Serotonin-stimulated protein phosphorylation in aortic smooth muscle cells [1]

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Summary. The effects of serotonin on the formation of inositol phosphates and protein phosphorylation were examined in cultured smooth muscle cells. Serotonin stimulated the formation of [^{3}H]inositol monophosphate, [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate. This effect was prevented by 5-HT_2 specific antagonist, 6-methyl-1-(1-methylethyl)ergoline-8-carboxylic acid, 2-hydroxy-1-methylpropyl ester [*Z*]-2-butenedioate (LY53857). Serotonin stimulated the phosphorylation of many polypeptides, among which a 20 kDa polypeptide was the most prominent. The phosphorylation was also inhibited by LY53857. LY53857 alone produced no effects on protein phosphorylation. The 20 kDa polypeptides were also phosphorylated by the addition of 12-*O*-tetradecanoylphorbol-13-acetate. These results suggest that serotonin stimulates protein phosphorylation through 5-HT_2 receptors and possibly activates protein kinase C in intact vascular smooth muscle cells.
Key words. Serotonin; protein phosphorylation; smooth muscle cells; LY53857; inositol trisphosphate.

Serotonin has been implicated as a vasoconstrictor and neurotransmitter. A newly discovered action of serotonin is the stimulation of mitogenesis of aortic smooth muscle cells[2]. This action of serotonin is potentially crucial for the genesis of atherosclerosis, because proliferation of smooth muscle cells has been implicated as a key event in the pathogenesis of the disease[3]. Serotonin is known to activate polyphosphoinositide hydrolysis by phospholipase C in smooth muscles[4-8]. Since diacylglycerol, an endogenous activator of protein kinase C, is generated as a result of polyphosphoinositide hydrolysis, we have studied protein phosphorylation following stimulation with serotonin in cultured smooth muscle cells.

Materials and methods

6-Methyl-1-(1-methylethyl)ergoline-8-carboxylic acid, 2-hydroxy-1-methylpropyl ester [*Z*]-2-butenedioate (LY53857) was donated by Eli Lilly & Company, Indianapolis, IN, USA. Markers for the molecular weight determination of proteins were purchased from Bio-Rad, Richmond, CA, USA and Pharmacia, Piscataway, NJ, USA; 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) was from Seikagaku Kogyo Co. Ltd., Tokyo, Japan; 12-O-tetradecanoylphorbol-13-acetate (TPA) was from Sigma Chemical Company, St. Louis, MO, USA; *myo*-[2-^{3}H]inositol (17 Ci/mmol; 1 Ci = 37 GBq) and carrier-free ^{32}P were from New England Nuclear, Boston, MA, USA; serotonin creatininesulfate was from Merck; fetal calf serum was from Gibco, Grand Island, NY, USA.

The primary cultures of aortic smooth muscle cells were prepared from 6-week-old male Sprague-Dawley rats according to the method of Smith and Brock[9]. The cells were seeded in 35-mm petri dishes at a concentration of 1×10^5 cells/dish. The medium was changed one week later and every four days thereafter. They grew in the presence of 10% fetal calf serum and were used for experiments after they reached confluency, which occurred two weeks after plating.

The growth medium was aspirated off and each dish was washed twice with a modified Hanks' solution containing 137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.3 mM $CaCl_2$ and 4.2 mM $NaHCO_3$. Cells were incubated in 1 ml of the same buffer for 30 min at 37 °C, followed by incubation for 5 min in 1 ml of the buffer containing carrier-free ^{32}P (1 mCi/ml). Longer incubation with ^{32}P increased the total background of radioactive proteins which are not responsive to the amine. Therefore we used a short loading time compared to that for other cell types[10]. Cells were then washed twice with the same buffer without ^{32}P.

Each dish received 1 ml of a buffer containing serotonin or TPA. Some dishes received a 5-HT_2 receptor-specific antagonist LY53857 (10 μM) or a protein kinase C inhibitor H-7 (50 μM) together with serotonin. The incubation was terminated 30 s or 10 min later by aspiration of the buffer and addition of 0.5 ml of 125 mM Tris/HCl (pH 6.8), 3% SDS, 0.01% bromphenol blue and 10% glycerol to the dish. The cell lysate was heated for 3 min in the presence of 5.2% mercaptoethanol. Portions (50 μl) of the cell lysate containing about 10 μg protein in each sample were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography as described previously[11].

Cells were loaded with *myo*-[^{3}H]inositol (10 μCi/ml) for 5 days. The growth medium was then aspirated and cell sheets were washed with Hanks'-Hepes buffer containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, 4.2 mM $NaHCO_3$ and 10 mM Hepes. Cells were preincubated with 10 mM LiCl in the Hanks'-Hepes buffer for 20 min at 37 °C. Some dishes received serotonin (100 μM) and were further incubated for 2 min at 37 °C. To some dishes the 5-HT_2-receptor specific antag-

onist LY53857 was added 5 min before the addition of serotonin. At the end of the 2-min incubation period the buffer was aspirated and 500 µl of 10% trichloroacetic acid was added. Samples were processed as described previously[12] for the determination of [^{3}H]InsP_1, InsP_2, and InsP_3, by the method of Batty et al.[13]. The counting efficiency for the tritiated samples were about 35%.

Results

The table shows that incubation of cells with serotonin (100 µM) stimulated the formation of [^{3}H]InsP_1, InsP_2, InsP_3 in accordance with a recent report by Go et al.[14]. This action of serotonin was also antagonized by a 5-HT_2 receptor antagonist LY53857. Therefore, stimulation of 5-HT_2 receptor results in activation of phospholipase C, which is consistent with the data on a ring preparation of rat aortic smooth muscles[4-8], and on 5-HT_2 receptor-mediated increase in calcium concentration[15].

Serotonin at a concentration of 100 µM, which causes maximal mitogenesis of aortic smooth muscle cells[2], stimulated the phosphorylation of many polypeptides, among which a 20 kDa polypeptide was the most prominent and was stimulated 2.8-fold (fig. 1). Incubation with serotonin for 10 min stimulated protein phosphorylation to a greater extent than incubation for 30 s. LY53857, a specific antagonist for 5-HT_2 receptors[16], inhibited the serotonin-stimulated phosphorylation (fig. 2), which indicates that the effect of serotonin was mediated through the 5-HT_2 receptor (fig. 2). LY53857 alone had no effect on endogenous protein phosphorylation (data not shown). A protein kinase C inhibitor H-7[17] reduced serotonin-stimulated phosphorylation of the 20 kDa polypeptides but H-7 alone also reduced the basal level of the phosphorylation (data not shown). In order to demonstrate the direct involvement of protein kinase C in the phosphorylation of 20 kDa polypeptides, we examined the effects of TPA, an activator of protein kinase C. TPA stimulated the phosphorylation of the 20 kDa polypeptides by about 3-fold (fig. 3).

5-HT_2 receptor-mediated stimulation of InsP_1, InsP_2, InsP_3 formation in intact smooth muscle cells. Cells were loaded with *myo*-[^{3}H]inositol (10 µCi/ml) for 5 days, and were preincubated with 10 mM LiCl in Hanks'-Hepes buffer for 20 min at 37 °C. Some dishes received serotonin and were further incubated for 2 min at 37 °C. LY53857 was added 5 min before the addition of serotonin. Data are the mean ± SE of the means from four different dishes.

Treatment	[^{3}H]InsP_1	[^{3}H]InsP_2	[^{3}H]InsP_3
		cpm per dish	
Control	18600 ± 670	1630 ± 120	980 ± 130
LY53857, 10 µM	19200 ± 710	1460 ± 150	940 ± 140
Serotonin, 100 µM	22200 ± 920*	2340 ± 210*	2770 ± 60*
Serotonin + LY53857	18100 ± 1000	1540 ± 200	830 ± 160

Abbreviation: InsP_1, inositol monophosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate. *$p < 0.05$ vs control, LY53857 alone and serotonin plus LY53857 group.

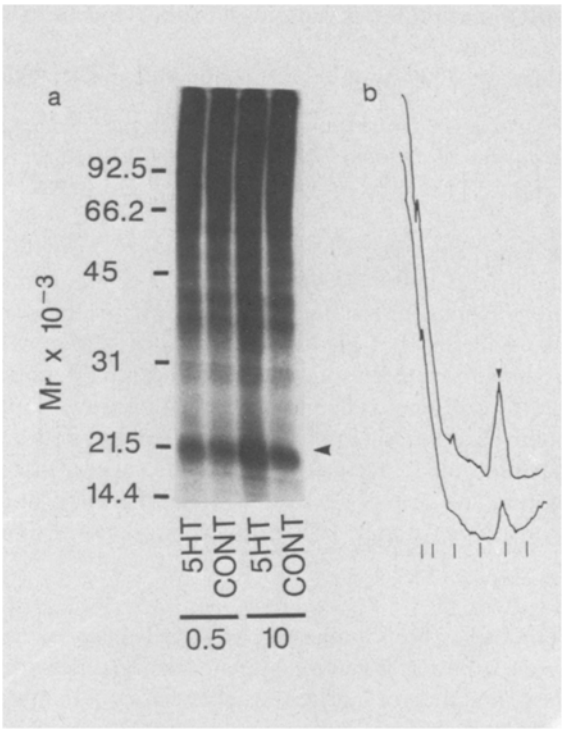

Figure 1. Autoradiogram (*a*) and densitometric scan (*b*) showing endogenous protein phosphorylation stimulated by serotonin in cultured smooth muscle cells prepared from rat aorta. ^{32}P-labelled intact cells were stimulated by serotonin (100 µM) for 0.5 or 10 min. Phosphorylated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The abbreviations used are: CONT, control; 5HT, serotonin. The numbers under the horizontal lines in panel *a* indicate the incubation period with serotonin (0.5, 30 s; 10, 10 min). The vertical lines at the bottom of the densitometric scan in panel *b* show molecular mass of marker protein: from the left to the right 92.5, 66.2, 45, 31, 21.5, 14.4 kDa. Experiments were repeated three times with similar results. The arrow at the right indicates the position of 21.5 kDa protein standard.

Discussion

Our data on phosphorylation are consistent with the conclusion that the subtype of the serotonin receptor which is involved in vasoconstriction and mitogenesis of aortic smooth muscle cells is the type 2 serotonin receptor[2].

The serotonin-stimulated phosphorylation of 20 kDa polypeptides in intact cells is possibly mediated, at least in part, through protein kinase C. The evidence for this is as follows. First, protein kinase C is present in rat aortic smooth muscles[11,18]. Second, the 20 kDa polypeptide is an endogenous substrate for protein kinase C in a cell-free system[11]. Third, an activator of protein kinase C, TPA, stimulated the phosphorylation of the 20 kDa polypeptides. Fourth, serotonin stimulated phospholipase C activity in intact cells, which indicates that serotonin generates diacylglycerol, the endogenous activator of protein kinase C[19]. This effect of serotonin is mediated through 5-HT_2 type receptors, which are also involved in the serotonin-stimulated phosphorylation. Fifth, phosphorylation of the 20 kDa polypeptide in intact cells was inhibited by 50 µM of a protein kinase C inhibitor H-7; however, this evidence is circumstantial –

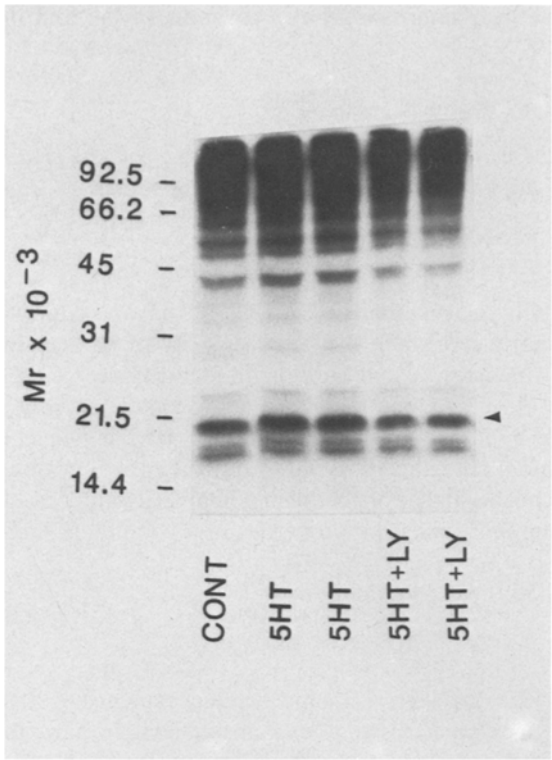

Figure 2. Effects of a serotonergic antagonist on serotonin-stimulated endogenous phosphorylation in cultured smooth muscle cells. ^{32}P-labeled cells were incubated with serotonin (100 μM) for 10 min in the presence or absence of LY53857 (10 μM). Each lane corresponds to a sample derived from a different dish. CONT, control; 5HT, serotonin; LY, LY53857. Samples were processed as described in the legend for fig. 1.

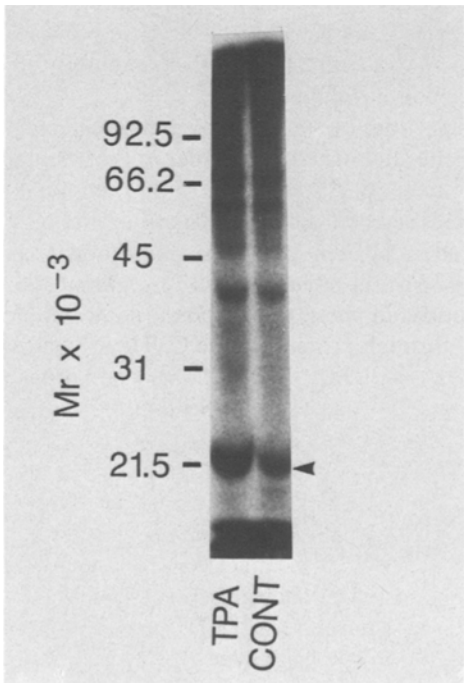

Figure 3. Effects of a phorbol ester on phosphorylation of proteins in cultured smooth muscle cells. ^{32}P-labelled cells were stimulated by TPA (50 μM) for 10 min or incubated with 0.1% dimethyl sulfoxide as a vehicle. Experiments were repeated twice with similar results.

H-7 may affect not only protein kinase C (K_i = 6 μM) but also other protein kinases[17]. We still cannot exclude a possibility that protein kinase other than protein kinase C are also involved in the serotonin-induced phosphorylation.

Protein kinase C activation by diacylglycerol has been suggested as an intermediate step in the expression of a cellular oncogene c-*myc*[20, 21], which has been shown to be involved in cellular proliferation[22]. It is also known that 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C[23], induces mitogenesis of cultured aortic smooth muscle cells[24]. Vasoconstriction induced by serotonin possibly involves the protein kinase C, too[4–8]. Therefore, it is not unexpected that serotonin stimulates protein kinase C in intact smooth muscle cells. The identity of the 20 kDa polypeptide is unknown, although a possible candidate is myosin light chain, which is phosphorylated by purified protein kinase C in vitro[25].

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