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**U-73122, an aminosteroid phospholipase C inhibitor, may also block Ca\(^{2+}\) influx through phospholipase C-independent mechanism in neutrophil activation**

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**Abstract** 1-(6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122) has been proven to be a useful tool in investigation of phospholipase C (PLC)-coupled signal transduction during cell activation. In the present studies, the inhibition by U-73122 of cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of neutrophils was investigated. U-73122 suppressed the [Ca\(^{2+}\)]\(_i\) elevation of neutrophils suspended in Ca\(^{2+}\)-containing medium challenged by N-formyl-Met-Leu-Phe (fMLP), cyclopiazonic acid (CPA) and ionomycin. The concentrations of U-73122 required for inhibition of CPA- and ionomycin-induced changes with IC\(_{50}\) values 4.06 ± 0.27 µM and 4.04 ± 0.44 µM, respectively, is almost 10-times that required for inhibition of the fMLP-induced response (IC\(_{50}\) value 0.62 ± 0.04 µM). U-73122 also reduced the intracellular Ca\(^{2+}\) mobilization of neutrophils suspended in Ca\(^{2+}\)-free medium stimulated by fMLP and CPA, but not by ionomycin, with IC\(_{50}\) values 0.52 ± 0.02 µM and 6.82 ± 0.74 µM, respectively. 1-(6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U-73343), a close analog of U-73122 that does not inhibit PLC activity, suppressed the [Ca\(^{2+}\)]\(_i\) elevation of neutrophils challenged by fMLP in Ca\(^{2+}\)-containing medium, but not in Ca\(^{2+}\)-free medium, with IC\(_{50}\) value 22.30 ± 1.61 µM. In Mn\(^{2+}\)-quench studies, U-73122 suppressed the Mn\(^{2+}\) influx in CPA-activated neutrophils (IC\(_{50}\) value was 7.16 ± 0.28 µM) as well as in resting neutrophils (IC\(_{50}\) value was 6.72 ± 0.30 µM). U-73343 also suppressed the Mn\(^{2+}\) influx in resting neutrophils in a concentration-dependent manner. These results suggest that the inhibitory effect of U-73122 on [Ca\(^{2+}\)]\(_i\) of activated neutrophils is attributed partly to the suppression of Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores through PLC inhibition, and partly to the blockade, especially at higher concentrations, of Ca\(^{2+}\) entry from the extracellular space through PLC-independent processes.

**Key words** Neutrophil (rat) • Ca\(^{2+}\) influx • U-73122 • Phospholipase C inhibition • U-73343 • Cytosolic Ca\(^{2+}\) concentration • Mn\(^{2+}\)-quench

**Introduction** As a professional phagocyte, neutrophil is one of the principal cellular component of host defense mechanism. For this purpose, neutrophils are equipped for chemotaxis, phagocytosis, degranulation and generation of toxic oxygen metabolites (Baggiolini and Dewald 1985; Borregaard 1988). A common feature of receptor-mediated neutrophil activation consists of a G protein-dependent activation of phospholipase C (PLC) that catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP\(_2\)) to produce inositol trisphosphate (IP\(_3\)) and diacylglycerol (Di Virgilio et al. 1985; Verghese et al. 1986). This receptor-mediated activation is accompanied with the elevation of [Ca\(^{2+}\)]\(_i\) which result from both the release of Ca\(^{2+}\) from internal stores and the influx of Ca\(^{2+}\) across plasma membrane (Pozzan et al. 1983; von Tschcharner et al. 1986). It has been clearly demonstrated that IP\(_3\) mediated the release of intracellular Ca\(^{2+}\) from IP\(_3\)-sensitive Ca\(^{2+}\) stores (Burgess et al. 1984; Andersson et al. 1986), probably calciosomes (Krause et al. 1989). However, the mechanisms of the regulation of Ca\(^{2+}\) influx have remained elusive. In many cell types, the depletion of intracellular Ca\(^{2+}\) stores which results in the Ca\(^{2+}\) entry has been shown as one of the mechanisms of influx control (Putney 1990; Demaurex et al. 1992). The empty state of the stores appears to be...
transduced to the plasma membrane by means of a phosphatase and a diffusible small messenger termed Ca²⁺-influx factor has been proposed (Randriamampita and Tsien 1993; Parekh et al. 1993).

1-[6-[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl] amino][hexyl]-1H-pyrole-2,5-dione (U-73122), an amino- steroid which inhibits PLC-coupled processes in several cell types including platelets, neutrophils, pancreatic acinar cells, insulinoma cells, neuronal cells and thyroid cells (Bleasdale et al. 1990; Smith et al. 1990; Yule and Williams 1992; Chen et al. 1994; Jin et al. 1994; Wang et al. 1994a). U-73122 has proven to be an useful tool for the study of receptor-PLC coupled signal transduction in cellular responses (Smith et al. 1990; Jin et al. 1994). The mechanism of action of U-73122 is unclear. The inhibition of PLC by U-73122 has been proposed to be due to the inhibition of G protein regulation (Thompson et al. 1991) and to the decrease of substrate availability rather than direct enzyme inhibition (Vickers 1993).

In this study, the inhibition by U-73122 of [Ca²⁺]i of activated neutrophils was investigated. Chemo-attractant N-formyl-Met-Leu-Phe (fMLP)-induced [Ca²⁺], elevation, a process known to be mediated by PLC (Di Virgilio et al. 1985), and cyclopiazonic acid (CPA)- and ionomycin-induced non-receptor-coupled responses were also used to characterize the inhibitory effect of U-73122.

**Methods**

Isolation of neutrophils. Rat peripheral neutrophils were isolated and purified by use of a modification (Wang et al. 1994b) of the procedures described previously (Böyum 1968). Briefly, EDTA-mixed fresh blood was obtained from the abdominal aorta of anesthetized rats (Sprague Dawley, 300-350 g). After sedimentation on dextran and centrifugation on Ficoll-Hypaque, the residual erythrocytes were removed by hypotonic lysis. Purified neutrophils containing > 95% viable cells (trypan blue exclusion) were normally resuspended in HBSS containing 4 mM NaHCO₃ and 10 mM Hepes, pH 7.4 to a final concentration of 1 x 10⁷ cells/ml, and kept in ice bath before use.

[Ca²⁺], measurements. Neutrophils (1 x 10⁷ cells/ml) were suspended in Hepes buffer of the following composition: 124 mM NaCl, 4 mM KCl, 0.64 mM Na₂HPO₄, 0.66 mM KH₂PO₄, 15.2 mM NaHCO₃, 5.56 mM dextrose and 10 mM Hepes, pH 7.4, and loaded with 5 μM fura-2-AM at 37°C for 15 min as described previously (Wang et al. 1995). The cells were then diluted 5 fold with same buffer and incubated for an additional 15 min. Cell suspensions were then centrifuged at 900 x g for 10 min at 4°C, and the pellets resuspended in Hepes buffer with 0.05% BSA in the presence or absence of 1 mM CaCl₂. In some experiments, EDTA (1 mM) was present in the reaction mixture in which CaCl₂ was absent. Cell suspensions were placed in a double wavelength fluorescence spectrofluorometer (PTI, DeltaScan 4000) equipped with a thermostated 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 Ca²⁺ concentration was performed by using fura-2-Ca²⁺ standards according to a previously described method (Crynkliewicz et al. 1985).

**Assessment of Mn²⁺ influx.** Entry of Mn²⁺ into cells was measured by the fura-2 fluorescence quenching technique. Fluorescence quenching is triggered by the influx of Mn²⁺ through Ca²⁺-permeable channels (Sage et al. 1989). Fluorescence was monitored in fura-2-loaded cells in the Ca²⁺-containing medium at excitation 360 nm, the isosbestic point where fura-2 is insensitive to changing Ca²⁺, and emission 510 nm. Mn²⁺ (0.5 mM) was added following the preincubation of the neutrophils with 10 μM CPA for 1 min (Demurex et al. 1992). Mn²⁺-mediated quenching of fluorescence was also assessed in fura-2-loaded cells suspended in Ca²⁺-free medium. Fluorescence intensity was declined as Mn²⁺ was added to the resting cells without pretreatment with CPA (Wong et al. 1992). DTPA (2 mM) was added at the end of an experiment, which indicated that less than 5% of the total fluorescence quenched by Mn²⁺ was due to leakage of fura-2.

**Statistical analysis.** Results are expressed as means ± SEM. Newman-Keuls test was used, and differences were considered significant when P < 0.05. Analysis of the regression line test (Tallarida and Murray 1987) was used to calculate IC₅₀ values.

**Materials.** The substances employed were the following: 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino][hexyl]-1H-pyrole-2,5-dione (U-73122), cyclopiazonic acid (CPA) (Biomol Research Lab. Plymouth, Pa, USA); 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino][hexyl]-2-5-pyrrolidinedione (U-73343) (RBI Research Biochemical International, Natick, Mass, USA); ionomycin, N-formyl-Met-Leu-Phe (fMLP), ethylenediaminetetraacetic acid (EDTA), Ficoll-400, sodium diatrizoate, diethylenetriamine pentaacetic acid (DTPA), [N-[2-hydroxylethyl] piperoxane-N'-[2-ethanesulphonic acid] (Hepes), bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo., USA); dextran T-500 (Pharmacia Biotech Asia Pacific Ltd., Taipei, Taiwan, ROC); MnCl₂, CaCl₂, NiCl₂, dimethyl sulfoxide (DMSO) (Merck Taiwan Ltd., Taipei, Taiwan, ROC); Hanks' balanced salt solution (HBSS) (Gibco Lab., Grand Island, USA); fura-2-AM (Molecular Probes Inc., Eugene, USA).

**Results**

Effect of U-73122 on [Ca²⁺], of neutrophils activated in Ca²⁺-containing medium

U-73122 caused a concentration-dependent inhibition of fMLP (0.1 μM)-induced [Ca²⁺], elevation in Ca²⁺ (1 mM)-containing medium (Fig. 1A). Significant inhibition (P < 0.01) of the maximum [Ca²⁺] level was observed at the concentrations of U-73122 ≥ 0.5 μM. The IC₅₀ value was 0.62 ± 0.04 μM (95% CL, 0.50-0.74 μM) for fMLP-induced response (Fig. 1D). U-73122 at higher concentrations range (3-10 μM) also produced a concentration-dependent inhibition of CPA (5 μM)- and ionomycin (0.06 μM)-induced [Ca²⁺], elevation (Fig. 1B,C). Significant inhibitions of the maximum [Ca²⁺], level were observed at the concentrations of U-73122 ≥ 5 μM for CPA (P < 0.01), and 3 μM (P < 0.05) and 5 μM (P < 0.01) for ionomycin. The IC₅₀ values were 4.06 ± 0.27 μM (95% CL, 3.29-4.83 μM) for CPA- and 4.04 ± 0.44 μM (95% CL, 2.81-5.27 μM) for ionomycin-induced responses (Fig. 1D). These IC₅₀ values are higher than that calculated in fMLP reaction.