Effects of Isoflavone Compounds on the Activation of Phospholipase C

Kyoichiro HIGASHI* and Hiroshi OGAWARA

Department of Biochemistry, Meiji College of Pharmacy, 1–35–23 Nozawa, Setagaya-ku, Tokyo 154, Japan. *Received May 16, 1991*

The effect of isoflavone compounds, genistein and daidzein, on the breakdown of inositol phospholipids in 3T3 cells was studied. Genistein (100 μg/ml) inhibited the stimulation of the production of inositol phosphates by bombesin. The stimulated production of inositol phosphates by AlF_4^- was also inhibited by genistein ($IC_{50}=0.6$ μg/ml) and daidzein ($IC_{50}=2$ μg/ml). However, the catalytic activity of phospholipase-C (PLC) in 3T3 cell extracts was not inhibited by these isoflavones. These results suggest that the isoflavones inhibited the activation of PLC at the G-protein or downstream of the sequences in signal transduction.

In permeabilized 3T3 cells, the inhibition of AlF_4^- plus adenosine triphosphate (ATP)-dependent PLC was recovered by increasing ATP but not AlF_4^-. Genistein also inhibited the activity of adenosine 5′-[3-*O*-thiotriphosphate] (ATP[S])-dependent PLC. The effect of genistein and other inhibitors of protein tyrosine kinases and phosphatases suggests that protein tyrosine phosphorylation is not involved in the activation of PLC in 3T3 cells and that AlF_4^-- and ATP[S]-mediated activation of PLC involves a different mechanism from the tyrosine kinase-mediated activation of PLC.

Daidzein and genistein seem to interrupt the ATP-dependent step of PLC activation by a putative G-protein.

Keywords genistein; isoflavone; phospholipase-C; ATP-dependent activation; G-protein

Introduction

It is widely accepted that Ca^{2+}-mobilizing receptors stimulate phosphoinositide hydrolysis and provide two potent second messengers, *i.e.*, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DG).[1] Recent studies suggested the involvement of unidentified G-proteins (Gp) in the coupling of receptor function to phospholipase-C (PLC).[2] However, little is known as to how the receptors are able to activate PLC.

We have previously shown that in permeabilized 3T3 cells adenosine triphosphate (ATP) is required for Gp-mediated activation of PLC in addition to Gp activators, that adenosine 5′-[3-*O*-thio triphosphate] (ATP[S]) directly activates PLC, bypassing the Gp activation, and that the activation by ATP[S] is not the effect of phosphatidyl inositol 4,5-bisphosphate (PIP_2) maintenance, because the levels of phosphatidyl inositol 4-phosphate (PIP) and PIP_2 are not increased by ATP[S], but decreased.[3] From these results, we proposed that a protein was involved in ATP[S]-dependent activation of PLC. However, the properties of the protein mediating the activation by ATP[S] is still obscure.

To clarify these points further, the inhibitory effect of the isoflavone compounds was studied on phosphoinositide metabolism in intact and chemically permeabilized 3T3 cells. The results suggest that the isoflavone compounds inhibited the ATP-dependent step of PLC activation.

Materials and Methods

Materials Myo[^{3}H]inositol was purchased from Amersham, daidzein from Funakoshi, Sci., Co., poly(Glu–Tyr) 4:1 from Sigma, Co., and digitonin from Merck. Genistein was prepared following the method previously reported.[4] Lavendustin-A, erbstatin, herbimycin-A and okadaic acid were generous gifts from Dr. K. Umezawa, Dr. Y. Uehara and Dr. H. Fujiki, respectively. All other reagents used in this study were of the highest grades available.

Cell Culture Swiss 3T3 cells were grown in 33 mm plastic dishes with Dulbecco's modified Eagle's medium (DME medium) supplemented by 10% calf serum, in humidified 5% CO_2 air at 37 °C. After confluent growth, the medium in each dishes was changed to an inositol-deprived DME medium, and the cells were labelled with 2 μCi of myo[^{3}H]inositol for 16—20 h.

Phosphoinositide Metabolism Assay [^{3}H]-Labelled cells were washed once with 0.5 ml of phosphate buffered saline (PBS), then 0.5 ml of hydroxyethylpiperazine-*N*′-2-ethanesulfonate (Hepes) buffered saline supplemented with 0.1% glucose was added to the cells. In the case of bombesin-stimulation, after 5 min of preincubation, stimulation was started by the addition of 5 μl of bombesin (1 μM). In the case of AlF_4^- stimulation, cells were previously loaded with 20 μM of $AlCl_3$ for 30 min. Stimulation was started by the addition of 30 μl of NaF (500 mM). At the end of stimulation, cells were fixed by the addition of 0.5 ml of 10% trichloroacetic acid. [^{3}H]IP_2 and -IP_3 in acid-soluble extracts were separated using Dowex1 ion exchange resin by the method of Berridge *et al.*[5] and determined using a liquid scintillation counter.

Permeabilization and Stimulation of 3T3 Cells Subconfluently grown cells were labeled with myo[^{3}H]inositol (2 μCi/dish) for 16—20 h in the inositol-depleted DME medium. Thereby, cell permeabilization was performed as previously described.[3] The composition of the permeabilization medium was as follows: 120 mM KCl, 10 mM NaCl, 20 mM Hepes-Na, 5 mM $MgCl_2$, 3 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid sodium salt (EGTA-Na), 0.6 mM $CaCl_2$, 20 μM digitonin, 1 mM ATP-Mg (pH 7.0). The concentration of free Ca^{2+} was calculated as 50 nM at pH 7.0 from the equations of Bartfai.[6] Permeabilized cells were stimulated by 5 mM NaF/20 μM $AlCl_3$. Reactions were stopped at indicated times by the addition of 0.5 ml of 10% trichloroacetic acid (TCA) to the dishes. [^{3}H]IP_2 and [^{3}H]IP_3 in acid-soluble extracts were determined as described above.

PLC Assay An aliquot of cholate extract of 3T3 (5—10 μg protein) was incubated at 37 °C for 10—30 min in a volume of 50 μl of reaction mixture. The reaction mixture contained the following: 100 mM Hepes-Na (pH 7.0), 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% bovine serum albumin, 1% cholate-Na, 0.2—980 μM [^{3}H]PIP_2 (10 nCi), 0.8 mg/ml phosphatidylcholine. The reaction was terminated by the addition of 100 μl of 10% TCA. The reaction mixture was allowed to stand for 30 min on ice, then an acid-soluble fraction containing [^{3}H]IP_3 was separated from residual PIP_2 by centrifugation at 16000 rpm for 5 min. Radioactivity of [^{3}H]IP_3 was determined by liquid scintillation counting.

Results and Discussion

In 3T3 cells (Swiss albino), bombesin (10 nM) increased the levels of IP_2 and IP_3 within 30 s (Table I). When genistein had been preloaded to the cells before the 30 min of stimulation by bombesin, the stimulation was profoundly decreased. A typical time course of bombesin-stimulated production of inositol phosphates is shown in Fig. 1. The time course of phosphoinositide breakdown was practically identical in both genistein-untreated and

TABLE I. Inhibition of Bombesin-Stimulated Accumulation of Inositol Phosphate by Genistein

Addition	[^{3}H]IP_2+IP_3 dpm/dish	
	IP	IP_2+IP_3
Control		
Resting	2025±109	837±133
+Bombesin (10 nM)	4283±391	4612±497
+Genistein (100 μg/ml)		
Resting	1810±296	1596±103
+Bombesin (10 nM)	1835±203	1383±300
+Daidzein (100 μg/ml)		
Resting	—	802±155
+Bombesin (10 nM)	—	841±141

Confluently grown 3T3 cells in 33 mm dishes were labeled with 2 μCi of myo[^{3}H]inositol for 16 h. During the last 30 min of the labeling periods, genistein (100 μg/ml) was added to the dishes. Then, the medium was changed to 1 ml of Hepes-buffered Hanks solution containing the same concentrations of genistein, and the cells were preincubated for 5 min at 37°C. The cells were stimulated for 30 s by the addition of bombesin (10 nM) to the dish, then the medium was rapidly aspirated and the cells were fixed by the addition of 0.5 ml of 5% trichloroacetic acid. [^{3}H]IP_2 and -IP_3 were separated and counted as described in the Materials and Methods section. Values were expressed as means±S.E.M. ($n=5$).

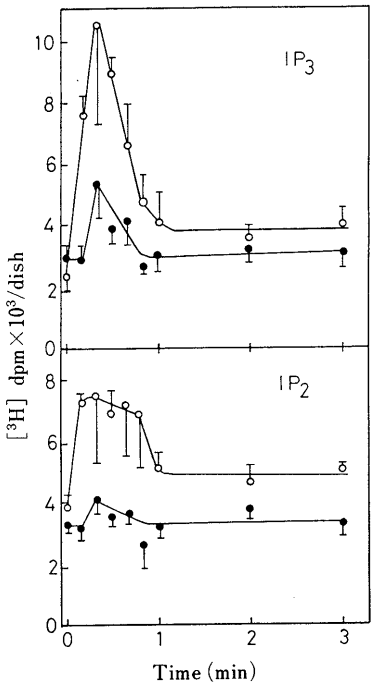

Fig. 1. Effect of Genistein on Bombesin-Stimulated Phosphoinositide Breakdown of 3T3 Cells

Swiss-3T3 cells in a 55 mm dish, labeled with 2 μCi of myo[^{3}H]inositol for 24 h, were washed once with 2 ml of PBS^-, and the medium was changed to 0.75 ml of PBS^+. Stimulation of the cells was started by the addition of bombesin (0.1 μM) at 37°C, and continued until the time indicated in the figure. At the end of incubation, 0.5 ml of 10% of TCA was added. The inositol phosphates thus formed were separated, followed by the method of Berridge *et al.*[5] Genistein was added at the period of the last 1 h of [^{3}H]-labeling and bombesin-stimulation. —○—, without genistein; —●—, with genistein (100 μg/ml). Values were expressed as the means of duplicate experiments.

genistein-treated cells. This transient accumulation of IP_2 and IP_3 was similar to that in the activation of PLC by Gp-coupled receptors.[7]

3T3 cells responded also to AlF_4^-, a potent activator of Gp.[8] The stimulation of phosphoinositide breakdown by AlF_4^- was inhibited by genistein or by daidzein, a 5-deoxy derivative of genistein (Fig. 2). Half-maximal inhibition

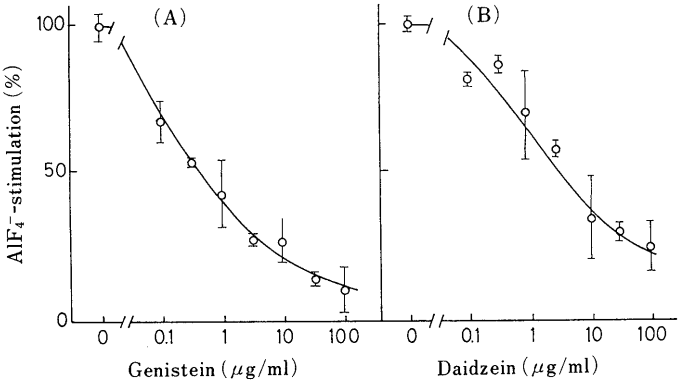

Fig. 2. Inhibition of AlF_4^--Stimulation of Phosphoinositide Breakdown by Genistein (A) and Daidzein (B)

Myo[^{3}H]inositol labeled cells were stimulated with 30 mM of NaF and 20 μM of $AlCl_3$ for 15 min at 37°C. Other conditions were the same as described in Fig. 1. In the absence of inhibitors, formed IP_3 and IP_2 were 23600 dpm/dish with AlF_4^- (stimulated), and 3100 dpm/dish without AlF_4^- (unstimulated). Values were expressed as the means of duplicate experiments.

TABLE II. Failure of Inhibition of PLC Activity of 3T3 Cell Lysate

Addition		Phospholipase-C activity nmol/min/mg protein
None		34.3±2.5
Genistein	10 μg/ml	33.6±3.0
	100 μg/ml	35.7±1.8
Daidzein	10 μg/ml	34.7±1.0
	100 μg/ml	36.1±0.8

PLC activity in cholate extracts of 3T3 (7.4 μg protein) was assayed with or without the isoflavones for 30 min at 37°C in the reaction mixture containing [^{3}H]PIP_2 as substrate. Further detailed procedures were described in the Materials and methods section. Values were expressed as means±S.E.M. ($n=3$).

was observed at 0.6 μg/ml of genistein and at 2 μg/ml of daidzein, respectively. Not only bombesin- but also AlF_4^--stimulation was inhibited by these isoflavones (Figs. 1 and 2). However, genistein and daidzein (100 μg/ml) did not inhibit the catalytic activity of PLC in the cholate extracts of 3T3 cells (Table II), indicating that PLC is not a direct target of these isoflavones. These results suggest, therefore, that the blocking point of these isoflavones is located on the Gp or downstream from Gp, but upstream of PLC in the sequences of signal transduction, and that both inhibitors interrupted Gp/PLC coupling.[7]

In order to determine the blocking point of the isoflavones, the effect of genistein in permeabilized cells was studied. In the permeabilized cell preparations, ATP was also necessary for Gp-mediated activation of PLC in addition to AlF_4^- (Fig. 3A), although ATP itself did not activate PLC without AlF_4^-. As shown in Fig. 3A, genistein changed the concentration of ATP for the half-maximal activation from 1 to 3 mM in the presence of 100 μg genistein per ml. In other words, augmenting the ATP diminished the inhibitory effect of genistein. On the other hand, by varying AlF_4^- concentrations while maintaining a constant ATP concentration of 1 mM, genistein pronouncely reduced the maximal activation levels, but the concentration of AlF_4^- necessary for the half-maximal activation did not change (Fig. 3B). In permeabilized cells, genistein also inhibited ATP[S]-dependent activation of PLC in the absence of AlF_4^- (Table III). It seems likely,

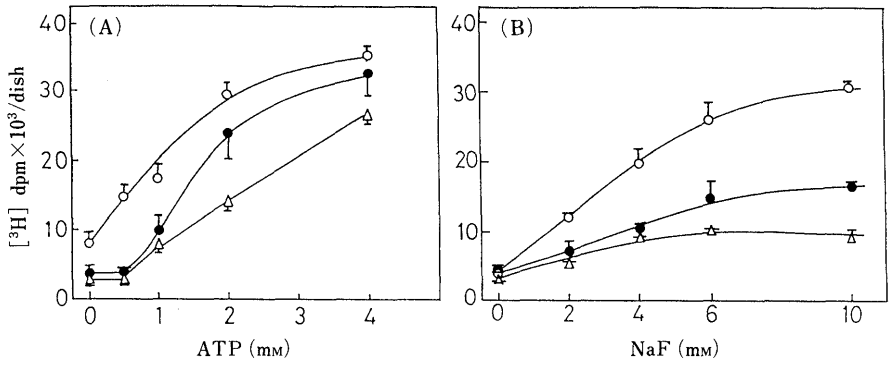

Fig. 3. Inhibition of ATP-Dependent AlF_4^- Activation of PLC of Permeabilized-Cells by Genistein

Myo[^{3}H]inositol labeled cells were permeabilized with a medium containing 20 μM digitonin and genistein at the concentrations indicated in the figures. After 5 min of preincubation at 37 °C, the indicated concentrations of ATP–Mg and NaF, and 20 μM of $AlCl_3$ were added to the cells to activate PLC.

(A) 5 mM of NaF and the indicated concentration of ATP–Mg, (B) 1 mM of ATP–Mg and indicated concentration of NaF were added respectively for the activation of PLC. Then, permeabilized cells were incubated for 10 min at 37 °C. —○—, without genistein; —●—, 10 μg/ml of genistein; —△—, 100 μg/ml of genistein. Values were expressed as the means of duplicate experiments.

Table III. Effect of Tyrosine Kinase Inhibitors and Phosphatase Inhibitors on PLC Activation of permeabilized 3T3 Cells

Inhibitor addition	$[^3H]IP_2+IP_3$ dpm × 10^3/dish Assayed with ATP	ATP+AlF_4^-	ATP[S]
None	2.0±0.2	13.3±0.2	12.1±1.4
Genistein (100 μg/ml)	1.1±0.2	4.8±1.5	3.9±1.1
Erbstatin (100 μg/ml)	—	17.0±3.5	—
Herbimycin-A (10 μg/ml)	—	14.7±0.5	—
Lavendustin-A (10 μg/ml)	2.7±0.1	17.6±5.5	14.7±0.9
None[a]	2.4±0.3	16.1±0.6	—
Genistein[a] (100 μg/ml)	—	6.2±0.8	—
Daidzein[a] (100 μg/ml)	—	6.2±0.1	—
None[a]	2.4±0.2	11.0±0.9	9.2±0.5
Poly(Glu–Tyr)[a] (10 μM)	2.0±0.2	—	11.2±1.4
Heparin[a] (0.3 mg/ml)	2.2±0.0	—	11.4±0.8
Okadaic acid[a] (1 μM)	2.2±0.2	—	—

Several protein kinase or phosphatase inhibitors were added to the permeabilization medium at the concentrations indicated in the table. Cells were incubated for 10 min at 37°C with 1 mM ATP (resting) or with 1 mM of ATP plus AlF_4^- (activated) or with 1 mM of ATP[S] (activated). The resulting IP_2 and IP_3 were determined as described in the Materials and Methods section. Values were expressed as the means of duplicate (*a*) or triplicate experiments ± S.E.M.

therefore, that genistein inhibited the ATP-dependent step of Gp/PLC coupling, but did not inhibit the activation of Gp. The result also coincides with the evidence that genistein inhibits tyrosine kinase activity in competition with ATP but not with a tyrosine-containing peptide substrate.[9]

As we showed previously, ATP[S] did not augment but rather decreased PIP and PIP_2 levels in the same assay condition as that of the PLC activation in permeabilized cells.[3] It suggests that stimulation of inositol phosphate formation by ATP[S] is due to the activation of PLC but not to the maintenance of PIP_2 production. The ATP[S]-dependent activation of PLC indicates the possibility of the involvement of kinase/phosphatase systems in the regulation of PLC.[10] As genistein is an inhibitor of tyrosine kinases,[9] it may inhibit the tyrosine kinase activity coupled with PLC. Several papers reported the involvement of tyrosine kinases in PLC activation.[11] Thus, we examined the possibility of involvement of tyrosine kinases in the activation of PLC of 3T3 cells by using inhibitors of tyrosine kinases and phosphatases in permeabilized 3T3 cells. Permeabilized cells eliminated the effect according to the difference in permeability of the tested reagents. Table III shows that genistein inhibited the activation of PLC accompanied by both ATP plus AlF_4^- and by ATP[S], but lavendustin-A, erbstatin and herbimycin did not. Furthermore, heparin and poly(Glu–Tyr), inhibitors of protein tyrosine phosphatases,[12] did not activate PLC in the presence of ATP. These results, together with the inhibition by daidzein (Table III, Fig. 2) which was reported not to inhibit tyrosine kinases,[9] strongly suggest that the inhibitory effect of the isoflavones is not due to the inhibition of tyrosine kinases, but to unknown ATP-dependent processes. In addition, cyclic adenosine monophosphate (AMP), cyclic guanosine monophosphate (GMP), and phorbol myristate acetate failed to activate PLC (data not shown), and okadaic acid[13] did not activate PLC in the presence of ATP without AlF_4^- (Table III). It seems, therefore, that the target is not cyclic-AMP, cyclic-GMP, or calcium/phospholipid-dependent protein kinases.

Daidzein and genistein are superior reagents for the investigation of an ATP-dependent process in PLC activation. Recently, Samuelson *et al.* reported that herbimycin, a tyrosine kinase inhibitor, inhibited T cell receptor-mediated activation of PLC but did not inhibit AlF_4^--mediated activation.[14] Inhibition of T cell receptor activation by genistein was also reported.[11] We showed that herbimycin did not inhibit AlF_4^--mediated activation of PLC (Table III), so that AlF_4^-- and ATP[S]-mediated activation of PLC seems to involve a different mechanism from the tyrosine kinase-mediated activation of PLC.

It seems that ATP[S]-dependent activation of PLC is not a rare case in 3T3 cells because we observed ATP[S]-dependent activation of PLC in permeabilized A431 cells and H-*ras*-transformed NRK cells (unpublished data). This activation required fairly high concentrations of ATP (Fig. 3A) or ATP[S]. The ATP concentration in 3T3 cells

was determined as 9 nmol per 3×10^5 cells by enzymatic assay using the combined reaction of hexokinase and glucose-6 phosphate dehydrogenase (data not shown). According to the size of trypsinized spherical 3T3 cells (about 20 μm of diameter), the ATP concentration in a 3T3 cell was calculated as 5.4 mM. Therefore, the physiological concentration of ATP seems to be sufficient for this ATP-dependent activation of PLC. Genistein (100 μg/ml) reduced the ATP level of intact cells to 50% of the control level within 30 min. But it must be noted that genistein also inhibited the PLC activation of permeabilized cells (Figs. 3A, B), in which ATP had been supplied from a permeabilization medium. The reduction of ATP level by genistein did not sufficiently provide a direct reason for the inhibition of PLC. But, a decrease in the ATP of intact cells may synergistically reduce the PLC activation. Since phosphoinositide metabolism is a cyclic reaction, one molecule of inositol triphosphate, hydrolyzed from PIP_2, must be recycled to phosphatidylinositol and further phosphorylated to PIP and PIP_2 using four molecules of ATP. Further sequences of cell response such as cell proliferation may also require vast amounts of ATP. Therefore, it is possible to speculate that ATP may play a regulatory role in PLC activation, as an "emergency brake," for example, or in the nutritional regulation of PLC activation. The permeabilized cell system seems to be the only choice as a reliable assay system of Gp/PLC coupling at this moment, although it still involves some complexity of multiple biochemical reactions. Physically scraped cells or cell lysate completely lost or decreased greatly their responsiveness to the activation of PLC by ATP[S] or ATP/AlF_4^- (data not shown). For the study of a complete *in vitro* system of Gp/PLC coupling, it is important to identify the ATP[S]-dependent activator of PLC. In this point, daidzein and genistein would function as important probes for the investigation of Gp/PLC coupling.

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