested. However, the magnitude of the synergism was more sensitive to changes in the concentration of PDGF. When smooth muscle cells were exposed to 2 ng of PDGF per ml, the serotonin concentration had to be increased from 0.1 nM to 0.1 μM to elicit a 3-fold increase in DNA synthesis. A comparable mitogenic response was obtained by increasing the PDGF concentration from 2 ng/ml to 8 ng/ml, while holding the serotonin level constant at 0.1 nM.

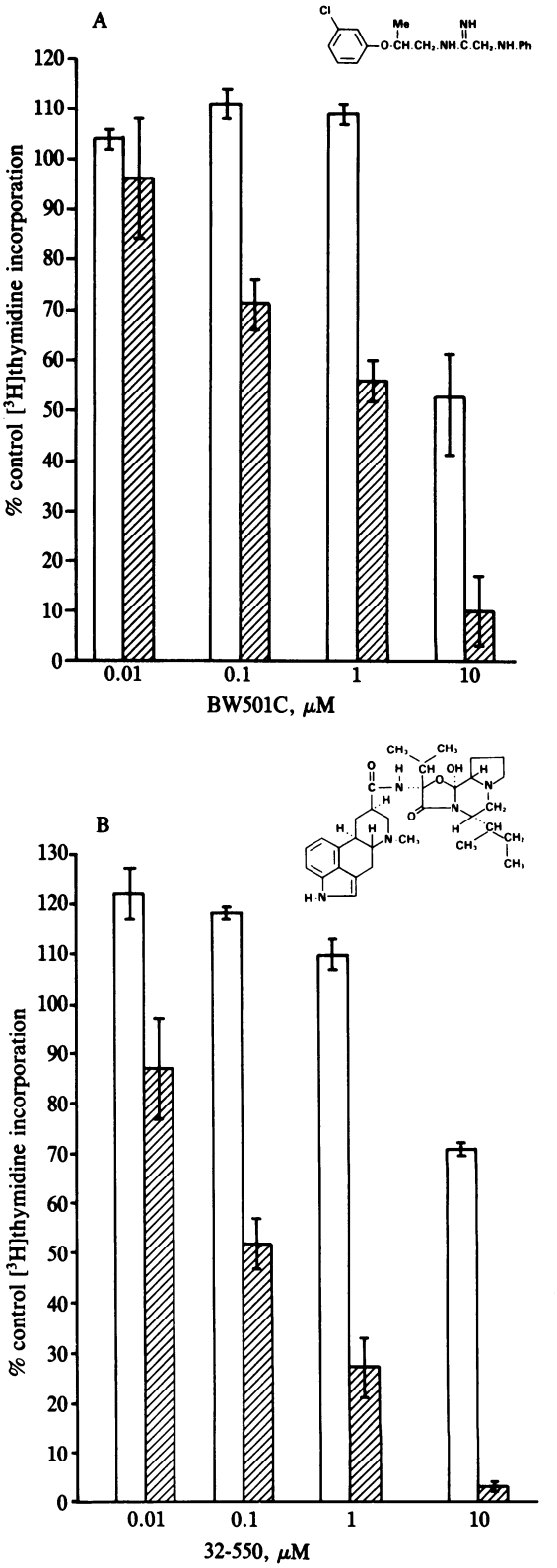

FIG. 4. Inhibition of PDGF- and serotonin-stimulated smooth muscle cell mitogenesis. The serotonin D receptor antagonists BW501C (*A*) and 32-550 (*B*) were added to cultured bovine aortic smooth muscle cells in quiescing medium 1 hr before half-maximal concentrations of PDGF (open bars; 10 ng/ml) or serotonin (hatched bars; 0.3 μM). [^{3}H]Thymidine incorporation into DNA was subsequently determined. Values are means ± SEM for determinations on three different cultures. The data are representative of results from four separate experiments. Control [^{3}H]thymidine incorporation: 376 ± 20 (n = 10) cpm per well; PDGF-stimulated [^{3}H]thymidine incorporation: 27,518 ± 1268 (n = 7) cpm per well; serotonin-stimulated [^{3}H]thymidine incorporation: 2282 ± 108 (n = 6) cpm per well.

Table 2. Effects of various compounds on serotonin-stimulated smooth muscle cell mitogenesis

Addition to incubation medium	[^{3}H]Thymidine incorporation, % control
1-Methyl-3-isobutylxanthine	
1 μM	85 ± 6
10 μM	57 ± 7
Forskolin	
1 μM	85 ± 7
10 μM	78 ± 1
Propranolol	
1 μM	111 ± 6
10 μM	90 ± 7
Phentolamine	
1 μM	56 ± 2
10 μM	36 ± 8
Atropine	
1 μM	115 ± 13
10 μM	99 ± 8
Cimetidine	
1 μM	126 ± 5
10 μM	131 ± 2

The indicated compounds were added to cultures of bovine aortic smooth muscle in quiescing medium 1 hr before addition of 0.1 μM serotonin. [^{3}H]Thymidine incorporation into DNA was subsequently estimated as described. Values are means ± SEM for determinations on three different cultures.

DISCUSSION

The data presented in this report indicate that serotonin exerts a structurally specific mitogenic effect on cultured vascular smooth muscle cells. The observed mitogenic response to serotonin was maximal above 1 μM and showed an ED_{50} of ≈80 nM. The increase in smooth muscle cell [^{3}H]thymidine incorporation in response to 1 μM serotonin was comparable to that seen with PDGF at 12 ng/ml. If the molecular weight of PDGF is taken to be 30,000–33,000 (15–18), 12 ng/ml corresponds to a concentration of ≈0.5 nM. Serotonin was therefore a less potent and less effective mitogen for smooth muscle cells *in vitro* than PDGF. However, serotonin showed mitogenic activity at concentrations that are comparable to those found in human platelet-poor plasma (19). Thus, serotonin could be an important *in vivo* determinant of vascular smooth muscle cell proliferation. In this regard, it is interesting that serotonin-producing neoplasms (argentaffinomas) in man are associated with fibrous cardiovascular lesions, which are composed largely of smooth muscle cells (20).

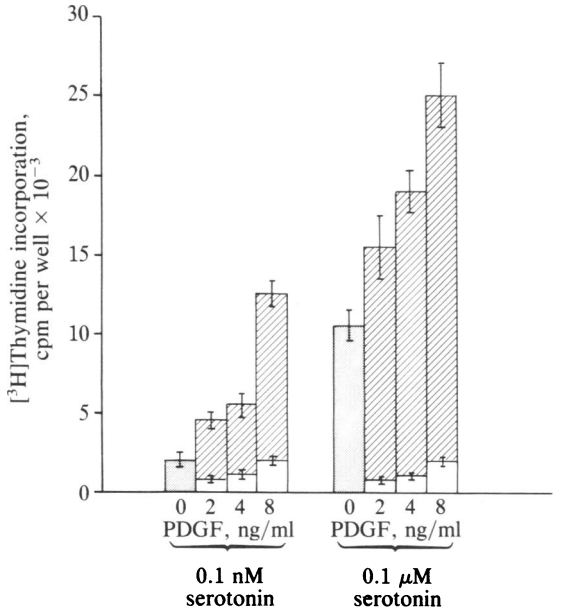

FIG. 5. Synergistic effects of PDGF and serotonin on smooth muscle cell mitogenesis. [^{3}H]Thymidine incorporation into DNA was determined in cultured bovine aortic smooth muscle cells exposed to PDGF alone (open bars), serotonin alone (stippled bar), or PDGF plus serotonin (hatched bars). Values are means ± SEM for determinations on three different cultures. Control [^{3}H]thymidine incorporation: 1106 ± 49 (n = 3) cpm per well. Data are representative of results from three separate experiments.

Significant *in vitro* stimulation of smooth muscle cell DNA synthesis by serotonin depended on prolonged activation of specific membrane receptors. PDGF-stimulated mitogenesis in human foreskin fibroblasts has also been found to require the interaction of the growth factor with its receptor for the major part of the G_0/G_1 phase (C. H. Heldin, personal communication). In contrast, BALB/c 3T3 fibroblasts proliferate after only transient exposure to PDGF if "progression factors" such as epidermal growth factor and somatomedin C are included in the incubation medium (22, 23). Antagonism of the smooth muscle cell mitogenic response to serotonin by BW501C or 32-550 implies that it is mediated by type 2 serotonin D receptors. This conclusion is supported by data that showed the stimulatory effect of serotonin on smooth muscle cell mitogensis was not mimicked by agents that increase intracellular cyclic AMP levels and was not blocked by serotonin M receptor antagonists.

The synergistic effects of submaximal concentrations of serotonin and PDGF on smooth muscle cell mitogenesis and, as previously reported by Coughlin *et al.* (6), on smooth muscle cell prostaglandin biosynthesis suggest that the proliferative responses may be mediated, at least in part, by metabolites of arachidonic acid. In this regard, it is noteworthy that smooth muscle cells exposed to serotonin show dramatic increases in prostacyclin biosynthesis long before they initiate DNA synthesis (24). Moreover, the prostacyclin response to serotonin, like the mitogenic response to the indole, appears to involve activation of type 2 serotonin D receptors (24). Some experimental support for the idea that arachidonic acid metabolites may serve as intracellular signals for mitogens comes from the work of Shier and Durkin (21). These investigators obtained similar IC_{50} values for phenylglyoxal inhibition of PDGF-stimulated arachidonate metabolism, prostaglandin release, and DNA synthesis in 3T3 fibroblasts. However, we have not observed significant effects of 10–100 μM prostacyclin, 0.1–5 μM indomethacin, 0.1–1 μM 5,8,11,14-icosatetraenoic acid, or 100 μM phenylglyoxal on the smooth muscle cell mitogenic response to PDGF (unpublished data). Ongoing studies have indicated that serotonin (0.1 nM to 1 μM) does not enhance the specific binding of ^{125}I-labeled PDGF to smooth muscle cells (unpublished data).

Synergistic mitogenic responses to serotonin and PDGF *in vivo* could have important implications with regard to the development and progression of atherosclerosis. Both serotonin and PDGF may be released from platelets that adhere to the vessel wall during the early stages of atherogenesis (2, 3). Ross *et al.* (4) have hypothesized that PDGF initiates myointimal lesion formation by stimulating the proliferation of vascular smooth muscle cells. Potentiation of the mitogenic effect of PDGF by serotonin would be expected to facilitate the disease process by increasing the sensitivity of vascular smooth muscle to low local concentrations of the growth factor.

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