

Phorbol ester modulates serotonin receptor-mediated increases in inositol phosphate production and calcium mobilization in cultured rat vascular smooth muscle cells

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Received 5 May 1988

The effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on serotonin-induced inositol phosphate (IP) accumulation and intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) was investigated in cultured rat vascular smooth muscle cells. Pretreatment with TPA had no effect on basal levels of both IP production and $[\text{Ca}^{2+}]_i$, whereas it significantly attenuated serotonin-induced increases in both IP production and $[\text{Ca}^{2+}]_i$. These data suggest that protein kinase C is involved in the negative feedback control of serotonin-induced rises in both IP production and $[\text{Ca}^{2+}]_i$.

Serotonin; Inositol phosphate; Protein kinase C; Intracellular Ca^{2+} concentration; Phorbol ester; (Vascular smooth muscle cell)

1. INTRODUCTION

Since Page [1] unravelled the chemistry of serotonin or 5-hydroxytryptamine (5-HT), a variety of its actions on blood vessels have been the subject of intensive research. It has been reported that 5-HT has a direct vasoconstrictive effect through a 5-HT₂ receptor recognition mechanism [2]. After binding to its specific receptors on vascular smooth muscle cells (VSMCs), inositol, 1,4,5-trisphosphate (IP₃), a product of the hydrolysis of the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C, appears to act as a second messenger to release Ca^{2+} from intracellular store sites in VSMCs [3]. This receptor-linked breakdown of phosphatidylinositol gives not only IP₃ but also diacylglycerol (DG) to activate protein kinase C (PKC) [4].

However, the interaction between PKC activation and Ca^{2+} mobilization induced by 5-HT in

VSMCs has not been reported. Therefore, we investigated whether PKC activation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumor promoting phorbol ester [5], affects 5-HT-stimulated IP production and Ca^{2+} mobilization in VSMCs.

2. MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Flow Laboratories (McLean, VA); fura-2 acetoxymethyl ester (AM) from Dojin Chemical (Kumamoto, Japan); TPA from Sigma St. Louis, MO; serotonin (5-hydroxytryptamine creatinine sulfate) from Nakarai Chemical (Kyoto, Japan); ketanserin tartrate was donated by Janssen-Kyowa Co. (Tokyo, Japan).

VSMCs were prepared from 9-week-old male Wistar rats by the method of Chamley et al. [6] were cultured and used between 11th and 25th passage as reported [7].

2.1. Measurement of inositol phosphate (IP)

The VSMCs were incubated in 1 ml serum-free DMEM containing 5 μCi myo-[³H] inositol (spec. act.; 54.5 Ci/mmol; New England Nuclear, Boston, MA) for 2–3 days. After washing with Hepes-buffered physiological salt solution (PSS: 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 20 mM Hepes, pH 7.4), cells were preincubated at 37°C for 10 min with PSS

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containing 10 mM LiCl. VSMCs were usually incubated with 5-HT at 37°C for 30 s, during which time interval the maximal stimulation of both IP_3 production and $[Ca^{2+}]_i$ occurred. The reaction was terminated by the addition of 1 ml of 15% trichloroacetic acid (TCA). The TCA extract was neutralized by 1 N NaOH and subjected to an anion-exchange resin (Dowex 1-X8, formate form; Dow Chemical, Midland, MI) column to separate total IP_3 by the method of Berridge et al. [8].

2.2. Measurement of $[Ca^{2+}]_i$

After incubation in serum-free medium for 24–48 h, dispersed cells were incubated at 37°C for 20 min in PSS with 4 μ M fura-2 AM. After loading, cells were washed and suspended in PSS at the concentration of $\sim 2 \times 10^6$ cells/ml. Fluorescence was measured at 37°C with Hitachi MPF-4 spectrofluorimeter (excitation, 340 nm and 380 nm, slit, 5.5 nm; emission, 505 nm, slit, 4.5 nm) equipped with a thermostated cuvette holder, stirring apparatus and chart recorder. $[Ca^{2+}]_i$ values were calculated as described [9].

3. RESULTS

5-HT dose-dependently (10^{-6} – 10^{-4} M) induced immediate and transient increases in both IP_3 production and $[Ca^{2+}]_i$ (fig.1), of which effects were completely blocked by the 5-HT₂-receptor antagonist, ketanserin (not shown); there is a close correlation ($r = 0.943$, $p < 0.001$) between $[Ca^{2+}]_i$ and total IP_3 accumulated in response to 5-HT.

Fig.2. depicts representative tracings of 5-HT-induced fluorescence changes of fura-2-loaded VSMCs without or with brief (3 min) pretreatment

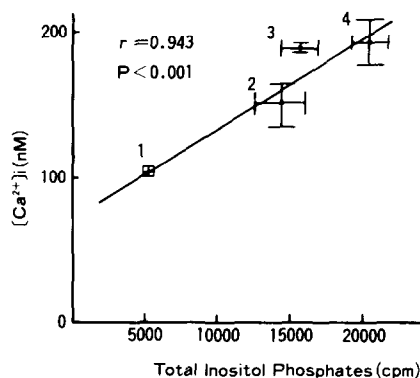


Fig.1. Correlation between accumulation of total inositol phosphates (IP_3) and $[Ca^{2+}]_i$ in response to serotonin (5-HT). Cultured VSMCs were unstimulated (1) or stimulated by 10^{-6} M (2), 10^{-5} M (3), 10^{-4} M (4) 5-HT. Total IP_3 accumulated and $[Ca^{2+}]_i$ are indicated on the abscissa and ordinate, respectively. Values are expressed as means of three experiments; bar shows SE.

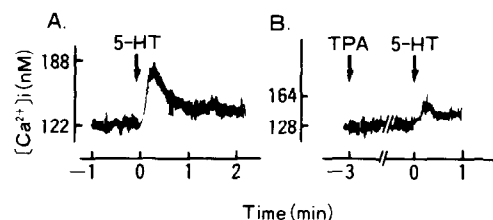


Fig.2. Effect of TPA on 5-HT-stimulated increase in $[Ca^{2+}]_i$. Fura-2-loaded VSMCs were pretreated without (A) or with 8×10^{-8} M TPA for 3 min (B) and exposed to 10^{-5} M 5-HT. Each panel represents a typical fluorescence tracing. Calculated values for $[Ca^{2+}]_i$ are indicated at the left.

with 8×10^{-8} M TPA. In control cells, 10^{-5} M 5-HT caused a rapid increase in $[Ca^{2+}]_i$, followed by a gradual decline to a sustained phase still higher than the prestimulation level (fig.2A), while pretreatment with TPA had no effect on resting $[Ca^{2+}]_i$, but markedly attenuated the 5-HT-induced $[Ca^{2+}]_i$ transient.

Table 1 summarizes the effect of TPA on 5-HT-induced increases in total IP_3 formation and $[Ca^{2+}]_i$. TPA significantly ($p < 0.05$) attenuated 5-HT-stimulated accumulation of total IP_3 and the $[Ca^{2+}]_i$ transient without affecting their basal levels.

Table 1

Effect of TPA on 5-HT-stimulated accumulation of total inositol phosphates (IP_3) and $[Ca^{2+}]_i$

Drug (M)	$[Ca^{2+}]_i$ (nM)	IP_3 (cpm)
Control	126 ± 33 (9)	2524 ± 79 (3)
TPA (8×10^{-8})	128 ± 7.8 (3) *	2647 ± 295 (3) ***
5-HT (10^{-5})	187 ± 6.2 (3)	3152 ± 118 (3)
TPA (8×10^{-8}) + 5-HT (10^{-5} M)	157 ± 12.4 (3) **	2817 ± 116 (3) **

VSMCs were incubated in the absence or presence of 10^{-5} M 5-HT, 8×10^{-8} M TPA and 5-HT plus TPA. Values of IP_3 and $[Ca^{2+}]_i$ are expressed as means \pm SE; in parentheses the number of samples tested is given. Asterisks show statistically significant differences between groups as indicated by arrows:

*, $p < 0.001$; **, $p < 0.005$; ***, $p < 0.005$

4. DISCUSSION

Endogenous vasoconstrictors, including 5-HT, norepinephrine, angiotensin II and arginine-vasopressin, induce phospholipase C-mediated PIP_2 hydrolysis through their specific receptors [3,10–12]. IP_3 and DG, two main products generated by PIP_2 breakdown, serve as putative second messengers for Ca^{2+} mobilization and PKC activation, respectively, and they act synergistically in a variety of cells, thus leading to positive physiological responses, such as hormone secretion and exocytosis of enzymes from endocrine and exocrine cells and cellular proliferation [4]. Recent evidence suggests that PKC activated by adrenergic α_1 -agonist and angiotensin II exerts negative feedback control on the receptors for these agonists in hepatocytes [13] or smooth muscle cells [14].

The present results show that pretreatment with TPA, a phorbol ester substituting for endogenous DG, presumably acting through stimulation of PKC, leads to inhibition of 5-HT-induced increases of both $[\text{Ca}^{2+}]_i$ and IP formation without affecting their basal levels, suggesting the possible involvement of PKC in the mechanism of 5-HT₂-mediated PI response and mobilization of intracellular Ca^{2+} in VSMCs. It has recently been shown that TPA inhibits IP formation and/or increases in $[\text{Ca}^{2+}]_i$ stimulated by AII [14] and norepinephrine [15], without any changes of these receptors in cultured VSMCs. In contrast, it has been demonstrated that phorbol esters cause phosphorylation of α_1 -adrenergic receptors in DDT MF-2 cells, a clonal cell line derived from hamster vas deferens smooth muscle cells, thereby uncoupling the receptor from phospholipase C activation [16]. However, it remains unknown whether PKC phosphorylates the 5-HT₂ receptor to uncouple the receptor from phosphoinositide metabolism to account for the observed TPA-induced attenuation of IP formation and $[\text{Ca}^{2+}]_i$ by 5-HT.

On the other hand, it has been demonstrated that pertussis toxin, that ADP-ribosylates the α -subunit of GTP-binding protein [17], attenuates the IP_3 -mediated increases in $[\text{Ca}^{2+}]_i$ induced by fMet-Leu-Phe and leukotriene B₄ [18] through the inhibition of the polyphosphoinositide breakdown by these agents in rabbit neutrophils [19], and that pertussis toxin-sensitive G-protein is phosphory-

lated by PKC in human platelets [20]. More recent study has shown that pertussis toxin inhibits 5-HT-induced increases of $[\text{Ca}^{2+}]_i$ in cultured VSMCs [21]. Therefore, it is possible to speculate that G-protein may also be involved in the mechanism of TPA-induced attenuation of IP production and $[\text{Ca}^{2+}]_i$ stimulated by 5-HT as observed in the present study.

Acknowledgements: We thank Ms S. Kaizuka for technical assistance. This study was supported in part by Grants-in-Aid from the Ministry of Health and Welfare (61C-2, 62A-1) and the Ministry of Education, Science and Culture (62304041, 62570530).

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