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

Shoichiro Takata[+°], Yukio Hirata[+°], Yasuyuki Takagi[+°], Hiroki Yoshimi[+°], Yuka Fukuda[+] and Takuo Fujita[°]

[+]*Hyptertension-Endocrine Division, National Cardiovascular Center Research Institute, Suita, Osaka 565* and [°]*Third Division, Department of Medicine, Kobe University School of Medicine, Kobe 650, Japan*

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

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-HT_2 receptor recognition mechanism [2]. After binding to its specific receptors on vascular smooth muscle cells (VSMCs), inositol, 1,4,5-trisphosphate (IP_3), a product of the hydrolysis of the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) 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 IP_3 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.

Correspondence address: S. Takata, Hypertension-Endocrine Division, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan

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); ketanserine 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*-[^{3}H] 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 $MgCl_2$, 1.5 mM $CaCl_2$, 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 IPs 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 μM fura-2 AM. After loading, cells were washed and suspended in PSS at the concentration of ~ 2×10^6 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-HT_2-receptor antagonist, ketanserine (not shown); there is a close correlation ($r = 0.943$, $p < 0.001$) between $[Ca^{2+}]_i$ and total IPs 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

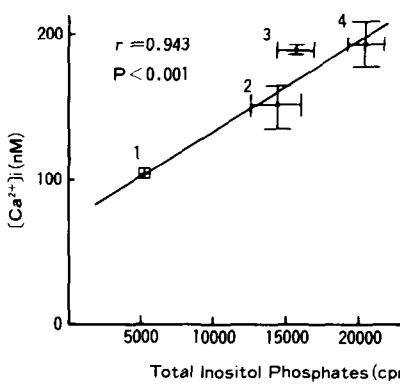

Fig.1. Correlation between accumulation of total inositol phosphates (IP) and $[Ca^{2+}]_i$ in response to serotonin (5-HT). Cultured VSMCs were unstimulated (1) or stimulated by 10^{-6} M (2), 10^{-5} M (3), 10^{-4} M (4) 5-HT. Total IPs accumulated and $[Ca^{2+}]_i$ are indicated on the abscissa and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

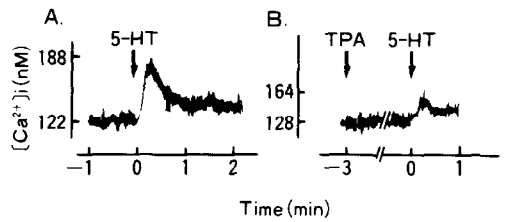

Fig.2. Effect of TPA on 5-HT-stimulated increase in $[Ca^{2+}]_i$. Fura-2-loaded VSMCs were pretreated without (A) or with 8×10^{-8} M TPA for 3 min (B) and exposed to 10^{-5} M 5-HT. Each panel represents a typical fluorescence tracing. Calculated values for $[Ca^{2+}]_i$ are indicated at the left.

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 formation and $[Ca^{2+}]_i$. TPA significantly ($p < 0.05$) attenuated 5-HT-stimulated accumulation of total IPs 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) and $[Ca^{2+}]_i$

Drug (M)	$[Ca^{2+}]_i$ (nM)		IPs (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 and $[Ca^{2+}]_i$ are expressed as means ± 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 $[Ca^{2+}]_i$ and IP formation without affecting their basal levels, suggesting the possible involvement of PKC in the mechanism of 5-HT_2-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 $[Ca^{2+}]_i$ 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-HT_2 receptor to uncouple the receptor from phosphoinositide metabolism to account for the observed TPA-induced attenuation of IP formation and $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 phosphorylated by PKC in human platelets [20]. More recent study has shown that pertussis toxin inhibits 5-HT-induced increases of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ stimulated by 5-HT as observed in the present study.

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