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Impairment of Phosphatidylinositol Signaling in Acetylshikonin-Treated Neutrophils

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ABSTRACT. In rat neutrophils, formylmethionyl-leucyl-phenylalanine (fMLP)-induced inositol phosphate formation was concentration-dependently inhibited by acetylshikonin as well as by a putative phospholipase C (PLC) inhibitor [6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122). The IC_{50} value of acetylshikonin for the inhibition of inositol trisphosphate (IP_3) formation was estimated to be 16.1 ± 1.5 μM. The reduction of inositol phosphate levels appeared to reflect inhibition of PLC activity because the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) catalyzed by a soluble fraction from neutrophils was also inhibited by acetylshikonin (IC_{50} value 21.4 ± 6.1 μM) over the same range of concentrations. Although acetylshikonin alone evoked Ca^{2+} and Mn^{2+} influx into neutrophils in Ca^{2+}-containing medium, acetylshikonin, like U73122, inhibited Ca^{2+} release (IC_{50} value ~5.3 ± 0.4 μM) from internal stores in Ca^{2+}-free medium. These results indicate that acetylshikonin inhibits phosphatidylinositol signaling in neutrophils. BIOCHEM PHARMACOL 53;8:1173–1177, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. acetylshikonin; neutrophils; inositol phosphates; phospholipase C; intracellular Ca^{2+} concentration

Various extracellular signaling molecules, when binding to their cell surface receptors, elicit transmembrane signal transduction mechanisms. These include the activation of cytosolic phosphatidylinositol-specific PLC[§] through a G-protein-dependent or -independent step, and catalysis of the hydrolysis of PIP_2 to generate two second messengers, diacylglycerol and IP_3 [1, 2]. Diacylglycerol is the physiological activator of protein kinase C, and IP_3 induces the release of Ca^{2+} from internal stores [3, 4]. This pathway is known to regulate several cellular processes, including metabolism, secretion, contraction, motility, and proliferation [5].

Acetylshikonin (Fig. 1), a naphthoquinone isolated from the Chinese herb tzu ts'ao, the dried purple roots of *Lithospermum erythrorhizon* **Sieb. et Zucc.**, *Arnebia euchroma* **(Royle) Johnst,** or *Macrotomia euchroma* **(Royle) Pauls** (Boraginaceae), has been found to possess antibacterial, antitumor, antipyretic, and analgesic activity [6, 7]. Recently, acetylshikonin was shown to inhibit rabbit platelet aggregation [8] and DNA topoisomerase I activity [9]. In addition, we demonstrated that acetylshikonin inhibits the release of chemical mediators from rat peritoneal mast cells *in vitro*, and suppresses mouse cutaneous plasma extravasation caused by inflammatory mediators *in vivo* [10]. It has been reported that many inflammatory mediators utilize phosphoinositide turnover as part of their signal transduction pathway [11, 12], and that inhibition of PLC partly accounts for the anti-inflammatory actions of manoalide [13]. In this study, we have examined the inhibitory effects of acetylshikonin on both inositol phosphate formation in neutrophils and PLC activity in crude cytosolic preparations. Furthermore, the effect of acetylshikonin on the intracellular Ca^{2+} concentrations of neutrophils was also investigated.

MATERIALS AND METHODS

Reagents

Acetylshikonin was isolated and purified from *Lithospermum erythrorhizon* **Sieb. et Zucc.** (Boraginaceae) as previously described [14]. All chemicals were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. except for the following: Dextran T-500 (Pharmacia Biotech Ltd., Uppsala, Sweden); Hanks' balanced salt solution (Life Technologies Gibco BRL Co., Gaithersburg, MD, U.S.A.); U73122 and CPA (Biomol Research Lab. Inc., Plymouth Meeting, PA, U.S.A.); AG 1-X8 resin (Bio-Rad Lab., Her-

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§ *Abbreviations:* CPA, cyclopiazonic acid; fMLP, formylmethionyl-leucyl-phenylalanine; IP, inositol monophosphate; IP_2, inositol biphosphate; IP_3, inositol trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIPES, 1,4-piperazinediethanesulfonic acid; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; and U73122, [6-[[17β-3-methyoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

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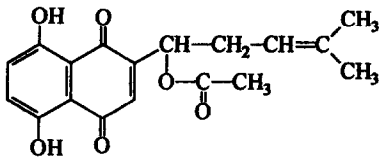

FIG. 1. Chemical structure of acetylshikonin.

cules, CA, U.S.A.); L-α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate (PIP_2) (Boehringer Mannheim, Mannheim, Germany); L-3-phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2) and *myo*-[^{3}H]inositol (Amersham International plc., Buckinghamshire, U.K.); and fura-2 AM (Molecular Probes Inc., Eugene, OR, U.S.A.).

Preparation of Neutrophils

Rat blood was collected from the abdominal aorta, and neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll–Hypaque [15]. Purified neutrophils containing >95% viable cells were resuspended in Hanks' balanced salt solution containing 4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4.

Determination of Inositol Phosphates

Neutrophils (3×10^7 cells/mL) were incubated with *myo*-[^{3}H]inositol (83 Ci/mmol) at 37° for 2 hr [16], then washed, and treated with inhibitors at various concentrations for 3 min at 37° prior to stimulation with 0.3 μM fMLP for 10 sec. Reaction was stopped by the addition of a $CHCl_3$:CH_3OH (1:1, v/v) mixture and acidification with 2.4 M HCl. The aqueous phase was removed, neutralized with 0.4 M NaOH, and then applied to an AG 1-X8 resin (formate) column. IP, IP_2, and IP_3 were eluted sequentially using 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid, respectively, as eluents, and then counted in dpm as described in detail elsewhere [17].

Measurement of Cytosolic PLC Activity

Neutrophils (4×10^7 cells/mL) were disrupted by sonication in relaxing buffer (115 mM KCl, 5 mM KH_2PO_4, 2 mM EGTA, 0.91 mM $MgSO_4$, 10 mM HEPES, pH 7.4) supplemented with 0.1 mM dithiothreitol, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF, and 3 mM benzamidine. The neutrophil lysate was centrifuged at 100,000 *g* for 1 hr at 4°, and the supernatant fluid was retained for PLC assays. Substrate stock was prepared by mixing PIP_2 and 5 μCi [^{3}H]PIP_2 (1 Ci/mmol) in $CHCl_3$:CH_3OH (1:1, v/v). The mixture was evaporated to dryness under N_2, redissolved in solvent mixture (20% sodium cholate, 250 mM 2-mercaptoethanol, 1 M PIPES, pH 6.8, 2.5 M NaCl) to produce a 0.361 mM PIP_2 solution, sonicated on ice, and then stored at −20°. PLC activity was assayed by measuring the hydrolysis of PIP_2 into inositol phosphates [18]. Briefly, assay tubes contained acetylshikonin or U73122 at various concentrations, 10 mM HEPES buffer, pH 7.4, and cytosolic PLC (about 0.04 mg protein). The assay mixture was mixed well and incubated at 37° for 3 min, and then 3 μL of 5 mM $CaCl_2$ and 20 μL of substrate stock were added to a total assay volume of 150 μL. Ten minutes later, reactions were quenched with 0.75 mL of ice-cold $CHCl_3$:CH_3OH:HCl (50:50:1, by vol.), and the mixture was acidified with 1 M HCl. After vigorous mixing and centrifugation at 2000 *g* for 5 min at 4°, a portion (400 μL) of the aqueous upper layer was collected for liquid scintillation counting.

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

Neutrophils (1×10^7 cells/mL) were suspended in HEPES buffer (124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 5.56 mM dextrose, and 10 mM HEPES, pH 7.4), and loaded with 5 μM fura-2 AM at 37° for 15 min as described previously [15]. Then the cells were diluted 5-fold with the same buffer and incubated for an additional 15 min. Next, cell suspensions were centrifuged at 900 *g* for 10 min at 4°, and the pellets were resuspended in HEPES buffer with 0.05% bovine serum albumin in the presence of 1 mM $CaCl_2$ or 1 mM EDTA. Cell suspensions were placed in a double wavelength fluorescence spectrophotometer (PTI, South Brunswick, NJ; Deltascan 4000) equipped with a thermostatically controlled cuvette holder and stirrer. The fluorescence was monitored at 510 nm with excitation wavelengths of 340 and 380 nm in the ratio mode. Calibration of the excitation ratio in terms of Ca^{2+} concentration was performed by using fura-2–Ca^{2+} standards according to a previously described method [19].

Assessment of Mn^{2+} Influx

Entry of Mn^{2+} into cells was measured by the fura-2 fluorescence quenching technique. Cell suspensions were placed in a fluorescence spectrophotometer (PTI, Deltascan 4000) equipped with a thermostatically controlled cuvette holder and stirrer. Fluorescence was monitored in fura-2-loaded cells in Ca^{2+}-containing medium at excitation 360 nm, the isobestic point where fura-2 was insensitive to changes in $[Ca^{2+}]$, and emission 510 nm. $MnCl_2$ (0.5 mM) was added following the preincubation of the neutrophils with 10 μM CPA [20] or various concentrations of acetylshikonin. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of an experiment, which indicated that less than 5% of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2.

Statistical Analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. A *P* value of less than 0.05 was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values.

RESULTS

Effect of Acetylshikonin on the Formation of Inositol Phosphates

Upon addition of 0.3 μM fMLP to *myo*-[^{3}H]inositol-loaded neutrophils, a significant increase in cellular IP_2 and IP_3 levels was observed in comparison with the resting levels (the levels of IP, IP_2 and IP_3 were 40.6 ± 6.0, 77.2 ± 8.2, and 150.6 ± 13.5 dpm, respectively, in the resting cells, and 45.8 ± 5.3, 129.3 ± 10.3, and 330.0 ± 18.7 dpm, respectively, in the fMLP-stimulated cells). Acetylshikonin, as well as a putative PLC inhibitor, U73122 [21], reduced both the IP_2 and IP_3 levels in a concentration-dependent manner (Fig. 2). Significant inhibitions of IP_2 and IP_3 formation were observed at concentrations of acetylshikonin ≥10 μM and concentrations of U73122 ≥3 μM. The IC_{50} values of acetylshikonin and U73122 for the inhibition of IP_3 formation were estimated to be 16.1 ± 1.5 and 13.4 ± 0.8 μM, respectively.

Effect of Acetylshikonin on Cytosolic PLC Activity

Upon the addition of $CaCl_2$ and PIP_2 to the reaction mixture, neutrophil cytosolic PLC was activated and used PIP_2 as substrate to produce inositol phosphates at the rate of 0.56 ± 0.05 nmol PIP_2 hydrolyzed/min/mg protein. Both acetylshikonin and U73122 suppressed the PLC activity in a concentration-dependent manner (Fig. 3). Significant inhibitions were observed at concentrations of acetylshikonin ≥10 μM with an IC_{50} value of 21.4 ± 6.1 μM and at concentrations of U73122 ≥10 μM with an IC_{50} value of 16.1 ± 1.8 μM.

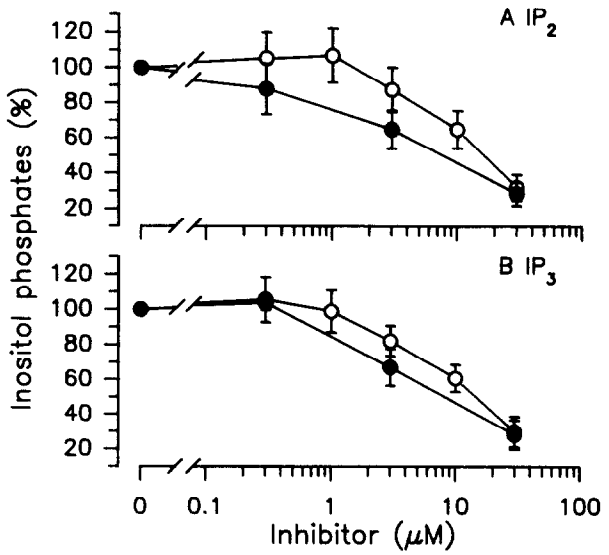

FIG. 2. Effect of acetylshikonin on the formation of inositol phosphates in neutrophils. The conditions of assay are described in Materials and Methods. In the presence of various concentrations of acetylshikonin (○) or U73122 (●), the IP_2 (A) and IP_3 (B) formations in fMLP-stimulated neutrophils are expressed as a percentage of the control (129.3 ± 10.3 vs 77.2 ± 8.2 dpm in the resting cells for IP_2; 330.3 ± 18.7 vs 150.6 ± 13.5 dpm in the resting cells for IP_3) in the absence of inhibitors. Values are means ± SEM of 5–6 independent experiments.

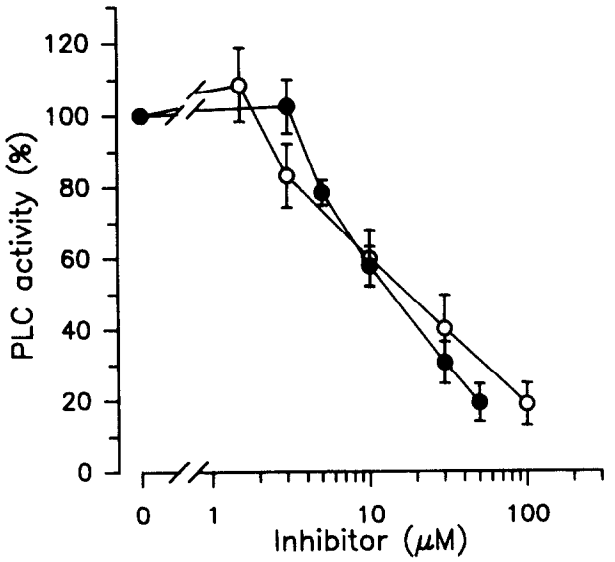

FIG. 3. Effect of acetylshikonin on neutrophil cytosolic PLC activity. The conditions of PLC assay are described in Materials and Methods. In the presence of various concentrations of acetylshikonin (○) or U73122 (●), the cytosolic PLC activity is expressed as a percentage of the control (0.56 nmol PIP_2 hydrolyzed/min/mg protein) in the absence of inhibitors. Values are means ± SEM of 4–5 independent experiments.

Effect of Acetylshikonin on $[Ca^{2+}]_i$

In fura-2-loaded neutrophils, 0.1 μM fMLP induced a rapid and transient elevation of $[Ca^{2+}]_i$ in Ca^{2+}-containing medium. Unexpectedly, there was a significant increase in $[Ca^{2+}]_i$ in cells treated with acetylshikonin (6 μM) in comparison with DMSO, with a slow and long-lasting profile of $[Ca^{2+}]_i$ elevation (Fig. 4A). Higher concentrations of acetylshikonin (>6 μM) induced even more $[Ca^{2+}]_i$ elevation (data not shown). In the presence of 1 mM EDTA to remove the extracellular Ca^{2+}, the acetylshikonin-induced increase in $[Ca^{2+}]_i$ was abolished. Moreover, acetylshikonin produced a concentration-dependent inhibition of $[Ca^{2+}]_i$ elevation in neutrophils stimulated with 0.1 μM fMLP in Ca^{2+}-free medium (Fig. 4B), and the IC_{50} value was ~5.3 ± 0.4 μM.

Mn^{2+}-mediated quenching of cytosolic fura-2 has proved to be a useful model system for investigating Ca^{2+} influx. Like 10 μM CPA, acetylshikonin at concentrations ≥15 μM significantly induced Mn^{2+} influx and subsequently increased the rate of fura-2 quenching (Fig. 5). Both CPA- and acetylshikonin-induced Mn^{2+} influx was abolished in the presence of 5 mM $NiCl_2$.

DISCUSSION

It has been demonstrated that the compound U73122 is capable of inhibiting several PLC-dependent processes in

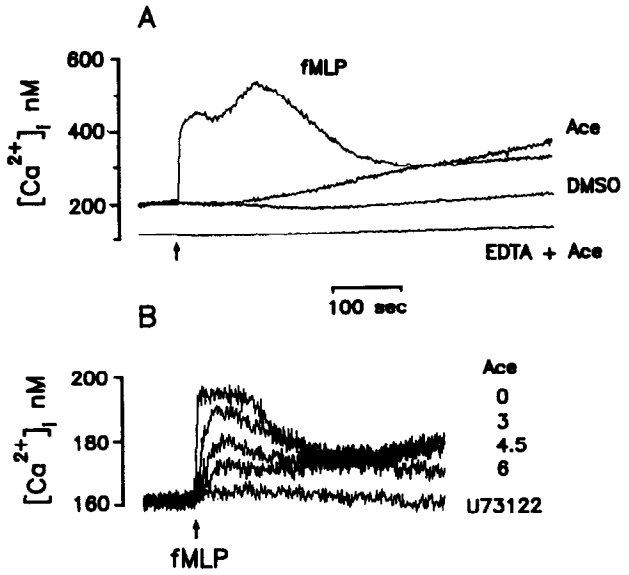

FIG. 4. Effect of acetylshikonin (Ace) on $[Ca^{2+}]_i$ of neutrophils. The $[Ca^{2+}]_i$ of neutrophils was measured as described in Materials and Methods. (A) Fura-2-loaded cells were challenged (arrow) with DMSO, 0.1 μM fMLP, or 6 μM acetylshikonin in the presence of 1 mM Ca^{2+} (the upper three traces), or with 6 μM acetylshikonin in the presence of 1 mM EDTA (the lower trace). (B) Fura-2-loaded cells were incubated with acetylshikonin (0–6 μM) or 1 μM U73122 for 3 min before stimulation with 0.1 μM fMLP (arrow) in the presence of 1 mM EDTA. Data shown are typical of 5 independent experiments.

neutrophils in response to receptor-specific agonists [21]. G proteins play a role in the transduction of signals from certain receptors to PLC-β [5]. The inhibition by acetylshikonin of fMLP-induced inositol phosphate (IP_2 and IP_3) formation in rat neutrophils was probably not a result of either a decrease in the agonist-receptor binding ability or interference with receptor–G protein coupling because the hydrolysis of PIP_2 catalyzed by the cytosol fraction of neutrophils was inhibited by acetylshikonin over the same

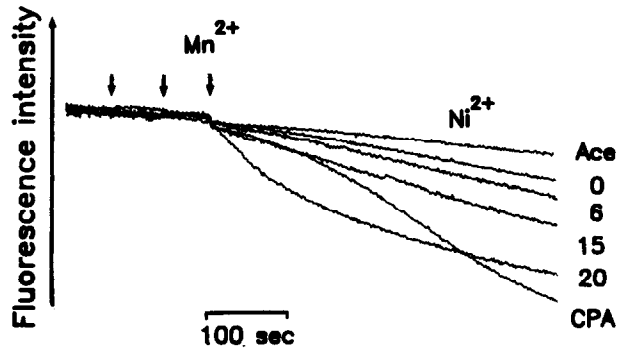

FIG. 5. Effect of acetylshikonin (Ace) on Mn^{2+} influx into neutrophils. The conditions of assay are described in Materials and Methods. Fura-2-loaded cells were preincubated with or without 5 mM $NiCl_2$ (first arrow) for 1 min, then subsequently exposed (second arrow) to acetylshikonin (0–20 μM) or 10 μM CPA, and 0.5 mM $MnCl_2$ (third arrow). Data shown are typical of 4 independent experiments.

range of concentrations as for the inhibition of inositol phosphate formation by intact cells. The observation that acetylshikonin and U73122 produced similar inhibitory profiles suggests that the inhibition by acetylshikonin of inositol phosphate formation in neutrophils was due to the suppression of PLC activity.

There are three phases of $[Ca^{2+}]_i$ changes elicited by activation of receptors coupled to PIP_2 hydrolysis: an initial spike, supported primarily by IP_3-induced release of Ca^{2+} from specific intracellular stores, followed by a plateau phase, which is sustained by Ca^{2+} influx from the extracellular medium [22], and then a Ca^{2+} pump-mediated return to resting Ca^{2+} levels [23]. In rat neutrophils, the first phase of fMLP-induced Ca^{2+} mobilization in the presence of 1 mM extracellular Ca^{2+} is attributable partly to internal Ca^{2+} release and largely to extracellular Ca^{2+} influx. In the absence of extracellular Ca^{2+}, the agonist-induced increase in $[Ca^{2+}]_i$ is the result mainly of the release of Ca^{2+} from IP_3-sensitive intracellular stores; therefore, a small first phase of Ca^{2+} mobilization is observed. The suppression of PLC activity resulted in a decrease in fMLP-induced IP_3 formation and $[Ca^{2+}]_i$ elevation. In support of the latter proposal was the finding that acetylshikonin as well as U73122 effectively suppressed the fMLP-induced $[Ca^{2+}]_i$ elevation in Ca^{2+}-free medium. Both acetylshikonin and U73122 are better able to inhibit $[Ca^{2+}]_i$ elevation than IP_3 formation in neutrophils challenged with fMLP, and these results can be explained by the cooperative characteristics of IP_3-dependent mobilization of Ca^{2+} [24]. However, thus far, we cannot explain the observation that acetylshikonin and U73122 inhibited inositol phosphate formation at similar IC_{50} values, but higher concentrations of acetylshikonin than of U73122 are needed for the inhibition of Ca^{2+} transient.

In this study, we found that acetylshikonin alone slowly increased the $[Ca^{2+}]_i$ in Ca^{2+}-containing medium, as reflected by the increase in fura-2 fluorescence. However, in the absence of extracellular Ca^{2+}, acetylshikonin alone had a negligible effect on the increase in cytosolic free Ca^{2+}. Since acetylshikonin alone increased the $[Ca^{2+}]_i$, experiments evaluating the effect of the novel PLC inhibitor on the suppression of fMLP-induced $[Ca^{2+}]_i$ elevation in the presence of extracellular Ca^{2+} could not be carried out. Like CPA, a Ca^{2+}-ATPase inhibitor [25], acetylshikonin induced Mn^{2+} influx, as measured by the fura-2 fluorescence quenching experiment. The finding that the acetylshikonin-induced response was abolished by Ni^{2+}, a specific Ca^{2+} channel blocker [26], suggests that the influx of Mn^{2+} occurred through Ca^{2+}-permeable channels. Thus, in addition to the inhibition of agonist-induced Ca^{2+} release from internal Ca^{2+} stores, acetylshikonin may also increase Ca^{2+} influx. Although the nature of the Ca^{2+} entry pathway is still unclear, it has been reported that the release of Ca^{2+} from internal Ca^{2+} stores can activate Ca^{2+} entry [4, 27]. The profile of a slow increase in $[Ca^{2+}]_i$ caused by acetylshikonin alone in Ca^{2+}-containing medium is similar to that

caused by the Ca^{2+}-ATPase inhibitor CPA or thapsigargin [25, 28]. The $[Ca^{2+}]_i$ changes caused by Ca^{2+}-ATPase inhibitor are not accompanied by increasing cellular IP_3 levels [20, 28]. In the absence of extracellular Ca^{2+}, CPA still increases $[Ca^{2+}]_i$ [29], whereas acetylshikonin failed to affect $[Ca^{2+}]_i$ under the same conditions. These results indicate that the Ca^{2+} influx induced by depletion of internal Ca^{2+} stores, either through the increase in IP_3 or the blockade of Ca^{2+}-ATPase, did not occur in response to acetylshikonin. Recently, a soluble cellular factor(s), extracted from neutrophils and a lymphocyte cell line, has been reported to directly stimulate Ca^{2+} entry [30, 31]. The exact mechanism underlying the effect of acetylshikonin on Ca^{2+} influx is not understood as yet and needs further investigation.

In conclusion, the present study indicates that the natural anti-inflammatory compound acetylshikonin inhibits phosphoinositide turnover in neutrophils with the same potency as the putative PLC inhibitor U73122. Inhibition of PLC activity contributes to the blockade of the phosphatidylinositol signaling pathway by acetylshikonin.

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