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ON THE MECHANISM OF CYTOSOLIC PHOSPHOLIPASE A_2 ACTIVATION IN CHO CELLS CARRYING SOMATOSTATIN RECEPTOR:

Wortmannin-Sensitive Pathway to Activate Mitogen-Activated Protein Kinase

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SUMMARY: We examined the mechanism of arachidonate release induced by somatostatin-14 (SS14) in CHO-K1 cells overexpressing rat hippocampal somatostatin receptor SSTR4. SSTR4 couples to pertussis toxin (PTX)-sensitive G-protein in CHO cells and does not lead to phosphoinositides breakdown or intracellular calcium ($[Ca^{2+}]i$) mobilization (Bito *et al.*: J. Biol. Chem. 269, 12722-12730, 1994). SSTR4 activated mitogen-activated protein (MAP) kinase and induced the phosphorylation of 85kDa cytosolic phospholipase A_2 ($cPLA_2$), in a PTX-sensitive manner. Furthermore, activations of both MAP kinase and $cPLA_2$ were inhibited by treatment with wortmannin, at almost identical IC_{50} values. Thus, SSTR4 appears to stimulate MAP kinase and $cPLA_2$ in a Gi-dependent, and through a wortmannin-sensitive pathway. We also showed that stimulation with SS14, in combination with calcium-ionophore, strongly enhanced arachidonate release from these cells.

Somatostatin has various physiological actions in the brain and in endocrine organs (1). The receptors for somatostatin consist of at least 5 subtypes SSTR1 to 5, and couple to various effectors, including adenylate cyclase, Ca^{2+} channel and K^+ channel (2). Functional differences of those receptor subtypes were not well understood. In the central nervous system, somatostatin modulates locomotor activity and cognitive functions (3, 4). In hippocampal pyramidal neurons, somatostatin augments a voltage-dependent K^+current (I_M), and those effects appear to be mediated by arachidonic acid or its metabolites (5, 6). The functional role of arachidonate metabolism has been widely discussed in the context of various pathological processes as well as synaptic plasticity (7, 8).

We recently cloned a hippocampal-specific type of somatostatin receptor (SSTR4) and stably expressed it in Chinese hamster ovary (CHO) cells (9). In CHO cell lines overexpressing

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Abbreviations: SS14, Somatostatin-14; MAPK, mitogen-activatied protein kinase; MAPKK, MAPkinase kinase; PTX, pertussis toxin; $cPLA_2$, cytosolic phospholipase A_2; PAF, platelet-activating factor; CHO, Chinese hamster ovary; PI-3 Kinase, phosphatidyl inositol-3 kinase.

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SSTR4, SS14 mediates adenylate cyclase inhibition, arachidonate release, and activation of mitogen-activated kinase (MAP) kinase kinase (MAPKK) /MAP kinase (MAPK) (9), and those effects were completely blocked by pertussis-toxin (PTX) treatment. We have also found that arachidonate release occurred by SS14 without affecting intracellular calcium mobilization or inositol 1, 4, 5-trisphosphate (IP_3) production (9).

In this study, we investigated the signaling pathways implicated in activating 85kDa cytosolic phospholipaseA_2 ($cPLA_2$) (10, 11) downstream of SSTR4. We also demonstrate that phosphorylation of $cPLA_2$ parallels MAPK activation by SS14, and that both arachidonate release and MAPK activation were completely inhibited by a phosphatidylinositol-3 (PI-3) kinase inhibitor, wortmannin.

Materials and Methods

Materials

SS14 was obtained from Nova-Biochem. Wortmannin and bovine serum albumin (fraction V, fatty-acid free) were from Seikagaku Kogyo, Tokyo, A23187 was from Calbiochem, and Ham's F12 was from Nissui, Tokyo. [^{3}H]Arachidonic acid (60-100 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Du Pont-NEN. cPLA2 antibody was a generous gift from Dr. J. Clark (Genetics Institute, Boston). All other reagents were from Sigma.

Cell culture

CHO-K1 cells stably expressing SSTR4 (9), or guinea-pig platelet-activating factor (PAF) receptor (12) were maintained in Ham's F12 containing 0.3 g/l Geneticin, 10 % (v/v) fetal calf serum (Gibco), 100 units /ml penicillin and 100 µg/ml streptomycin.

Immunoblot analysis of $cPLA_2$

Cells were lysed in the lysis buffer (0.5 % NP-40, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM sodium fluoride, and 2 mM sodium orthovanadate) at 4°C for 20 min, then centrifuged at 15,000 x g for 15 min. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (10 %) and transferred to nitrocellulose membranes. Blots were immunostained with a $cPLA_2$ antibody and developed using an Amersham ECL system.

Release of arachidonic acids

Arachidonic acid release was measured as described before (9). In brief, sub-confluent CHO cells in 24-well plates were prelabelled with 1µM/ml of [^{3}H]arachidonic acid in serum-free medium containing 0.1 % bovine serum albumin (BSA) for 20 hrs at 37°C. The cells were washed twice with HBS (Hepes-buffered saline) containing 0.1 % BSA, and re-fed with serum-free medium containing appropriate ligands. After indicated times, aliquots were taken, and the radioactivity was determined.

Assay of MAPK

MAPK activity was measured as described (13), using myelin basic protein (MBP) as a substrate, with some modifications (Waga, I. *et al.*. unpublished data). In brief, Q-Sepharose beads (Pharmacia) were mixed and incubated with cell lysates, prepared in the lysis buffer (20 mM Tris-HCl, pH8.0, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM EGTA, 2 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, 10 µg/ml aprotinin, and 100 mM NaCl). After several washes with the lysis buffer, MAPK containing fractions were eluted with the lysis buffer containing 300 mM NaCl. The eluates were incubated with 2.5 mg/ml MBP, 0.1 µCi/ml of [γ-^{32}P]ATP in 20 mM Tris-HCl, pH8.0, 0.25 mM $MnCl_2$, 2.5 mM $MgCl_2$, 50 µM ATP, and 25 µM protein kinase inhibitor (Sigma) at 30°C for 20 min. Reaction was stopped by adding 0.85 % phosphoric acid, and an aliquot was spotted onto P81 filter papers (Whatman). Filters were washed with 0.5 % phosphoric acid, then dried, and the incorporated radioactivity was determined by Cerenkov counting.

Results and Discussion

85kDa cPLA2 activation in CHO cells

We have previously shown that SS14 induced arachidonic acid release in SSTR4-tranfected CHO cells, without accumulation of inositol 1,4,5-trisphosphate (IP_3) or $[Ca^{2+}]_i$ mobilization (9). 85kDa soluble form of PLA_2 ($cPLA_2$) has recently been identified and cloned (10, 11), the activation being coupled to hormonally regulated release of arachidonic acid (14). Lin *et al.* found that $cPLA_2$ was phosphorylated at its Ser-505 residue by MAP kinase, and intracellular calcium increase induces translocation of the enzyme to the membrane (15). It has also been demonstrated that $cPLA_2$ protein migrates as a doublet, and that decrease in electrophoretic mobility following phosphorylation is associated with the increased catalytic activity (14). To determine $cPLA_2$ contribution in arachidonate release by SS14 in SSTR4-transfected CHO cells, we performed Western blot analysis of $cPLA_2$. SS14 stimulation induces a mobility-shift of the $cPLA_2$ protein band, and the effect was completely inhibited by pertussis-toxin (PTX) treatment (Fig. 1). Although the relative intensity of slowly migrating species induced by two ligands was similar, PAF liberated more arachidonate than did SS14 (data not shown). These findings suggest that phosphorylation of $cPLA_2$ is induced by SS14, but that phosphorylation of $cPLA_2$ alone was not enough to fully activate arachidonate release (see below). PAF induced arachidonate release from CHO cells expressing PAF receptor was partially inhibited by PTX treatment (16), whereas the SS14-induced arachidonate release was completely inhibited by PTX (9). As shown in Fig. 1, the mobility shift of $cPLA_2$ protein band elicited by SS14 was completely abolished by PTX treatment, while with PAF, the inhibition of the shift was only partial.

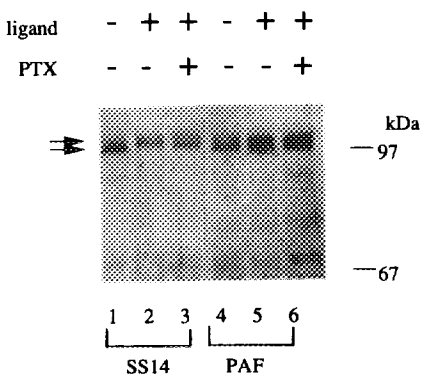

Fig. 1. Electrophoretic mobility shift of $cPLA_2$ induced by SS14 and PAF.
CHO cells were serum-starved for 20 hrs before stimulation. 10 min after application of ligands, cell lysates were prepared and analyzed by immunoblotting with a $cPLA_2$ antibody. Arrows indicate migration of the bands corresponding to $cPLA_2$. Lanes 1-3; CHO cells overexpressing SSTR4 treated with vehicle (lane 1), 1 μM SS14 (lanes 2, 3), or pretreated with PTX (100 ng/ml) for 20 hrs (lane 3). Lanes 4-6; CHO cells overexpressing PAF receptor, treated with vehicle (lane 4), 1 μM PAF (lanes 5, 6) or pretreated with PTX (lane 6).

Wortmannin inhibits MAPK activation and arachidonate release

In accordance wit arachidonate release, SS14 activated MAPK through a PTX sensitive pathway in SSTR4-transfected CHO cells (9). On the other hand, PAF-induced activation of MAPK was partially inhibited by this treatment (16). Recently, we found that PAF-stimulated MAPK activation in guinea-pig leukocytes is mediated by Ca^{2+}-dependent and Ca^{2+}-independent/wortmannin sensitive pathways (17) . To determine the wortmannin effect on the MAPK activation by SS14 in CHO cells, the kinase activity was measured after a 10-min pretreatment with wortmannin (Fig. 2-1). 0.5 μM wortmannin completely inhibited the MAPK activity induced by SS14 in CHO cells. In contrast, PAF- induced MAPK activity was partially inhibited at the same concentration (data not shown) . This indicates that in CHO cells, SS14 activates MAPK in a Ca^{2+} independent but wortmannin-sensitive manner. Fig. 2-2 shows the effect of wortmannin on arachidonate release induced by SS14. Arachidonate release was also completely inhibited with 0.5 μM wortmannin, whereas the PAF-stimulated release was partially inhibited (data not shown). The IC_{50} values ranged between 50 nM and 100 nM. These observations suggest that a wortmannin sensitive signaling molecule such as PI3-kinase may lie upstream of the MAPK activating pathway and downstream of PTX sensitive G-protein (s), as reported by others using hematopoietic cells (18, 19) .

Synergistic effect of $[Ca^{2+}]i$ to activation of $cPLA_2$

Somatostatin induced phosphorylation of $cPLA_2$ (Fig. 1), but it did not mobilize calcium ion (9). Hence, the absolute amount of arachidonate release was significantly smaller than that

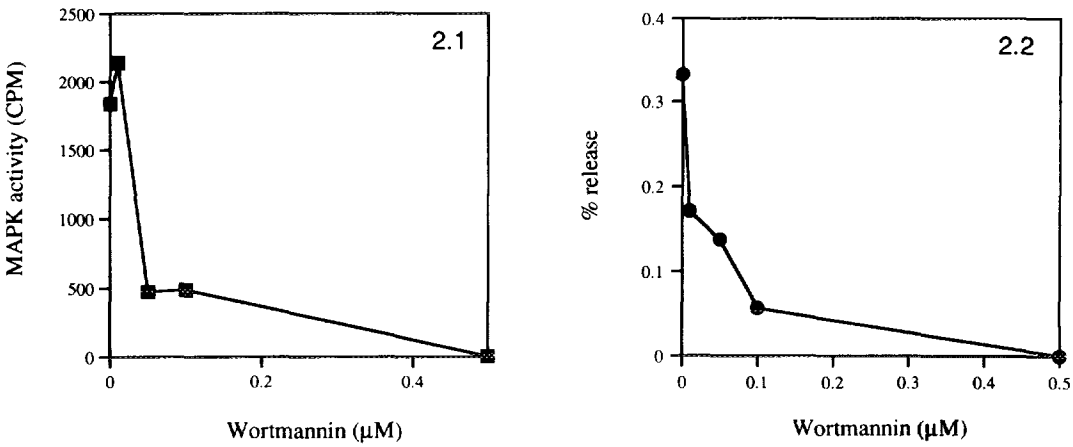

Fig. 2-1. Wortmannin inhibits MAPK activation induced by SS14.
CHO cells were pretreated for 10 min with wortmannin at indicated concentrations before stimulation. Cells were stimulated with 0.1 μM SS14 for 3 min, and cell lysates were assayed for MAPK activity as described under "Materials and Methods". The results are mean of duplicate determinations and a representative of three experiments.

Fig. 2-2. Wortmannin inhibits arachidonate release induced by SS14.
CHO cells were pretreated for 10 min with indicated concentrations of wortmannin before stimulation. Cells were stimulated with 0.1 μM SS14 for 15 min and the release of arachidonate was determined. The results are mean of duplicate determinations and a representative of three experiments.

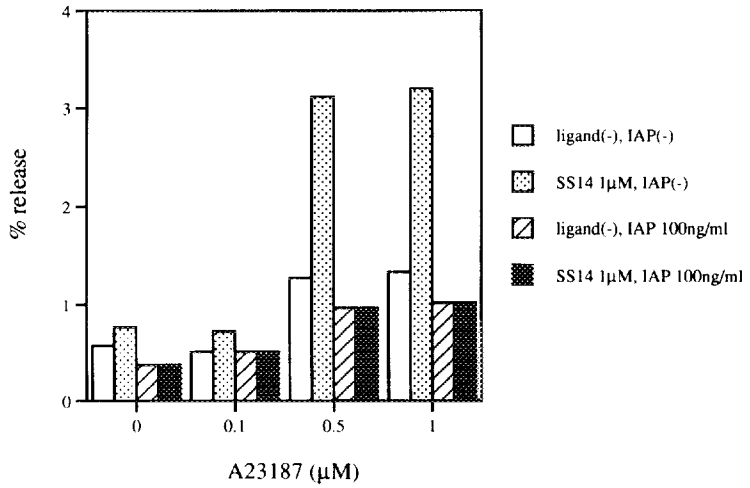

Fig. 3.SS14-induced arachidonate release in the presence of A23187.

CHO cells were treated with vehicle (☐) or SS14 (▩) for 15 min with A23187 at varying concentrations. The same experiments were done following pretreatment with 100 ng/ml PTX for 20 hrs with vehicle (▨) or SS14 (■).

induced by PAF stimulation. However, the SS14-induced release of arachidonate was dramatically augmented in the presence of A23187. As shown in Fig. 3, at a low concentration (10^{-7} M), A23187 itself has little effect on arachidonate release, while at higher concentrations (>$5x10^{-7}$ M), A23187 liberated arachidonate. At any concentrations used, A23187 showed synergistic effects with SS14. This incremental effect of A23187 was completely abolished by PTX treatment.

In summary, we obtained evidence that SS14-induced $cPLA_2$ phosphorylation in CHO cells, in a PTX sensitive manner. We also showed that wortmannin, a PI-3 Kinase inhibitor, inhibited both MAPK activation and arachidonate release at almost identical IC_{50} values. This supports the presence of PI-3 Kinase upstream of MAPK and $cPLA_2$ in CHO-K1 cells. Recent reports revealed the presence of a novel type of PI-3 Kinase activated by a G-protein βγ subunit in hematopoietic cells (18, 19). Whether an identical or similar activity is present in the fibroblastic cell line remains to be established. Further investigations are needed to explicate this point in CHO cells and also in various other cells.

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