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PHORBOL ESTER MODULATES SEROTONIN-STIMULATED PHOSPHOINOSITIDE BREAKDOWN IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

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Summary: Stimulation of cultured rabbit aortic vascular smooth muscle cells(VSMC) with serotonin(5HT) induced a rapid generation of inositol phosphates from receptor-mediated hydrolysis of inositol phospholipids. Pretreatment of these cells with 500ng/ml of pertussis toxin for 24h prior to addition of 5HT reduced 5HT-induced formation of inositol phosphates. Phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate(TPA) or phorbol-12,13-dibutyrate(PDBu), are known to activate protein kinase C(PKC), but their role on cultured VSMC stimulated by 5HT has not been defined. TPA exhibited a rapid inhibition of 5HT-stimulated phosphoinositide breakdown, although 4α-phorbol-12,13-didecanoate(4αPDD), an inactive phorbol ester, did not inhibit it. These data suggest that a guanine nucleotide inhibitory (Gi)protein couples 5HT receptor to phospholipase C and TPA modulates 5HT-stimulated hydrolysis of inositol phospholipids in cultured VSMC through activation of PKC.

Serotonin(5HT) is a vasoactive agent for vascular contraction. Recently, it has been shown that 5HT stimulates hydrolysis of membrane inositol phospholipids, particularly phosphatidylinositol-4,5-bisphosphate(PIP_2), which results in the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate(IP_3) and diacylglycerol(DAG) in intact vascular tissue and cultured VSMC(1-3). IP_3 is thought to activate specific intracellular receptor sites to release calcium from intracellular

Abbreviations used are: VSMC, vascular smooth muscle cells; 5HT, serotonin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; 4αPDD, 4α-phorbol-12,13-didecanoate; FCS, fetal calf serum; EMEM, Eagle's minimum essential medium; BSS, balanced salt solution; BSA, bovine serum albumin; TCA, trichloroacetic acid; PI, phosphoinositide; IP_1, inositol 1-monophosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate.

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stores(4,5) and play an important role on phasic contraction(6), while DAG can activate calcium-activated, phospholipid-dependent protein kinase(PKC)(7). The tumor-promoting phorbol esters, such as TPA, can substitute for DAG and activate this enzyme(8). Previous studies have shown that TPA induces a slowly developing contraction of rabbit(9) and rat(10) aortae and dog basilar artery(11). Phorbol esters have also been shown to inhibit α-adrenergic mediated contraction of rat aorta(10). Recent evidence obtained with types of cells other than smooth muscle cells has indicated that guanine nucleotide regulatory (Gs, Gi)proteins coupled a wide variety of receptors to phospholipase C in signal transduction system. Furthermore, treatment of human and guinea pig neutrophils(12,13) and rat mast cells(14) with Bordetella pertussis toxin(islet-activating protein) inhibited phosphoinositide(PI) breakdown induced by f-Met-Leu-Phe and compound 48/80, respectively. Islet-activating protein(IAP) has been shown to inactivate the Gi protein that is coupled to receptor-mediated inhibition of adenylate cyclase by catalyzing the NAD-dependent, ADP ribosylation of its α-subunit(15,16). In cultured VSMC, it has not yet well established whether a guanine nucleotide regulatory protein may participate in receptor-linked PI breakdown. In this present study, we demonstrated that a Gi protein coupled 5HT receptor to phospholipase C and TPA modulated 5HT-stimulated hydrolysis of inositol phospholipids in cultured VSMC from rabbit aorta through activation of protein kinase C, which might inactivate 5HT receptor or a Gi protein by phosphorylation.

EXPERIMENTAL PROCEDURES

Materials Japanese White rabbits were purchased from Kansai Animal Care Co., Ltd. FCS was from Gibco Laboratories. Myo-[2-^{3}H] inositol (specific activity, 19.6 Ci/mmol) was obtained from Amersham. Serotonin and 4αPDD were from Sigma. TPA and PDBu were from CCR InC. Pertussis toxin was from Kaken Seiyaku, Co., Ltd. Other materials and chemicals were obtained from commercial sources.

Cell culture Primary cultures of VSMC were obtained from male Japanese White rabbit thoracic aortae by the explant method of Ross(17). Briefly, thoracic aortae of 12-week-old Japanese White rabbit were dissected free of surrounding tissue and transferred to a 60-mm dish.

The aortae were cut longitudinally to expose the tunica intima. Intimal-medial tissues were dissected away from the tunica adventitia and cut into 1-2mm^2 sections, which were placed in a 75-cm^2 flask and grown in EMEM containing 10% FCS. After 2 weeks of incubation at 37°C in a humidified atomosphere of 5% CO_2 and 95% air, cultured cells that had migrated from the explanted tissue were harvested in 0.1% trypsin solution containing 0.05% EDTA, transferred into 100-mm dishes and grown in the same medium for 3 days. The cells in the secondary cultures were trypsinized, seeded into 35-mm dishes at a density of $2x10^5$ cells/dish and grown in 2.5 ml of the same medium containing 2.5μCi/ml of myo-[2-^{3}H] inositol for 72hr.

Assay for inositol phosphates The prepared cells were washed twice with serum-free EMEM and incubated in 1ml of the same medium containing 2.5μCi/ml of myo-[2-^{3}H] inositol for 48hr at 37°C. After incubation, the cells were washed twice with a warm BSS(130mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.5mM $CaCl_2$, 20mM HEPES at pH7.4) and incubated with BSS containing 10mM glucose, 1mg/ml BSA and 10mM LiCl for 15 min in the presence or absence of phorbol esters, which were dissolved in dimethyl sulfoxide(DMSO) and diluted to the appropriate concentration for experimental use with distilled water. In some experiments, the cells were pretreated with pertussis toxin for varied periods before stimulation of the cells with 5HT. The cells were then stimulated by 5HT at 37°C. The reaction was terminated by rapid aspiration of assay buffer and addition of 1ml of ice-cold 15% TCA and dishes were placed on ice for 30 min. After removal of TCA with diethylether, extracts were neutralized with 0.1N NaOH. The phosphorylated inositols were separated as described by Berridge(18). Each sample was applied to a column containing 1ml of Dowex AG 1x8(formate form). [^{3}H]inositol and [^{3}H] glycerophosphoinositol were eluted with 8ml of water and with 16ml of 5mM sodium tetraborate/60mM sodium formate, respectively. [^{3}H]IP_1 was eluted with 10ml of 0.1M formic acid/0.2M ammonium formate. The column was washed with 6ml of the same buffer and [^{3}H]IP_2 was eluted with 10ml of 0.1M formic acid/0.4M ammonium formate. The columun was washed with 6ml of the same buffer and [^{3}H]IP_3 was eluted with 10ml of 0.1M formic acid/1.0M ammonium formate. Fractions of 2ml each were collected. The radioactivity of each fraction containing IP_1, IP_2 and IP_3 was determined.

RESULTS

The kinetic profile of the accumulation of three inositol phosphates in cultured VSMC by 10μM 5HT is shown in Fig.1. 5HT was added to the cells after pretreatment of the cells with 10mM LiCl for 15 min. 5HT elicited an increase in the amount of radioactivity present in all three inositol phosphates. Accumulation of IP_1 increased linearly with 6 fold at 20 min. This occurred by the presence of LiCl, which inhibited the activity of inositol-1-phosphatase(19). IP_2 and IP_3 production was rapid and reached a peak at 10 min and 5 min with 10 fold and 3.5 fold increases, respectively. Stimulation with 5HT for 3 min produced IP_3 in a concentration-dependent manner, as shown in Fig.2.

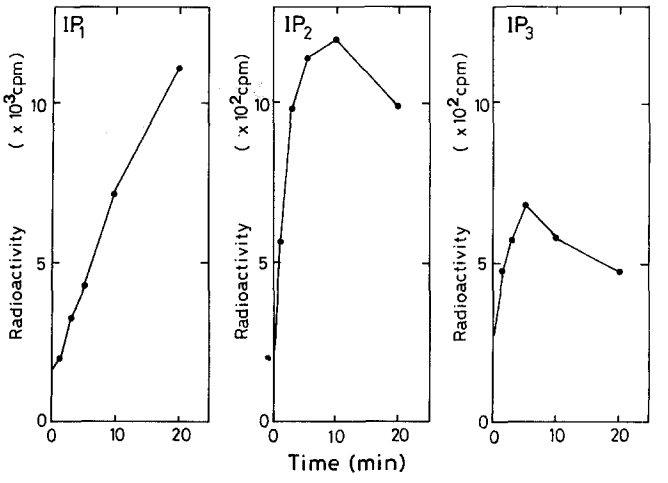

Fig. 1 Time course of 5HT-stimulated formation of inositol phosphates. VSMC prelabeled with [^{3}H] inositol for 72h were stimulated with 10μM 5HT for the time indicated after pretreatment with 10mM LiCl for 15 min. Experimental conditions were described under "EXPERIMENTAL PROCEDURES". Each point represents the average values of four determinations.

A slight amount of IP_3 was produced by stimulation with 1nM 5HT for 3 min. The mean half-maximal effective concentration(ED_{50}) value of the 5HT concentration-response curve was about $5x10^{-7}$M. Although the mechanism of signal transduction from receptor to PI hydrolysis is not clear, accumulating evidence strongly suggests that a guanine nucleotide

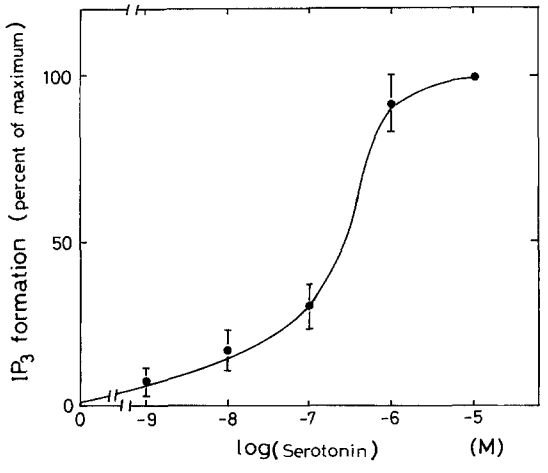

Fig. 2 The concentration-response curve to 5HT-stimulated IP_3 formation. VSMC prelabeled with [^{3}H] inositol for 72h were stimulated with the indicated concentrations of 5HT for 3 min after pretreatment with 10mM LiCl for 15 min. Experimental conditions were described under "EXPERIMENTAL PROCEDURES". Results are expressed as % maximal response to 5HT. Data represent the mean ± S.D. of three determinations.

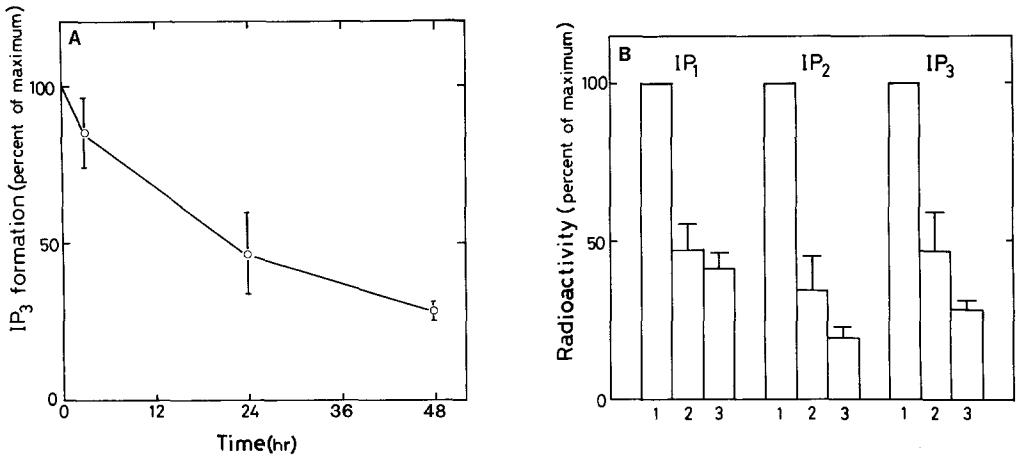

Fig. 3 Time course and concentration-response of the effect of pertussis toxin on 5HT-stimulated formation of inositol phosphates. A. Cells prelabeled with [^{3}H] inositol for 72h were pretreated with 500ng/ml pertussis toxin for the indicated period. Then the cells were stimulated with 1μM 5HT for 3 min after pretreatment with 10mM LiCl for 15 min. B. Prelabeled cells were pretreated with 500ng/ml or 2μg/ml pertussis toxin for 24h before stimulation with 1μM 5HT for 3 min in the presence of 10mM LiCl. Experimental conditions were described under "EXPERIMENTAL PROCEDURES". Results are expressed as % maximal response to 5HT. Data represent the mean ± S.D. of three determinations. 1: Control, 2: With 500ng/ml pertussis toxin, 3: With 2μg/ml pertussis toxin

regulatory protein may be involved in signal transduction of a wide variety of cells. We used IAP to investigate whether a guanine nucleotide regulatory protein is involved in 5HT receptor-mediated PI hydrolysis in cultured VSMC. Fig.3A demonstrates that pretreatment with 500ng/ml IAP inhibited 5HT-stimulated IP_3 production in a time-dependent manner. IAP had no effect on basal radioactivity of inositol phosphates. This inhibitory effect was also concentration dependent, as shown in Fig.3B. Phorbol esters, such as TPA, which are known to activate protein kinase C, have been shown to inhibit α-adrenergic mediated contraction of rat aorta(10). We therefore studied whether TPA induced an inhibition of 5HT-stimulated PI breakdown in cultured VSMC. Fig.4A shows that exposure of 100nM TPA reduced rapidly 5HT-stimulated IP_3 formation. This inhibitory effect was concentration dependent as shown in Fig.4B. Incubation with TPA or DMSO had no effect on basal radioactivity of inositol phosphates. Half-maximal inhibitory effect(IC_{50}) of TPA was at approximately 4nM,

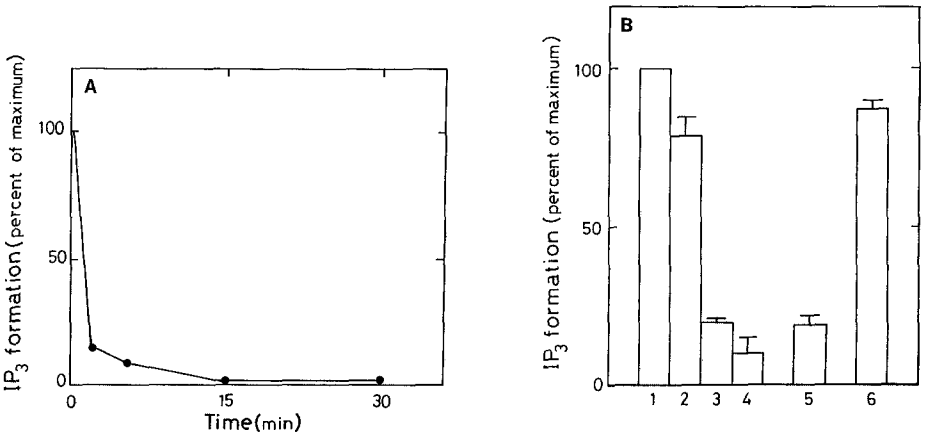

Fig. 4 Effect of phorbol esters on 5HT-stimulated IP_3 formation. A. Cells prelabeled with [^{3}H] inositol for 72h were pretreated with 100nM TPA for the indicated period before stimulation with 1μM 5HT for 3 min in the presence of 10mM LiCl. B. Prelabeled cells were pretreated with various phorbol esters for 2 min before stimulation with 1μM 5HT for 3 min in the presence of 10mM LiCl. Experimental conditions were described under "EXPERIMENTAL PROCEDURES". Results are expressed as % maximal response to 5HT. Data represent the mean ± S.D. of three determinations. 1: Control, 2: With 1nM TPA, 3: With 10nM TPA 4: With 100nM TPA, 5: With 10nM PDBu, 6: With 10nM 4αPDD

at which concentration TPA gave an enough activation of PKC in vitro(7). The ability of PDBu to inhibit the response to 5HT was almost equal to that of TPA. However, 4αPDD, an inactive phorbol ester, was without effect on 5HT-induced IP_3 formation(in Fig.4B).

DISCUSSION

The data presented here suggest that tumor-promoting phorbol esters, such as TPA, inhibit 5HT-stimulated IP_3 formation in cultured VSMC from rabbit thoracic aorta. TPA potently stimulates protein kinase C and mimics endogenously produced DAG, although 4αPDD which is inactive for tumor promotion is ineffective in activating this enzyme(8). This inhibitory effect of phorbol esters has been reported in various kind of cells, including astrocytoma cells(20), platelets(21,22), PC12 cells (23) and cultured VSMC(24). However, it has not been defined that phorbol esters exert an influence on 5HT receptor-mediated signal transduction systems in cultured VSMC. As shown in Fig.4A and 4B, TPA inhibited 5HT-induced IP_3 production in a time- and

concentration-dependent manner. 4αPDD was inactive in this capability. In short, the ability of phorbol esters to inhibit 5HT-stimulated PI breakdown corresponded to their potency to exhibit a tumor promotor action. Half-maximal inhibitory effect(IC_{50}) for TPA occurred at about 4nM at which concentration TPA was effective in rabbit aortic VSMC(7). Thus, it appears likely that these effects are mediated through activation of PKC. However, the mechanisms underlying the inhibitory effect of TPA on the response to 5HT in cultured VSMC are not yet fully understood. It has been demonstrated that phorbol esters induced the phosphorylation of insulin(25,26), somatomedin C(25), transferrin(27), epidermal growth factor(28), β-adrenergic(29,30) and $\alpha_1$adrenergic(31) receptors. Based on these observations, it may be possible that the action of TPA is mediated through the phosphorylation of 5HT receptor. It has been recently described that a Gi protein may be involved in receptor-mediated PI hydrolysis in mast cells(14) and neutrophils(12,13). IAP is known to ribosylate αi subunit of a Gi protein and uncouple receptor to phospholipase C-mediated PI hydrolysis. Fig.3A and 3B demonstrate that pretreatment with IAP prior to addition of 5HT inhibited 5HT-stimulated formation of inositol phosphates in a time- and concentration-dependent manner. Thus, a Gi protein may be involved in 5HT receptor-mediated PI hydrolysis. It has been also reported that protein kinase C phosphorylates a Gi protein in vitro(32). In conclusion, TPA modulates 5HT-stimulated PI hydrolysis in cultured VSMC from rabbit aorta through activation of protein kinase C, which may inactivate 5HT receptor or a Gi protein by phosphorylation.

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