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Magnolol induces cytosolic-free Ca^{2+} elevation in rat neutrophils primarily via inositol trisphosphate signalling pathway

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Abstract

In the present study, we describe the role of inositol trisphosphate in the signalling pathway that leads to the elevation of cytosolic-free Ca^{2+} in rat neutrophils stimulated with magnolol, a compound isolated from the cortex of *Magnolia officinalis*. Magnolol increased $[Ca^{2+}]_i$, by stimulating Ca^{2+} release from internal stores and Ca^{2+} influx across the plasma membrane, in a concentration-dependent manner. Ni^{2+} and [6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), but not pertussis toxin, inhibited the magnolol-induced Ca^{2+} influx. Measurement of cellular levels of inositol trisphosphate showed a clear increase upon exposure to magnolol. U73122 but not ryanodine suppressed the Ca^{2+} release from internal stores caused by magnolol. Pretreatment of cells with formyl–Met–Leu–Phe (fMLP) or cyclopiazonic acid greatly reduced the $[Ca^{2+}]_i$ changes caused by the subsequent addition of magnolol. Collectively, these findings suggest that a pertussis toxin-insensitive inositol trisphosphate signalling pathway is involved in the magnolol-induced $[Ca^{2+}]_i$ elevation in rat neutrophils. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Magnolol; Neutrophil, rat; Cytosolic-free Ca^{2+}; Inositol trisphosphate

1. Introduction

A variety of neutrophil functions which play a significant role in host defense mechanisms against invading microorganisms are regulated by fluctuations in the cytosolic-free Ca^{2+} concentration. Activation of neutrophils by chemotactic peptides causes Ca^{2+} release from internal stores and Ca^{2+} influx across the plasma membrane. This increase in $[Ca^{2+}]_i$ is mediated by the phosphoinositide cascade (Berridge and Irvine, 1989). Inositol 1,4,5-trisphosphate binds to an intracellular Ca^{2+} channel, the inositol trisphosphate receptor, and is an ubiquitous second messenger responsible for the release of Ca^{2+} from internal stores (Berridge and Irvine, 1989). The inositol trisphosphate-sensitive Ca^{2+} store is suggested to be the endoplasmic reticulum in non-muscle cells (Prentki et al., 1984; Somlyo et al., 1985). Several plant products have been reported to increase the cellular free Ca^{2+} concentration. Thapsigargin, a sesquiterpenoid lactone isolated from *Thapsia garganica* (Umbelliferae) (Patkar et al., 1979), and cyclopiazonic acid, an indole tetramic acid produced by certain fungi of *Aspergillus* and *Penicillium* (Dorner et al., 1983), are inhibitors of Ca^{2+}-ATPase (Goeger et al., 1988; Seidler et al., 1989) and release Ca^{2+} from inositol trisphosphate-sensitive stores without increasing cellular inositol trisphosphate levels (Demaurex et al., 1992). Besides this inositol trisphosphate-sensitive store, an inositol trisphosphate-insensitive type Ca^{2+} store has also been functionally characterized. Mobilization of Ca^{2+} from the inositol trisphosphate-insensitive store requires the concentration of cytosolic-free Ca^{2+} to be in the micromolar range (Bezprozvanny et al., 1991). The release of Ca^{2+} from this type of internal store can be induced by caffeine (Henzi and MacDermott, 1992) and cyclic ADP-ribose (Galione et al., 1991), and either elicited or blocked by ryanodine, a diterpenoid alkaloid insecticide constituent of *Ryania speciosa* (Flacourtiaceae) (Babin et al., 1965), depending on the concentrations used (Meissner, 1986). The two types of Ca^{2+} stores appear to be both functionally and spatially distinct (Tribe et al., 1994); however, Ca^{2+} can be transferred from one store to the other through the cytosol (Golovina and Blaustein, 1997). The activation of

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Ca^{2+} entry across the plasma membrane is a poorly understood process. Although the key signal for triggering Ca^{2+} influx is proposed to be the emptying of the Ca^{2+} store (Montero et al., 1992), a diffusible messenger signalling Ca^{2+} influx was also recently demonstrated in neutrophils (Davies and Hallett, 1995).

Magnolol, a hydroxylated biphenyl compound isolated from the Chinese herb Hou p'u, cortex of *Magnolia officinalis* (Magnoliaceae) (Fujita et al., 1973), has been found to relax rat vascular smooth muscle (Teng et al., 1990), inhibit platelet-activating factor production in human neutrophils (Yamazaki et al., 1994), and scavenge hydroxyl radicals (Fujita and Taira, 1994) in vitro, and suppress plasma extravasation caused by inflammatory mediators (Wang et al., 1993) and protect rat heart against injury during ischemia–reperfusion (Hong et al., 1996) in vivo. In a preliminary study of the effect of magnolol on neutrophil activation, we found that magnolol increased $[Ca^{2+}]_i$ in rat neutrophils. The current study attempted to assess the mechanism underlying this stimulatory effect. The results suggest that the inositol trisphosphate signalling pathway plays a dominant role in the $[Ca^{2+}]_i$ elevation in rat neutrophils following magnolol activation. This mechanism of action is utterly different from that of other plant products described to date.

2. Materials and methods

2.1. Materials

Magnolol was isolated and purified from the cortex of *M. officinalis* as previously described (Fujita et al., 1973). Briefly, the ethanol extract was dried, partitioned between water and $CHCl_3$, and the $CHCl_3$ fraction was then applied to a silica gel column eluted with a *n*-hexane:acetone (8:11, v/v) mixture. Magnolol was identified by TLC and mixed melting point determination by comparison with authentic magnolol (Wako, Osaka, Japan). The purity of magnolol was identified by HPLC with 3D photodiode array (> 99% purity) and by NMR (without impurity signals). All chemicals were purchased from Sigma (St. Louis, MO, USA), except for the following: dextran T-500 (Pharmacia Biotech, Uppsala, Sweden); Hanks' balanced salt solution (Life Technologies Gibco, Gaithersburg, MD, USA); fluo-3 acetoxymethyl ester (fluo-3/AM) and fura-2/AM (Molecular Probes, Eugene, OR, USA); [6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) and cyclopiazonic acid (Biomol Research, Plymouth Meeting, PA, USA); *myo*-[^{3}H]inositol (Amersham, Buckinghamshire, UK); AG 1-X8 resin (formate form) (Bio-Rad, Hercules, CA, USA). Dimethylsulfoxide (DMSO) was the solvent for magnolol and the final volume of DMSO in the reaction mixture was less than 0.5% (v/v).

2.2. 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 (Wang et al., 1995). 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 (HBSS), and kept in an ice bath before use.

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

Neutrophils (1×10^7 cells/ml) were suspended in HEPES buffer composition (mM): 124 NaCl, 4 KCl, 0.64 Na_2HPO_4, 0.66 KH_2PO_4, 15.2 $NaHCO_3$, 5.56 dextrose and 10 HEPES, pH 7.4, and loaded with 5 μM fluo-3/AM at 37°C for 45 min. After being washed, the cells were resuspended in HEPES buffer with 0.05% (w/v) bovine serum albumin. Fluorescence was monitored with a fluorescence spectrophotometer (PTI, Deltascan 4000) at 535 nm with excitation 488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$ where F is the observed fluorescence intensity (Merritt et al., 1990). The values F_{max} and F_{min} were obtained at the end of each experiment by the sequential addition of 0.33% (v/v) Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM (Kao et al., 1989).

2.4. Assessment of Mn^{2+} influx

The entry of Mn^{2+} into cells was measured with the fura-2 fluorescence quenching technique. Fluorescence was monitored in fura-2-loaded cells in Ca^{2+}-containing medium at excitation 360 nm, the isosbestic point where fura-2 was insensitive to changes in $[Ca^{2+}]_i$, and emission 510 nm (Wang et al., 1997). $MnCl_2$ (0.5 mM) was added following the stimulation of the neutrophils with cyclopiazonic acid or magnolol. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of each experiment; less than 5% of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2.

2.5. Determination of inositol phosphates

Neutrophils (3×10^7 cells/ml) were incubated with *myo*-[^{3}H]inositol (83 Ci/mmol) at 37°C for 2 h and then washed (Wang et al., 1994). After stimulation with formyl–Met–Leu–Phe (fMLP) or magnolol, the reaction was stopped by the addition of $CHCl_3/CH_3OH$ (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 a column of AG 1-X8 resin. Inositol mono-, bis- and trisphosphates were eluted sequentially

with 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid as eluent, respectively, and then counted in dpm.

2.6. Statistical analysis

Statistical analyses were performed by using the Bonferroni *t*-test method after analysis of variance. A P value less than 0.05 was considered significant for all tests.

3. Results

3.1. Effect of magnolol on $[Ca^{2+}]_i$ and Mn^{2+} influx

Magnolol (30–90 μM) induced a rapid and concentration-dependent increase of $[Ca^{2+}]_i$ in fluo-3-loaded rat neutrophils in the presence or absence of extracellular Ca^{2+}, with a maximal response being seen within 10 seconds. An initial rapid spike of $[Ca^{2+}]_i$ followed by a plateau phase was observed in magnolol-activated neutrophils in a Ca^{2+}-containing medium (Fig. 1A). In the absence of extracellular Ca^{2+}, magnolol induced a small and transient rise in $[Ca^{2+}]_i$ (Fig. 1B).

The effect of magnolol on Mn^{2+} influx was studied in fura-2-loaded cells. Like cyclopiazonic acid, magnolol

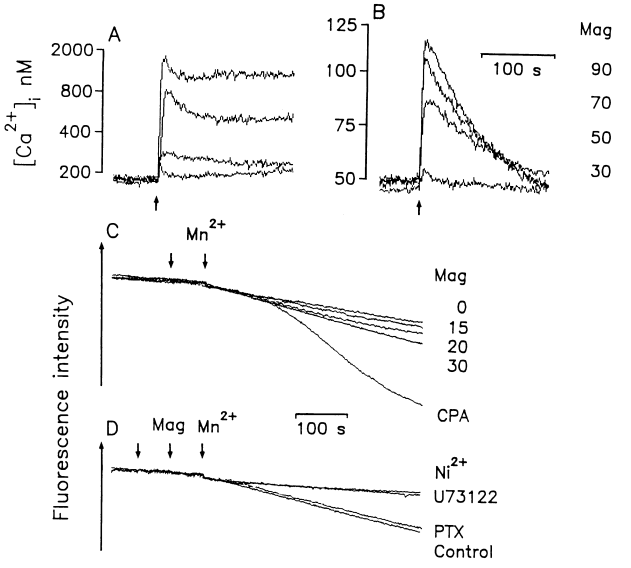

Fig. 1. Magnolol (Mag)-activated $[Ca^{2+}]_i$ elevation and Mn^{2+} influx in rat neutrophils. For the measurement of $[Ca^{2+}]_i$ changes, the fluo-3-loaded cell suspension was stimulated with various concentrations of magnolol (30–90 μM) (arrow) in the presence of (A) 1 mM $CaCl_2$ or (B) 1 mM EDTA. The traces are representative of four to five separate experiments. For the measurement of Mn^{2+} influx, fura-2-loaded rat neutrophils were (C) activated (first arrow) with 10 μM cyclopiazonic acid (CPA) or various concentrations of magnolol (15–30 μM) followed by 0.5 mM $MnCl_2$, or (D) pretreated (first arrow) with DMSO (as control), 5 mM $NiCl_2$ or 1 μM U73122 for 1 min at 37°C, and then activated with 30 μM magnolol. In some experiments, cells were pretreated with 1 μg/ml of pertussis toxin (PTX) for 2 h at 37°C before activation with magnolol. The traces are representative of four to five separate experiments.

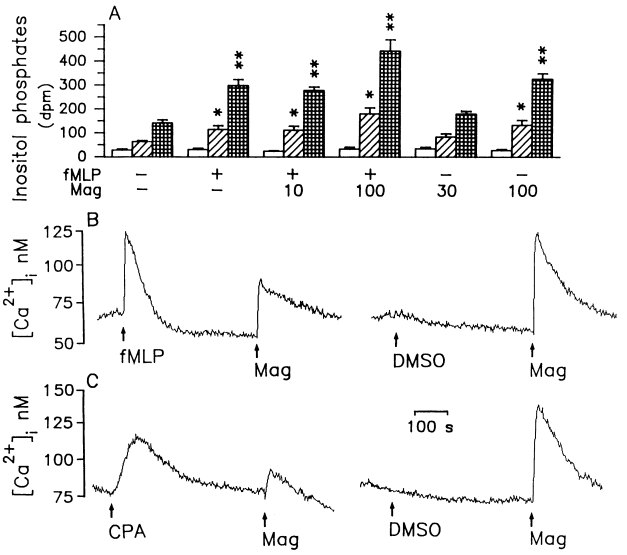

Fig. 2. Magnolol (Mag)-activated cellular inositol phosphate formation and the influence of fMLP and cyclopiazonic acid (CPA) on magnolol-induced $[Ca^{2+}]_i$ changes. (A) For the measurement of inositol phosphate formation, *myo*-[^{3}H]inositol-loaded cells were pretreated with DMSO or magnolol (10 and 100 μM) for 3 min at 37°C before stimulation with 0.3 μM fMLP for 10 s. In some experiments, cells were pretreated with DMSO for 3 min at 37°C before stimulation with magnolol (30 and 100 μM) for 10 s. Reaction was stopped by the addition of $CHCl_3/CH_3OH$ (1/1, v/v) mixture. Inositol phosphates were separated on an AG 1-X8 column, inositol mono- (blank columns), bis- (hatched columns) and trisphosphates (cross-hatched columns) were eluted, and the radioactivity was counted. Values are expressed as means ± S.E.M. of five separate experiments. $*P < 0.05$, $**P < 0.01$ compared to the corresponding values in the first group. For the measurement of $[Ca^{2+}]_i$, fluo-3-loaded cell suspensions were stimulated with (B) 0.1 μM fMLP or DMSO, (C) 5 μM cyclopiazonic acid or DMSO followed by 70 μM magnolol in the presence of 1 mM EDTA. The traces are representative of four separate experiments.

concentration dependently stimulated Mn^{2+} influx (the relative fluorescence intensity changes were 0.43 ± 0.1 for 30 μM magnolol vs. 0.31 ± 0.01 for vehicle control, $P < 0.05$) (Fig. 1C). Magnolol > 30 μM should not be used in Mn^{2+} quenching tests because at higher concentrations (≥ 50 μM) it affects the fluorescence of fura-2 at 360/510 nm. Magnolol-induced Mn^{2+} influx was abolished by Ni^{2+} and U73122 (Fig. 1D). Treatment of cells with 1 μg/ml of pertussis toxin at 37°C for 2 h, conditions which abolished the fMLP-induced $[Ca^{2+}]_i$ changes (data not shown), was found to have little effect on the magnolol-induced response.

3.2. Effect of magnolol on inositol phosphate formation

After addition of 0.3 μM fMLP to *myo*-[^{3}H]inositol-loaded neutrophils for 10 s at 37°C, a significant increase in the cellular formation of inositol bis- and trisphosphates was observed in comparison with their basal levels. Pretreatment of cells with 100 μM magnolol for 3 min at 37°C followed by fMLP further increased the cellular inositol bis- and trisphosphate levels ($P < 0.05$ and $P < 0.01$, respectively, in comparison with the fMLP alone-

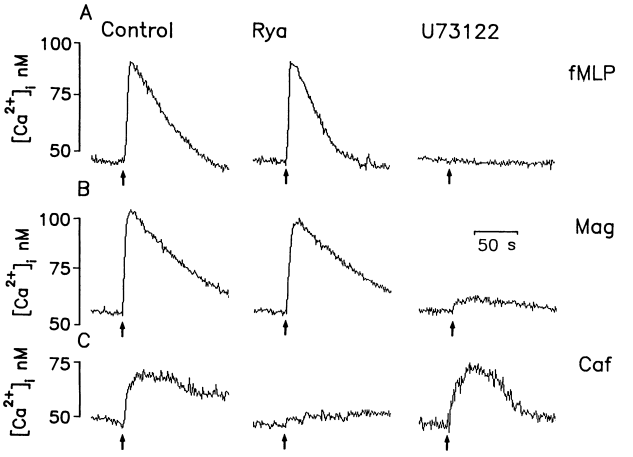

Fig. 3. Effect of ryanodine (Rya) and U73122 on the changes in $[Ca^{2+}]_i$ evoked by magnolol (Mag). Pretreatment of fluo-3-loaded neutrophil suspensions in the presence of 1 mM EDTA with DMSO (as control), 200 μM ryanodine or 3 μM U73122 for 1 min before stimulation (arrow) with (A) 0.1 μM fMLP, (B) 70 μM magnolol or (C) 10 mM caffeine (Caf). The traces are representative of four to five separate experiments.

treated values) (Fig. 2A). Moreover, addition of 100 μM magnolol alone to *myo*-[^{3}H]inositol-loaded neutrophils at 37°C for 10 s also significantly increased the formation of inositol bis- and trisphosphates.

3.3. Effect of fMLP and cyclopiazonic acid on magnolol-induced $[Ca^{2+}]_i$ changes

Addition of fMLP or cyclopiazonic acid to fluo-3-loaded neutrophils in a Ca^{2+}-free medium reduced the $[Ca^{2+}]_i$ changes caused by the subsequent addition of cyclopiazonic acid or fMLP, respectively (data not shown). The magnolol-induced $[Ca^{2+}]_i$ changes were significantly reduced by pretreatment of cells with fMLP or cyclopiazonic acid (41.7 ± 4.0 and 65.8 ± 2.5% inhibition, respectively, both $P < 0.01$) (Fig. 2B,C).

3.4. Effect of ryanodine and U73122 on magnolol-induced $[Ca^{2+}]_i$ changes

In fluo-3-loaded cells, the magnolol- and fMLP-induced $[Ca^{2+}]_i$ elevation was greatly reduced (5.2 ± 1.2 vs. 54.0 ± 6.4 nM cytosolic-free Ca^{2+} changes, $P < 0.01$) or abolished, respectively, by pretreatment with 3 μM U73122 (Fig. 3A,B). However, ryanodine affected neither magnolol- nor fMLP-induced responses. Under the same conditions, ryanodine but not U73122 effectively attenuated the caffeine-induced $[Ca^{2+}]_i$ changes (Fig. 3C).

4. Discussion

Magnolol evoked an increase in $[Ca^{2+}]_i$ in rat neutrophils. The profiles of the magnolol-induced responses were very similar to those for the chemotactic tripeptide fMLP but not to those for the Ca^{2+}-ATPase inhibitor cyclopiazonic acid and the Ca^{2+}-ionophore ionomycin, which showed a much slower increase in $[Ca^{2+}]_i$ (Wang, 1996). The rise in $[Ca^{2+}]_i$ caused by fMLP results from both Ca^{2+} release from internal stores and Ca^{2+} influx from the extracellular environment (Meldolesi et al., 1991). Therefore, we investigated the effect of magnolol on Ca^{2+} influx in neutrophils.

Mn^{2+}-mediated quenching of cytosolic fura-2 has proved to be a useful model system for investigating Ca^{2+} influx. Mn^{2+} permeates through the Ca^{2+} influx pathway in neutrophils activated by fMLP and cyclopiazonic acid (Demaurex et al., 1992) and subsequently quenches the fluorescence signal by its high-affinity binding of fura-2. Ni^{2+}, a specific Ca^{2+} channel blocker (Shibuya and Douglas, 1992), abolished the magnolol-induced response, suggesting that the influx of Mn^{2+} occurs through Ca^{2+}-permeable channels and magnolol evokes Ca^{2+} influx in neutrophils. Although the nature of the Ca^{2+} entry pathway is unclear as yet, it is generally accepted that the release of Ca^{2+} from internal Ca^{2+} stores can activate Ca^{2+} entry (Berridge and Irvine, 1989; Putney et al., 1989). Ligand binding to the fMLP receptor activates a pertussis toxin-sensitive G-protein-coupled phospholipase C (Mullmann et al., 1993), and the inositol trisphosphate produced mediates the release of Ca^{2+} from specific internal stores (Meldolesi et al., 1991) and Ca^{2+} entry. It is plausible that the decrease in phospholipase C activity blocked both inositol trisphosphate formation and Ca^{2+} entry. Magnolol-induced Mn^{2+} influx was not affected by pertussis toxin but was abolished by U73122, a phospholipase C inhibitor (Smith et al., 1990), which suggests that a pertussis toxin-insensitive inositol trisphosphate signalling pathway is probably involved in the magnolol-induced $[Ca^{2+}]_i$ elevation. That U73122 inhibited magnolol-induced $[Ca^{2+}]_i$ changes in the absence of extracellular Ca^{2+} provides further pharmacological evidence for the role of a inositol trisphosphate signalling pathway in magnolol-induced responses and rules out the possibility that magnolol acts as a Ca^{2+}-ionophore because U73122 failed to affect the ionomycin-induced response (Wang, 1996). The observation that magnolol increased the cellular formation of inositol trisphosphates provides direct evidence to support the proposal. Therefore, the mechanism of action of magnolol is different from that of cyclopiazonic acid because the Ca^{2+}-ATPase inhibitor increases $[Ca^{2+}]_i$ without increasing cellular inositol trisphosphate levels (Takemura et al., 1989; Demaurex et al., 1992).

Since fMLP and cyclopiazonic acid mobilize Ca^{2+} from the same inositol trisphosphate-sensitive stores, addition of fMLP or cyclopiazonic acid to neutrophil suspensions in a Ca^{2+}-free medium should greatly reduce the $[Ca^{2+}]_i$ changes caused by the subsequent addition of cyclopiazonic acid or fMLP, respectively, presumably by depleting the inositol trisphosphate-sensitive Ca^{2+} stores

(Demaurex et al., 1992). The finding that the magnolol-induced $[Ca^{2+}]_i$ changes was significantly reduced by pretreatment of cells with fMLP or cyclopiazonic acid reinforces the proposal that magnolol removed Ca^{2+} from the inositol trisphosphate-sensitive Ca^{2+} store.

It is now generally accepted that the inositol trisphosphate receptor resides on specialized regions of the endoplasmic reticulum, which in fact constitute inositol trisphosphate-sensitive Ca^{2+} stores (Pozzan et al., 1994). Inositol trisphosphate-induced Ca^{2+} release from internal stores is assumed to be a quantal process (Missiaen et al., 1997). Ca^{2+} stores in the endoplasmic reticulum are organized into small, spatially distinct compartments that function as discrete units (Golovina and Blaustein, 1997). So far, two types of endoplasmic reticulum Ca^{2+} stores (inositol trisphosphate-sensitive and -insensitive) have been functionally characterized. Cyclopiazonic acid and caffeine release Ca^{2+} from different, spatially separate compartments, suggesting that the cells can generate spatially and temporally distinct Ca^{2+} signals to control individual Ca^{2+}-dependent processes (Golovina and Blaustein, 1997). Neutrophils have recently been reported to contain ryanodine-sensitive Ca^{2+} stores (Elferink and De Koster, 1995). We, therefore, determined the contribution of inositol trisphosphate-insensitive type Ca^{2+} stores to the magnolol-induced responses. Ryanodine did not affect the magnolol-induced $[Ca^{2+}]_i$ changes, suggesting that inositol trisphosphate-insensitive type Ca^{2+} stores may not be involved. A relatively larger inositol trisphosphate (cyclopiazonic acid)-sensitive Ca^{2+} store observed in rat neutrophils is consistent with the observations that cyclopiazonic acid and thapsigargin evoke larger cytosolic-free Ca^{2+} transients than does caffeine in vascular smooth muscle cells and astrocytes (Tribe et al., 1994; Golovina et al., 1996).

The data of the present study indicate that a plant product, magnolol, induced $[Ca^{2+}]_i$ elevation in rat neutrophils, probably through a pertussis toxin-insensitive inositol trisphosphate signalling pathway which results in Ca^{2+} release from inositol trisphosphate-sensitive Ca^{2+} stores and then Ca^{2+} entry from the extracellular medium. This mechanism of action is utterly different from that of other plant products described previously. The cellular inositol trisphosphate levels could be increased either by the activation of phospholipase C activity or by the suppression of inositol trisphosphate degradation. However, the rapid Ca^{2+} spike upon exposure to magnolol makes the former more likely. The mechanism of action of magnolol on the formation of inositol trisphosphate needs further investigation.

Acknowledgements

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