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Requirement for both receptor-operated and store-operated calcium entry in *N*-formyl-methionine-leucine-phenylalanine-induced neutrophil polarization

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ABSTRACT

Tissue penetration of neutrophils is a key process in many inflammatory diseases. In response to inflammatory stimuli such as *N*-formyl-methionine-leucine-phenylalanine (fMLP), neutrophils polarize and migrate towards the chemotactic gradient of the stimulus. Elevated intracellular Ca^{2+} concentration is known to play a critical role in neutrophil polarization and migration; however, the exact mechanism remains elusive. Here, we demonstrated that fMLP stimulation caused not only store-operated calcium entry (SOCE), but also receptor-operated calcium entry (ROCE) in neutrophils by using both pharmacological and neutralizing monoclonal antibody approaches. We also investigated neither Rac2 nor Cdc42 activation could take place if either SOCE or ROCE was inhibited. This study thus provides the first evidence for coordination of Ca^{2+} influx by SOCE and ROCE to regulate neutrophil polarization.

1. Introduction

Neutrophils constitute a key component of the innate immune system and represent 50–60% of the total leukocytes in circulation. Accumulation of neutrophils in damaged tissues is a pathogenic signature of many inflammatory diseases. Polarization and migration of neutrophils toward the chemotactic gradient produced by an inflammatory stimulus is an essential part of this process. In the resting state, neutrophils adhere poorly and exhibit a spherical shape. However, in response to an inflammatory stimulus, neutrophils first become polarized, exhibiting a leading edge (pseudopod) at one end and a tail (uropod) at the other end, and then migrate towards the source of the stimulus.

Elevated cytoplasmic Ca^{2+} concentrations in neutrophils play a critical role in mediating the pro-polarizing and pro-motility effects induced by the inflammatory stimulus [1,2]. In neutrophils, *N*-formyl-methionine-leucine-phenylalanine (fMLP) stimulates activation of phospholipase C (PLC) via the fMLP receptor, leading to breakdown of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) [3,4]. IP_3 binds to its receptor on the endoplasmic reticulum (ER) and causes rapid Ca^{2+} release from ER stores. When the Ca^{2+} stored in the ER is depleted, it then activates the extracellular Ca^{2+} influx across the plasma membrane by store-operated calcium entry (SOCE) mechanisms. SOCE was originally proposed three decades ago by Putney and colleagues [5]. However, despite significant progresses, it is still not clear if SOCE is the sole mechanism responsible for the sustained phase of Ca^{2+} influx in neutrophils following inflammatory-mediator stimulation. Indeed, recent studies in a number of cell types have suggested the existence of a non-store operated or non-capacitative Ca^{2+}-entry pathway [6–8], referred to as receptor-operated calcium entry (ROCE), which may involve the DAG-activated protein kinase C (PKC) pathway [9,10]. These findings suggest that inflammatory-mediator-stimulated Ca^{2+} influx in neutrophils has two sources, SOCE and ROCE. Rho-family GTPases, including Rac and Cdc42, are small GTP-binding proteins with key roles in cytoskeletal rearrangement, cell adhesion and polarity. The present study investigated the involvements of SOCE and ROCE in fMLP-induced Ca^{2+} influx in human neutrophils, and compared their specific contributions to Rac2 and Cdc42 activation and neutrophil polarization in response to fMLP.

2. Materials and methods

2.1. Reagents and antibodies

fMLP from the bacterial formylpeptide, HEPES, the PLC inhibitor U73122, the PKC inhibitor calphostin C, $GdCl_3$, $CaCl_2$, $SrCl_2$ and anti-stromal interacting protein 1 (STIM1) antibody (N-terminal) were purchased from Sigma (Saint Louis, MO). Dextran T500 was from Amersham Biosciences (Piscataway, NJ), and Fluo-4 acetoxymethyl ester (Fluo-4/AM) and endotoxin-free HBSS (pH 7.4) without calcium and magnesium were from Invitrogen (Grand Island,

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NY). All protease inhibitors were obtained from Roche (Basel, Switzerland).

2.2. Neutrophil isolation

Human neutrophils were isolated from heparin anti-coagulated venous blood [11] from healthy consenting adults according to the appropriate ethical guidelines at Southern Medical University. Briefly, erythrocytes were removed by Dextran sedimentation (6% dextran/0.9% NaCl) followed by two rounds of hypotonic lysis using sterile ddH_2O. With the exception of dextran sedimentation, the entire isolation procedure was performed at 4 °C. Purified neutrophils were re-suspended in D-Hanks without $CaCl_2$ at a concentration of 1.0×10^6 cells/ml prior to use. More than 95% of the cells isolated were neutrophils, as assessed by Wright–Giemsa staining. Viability, determined by trypan blue exclusion, was >98%.

2.3. Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} was monitored using the Ca^{2+}-sensitive fluorescent indicator, Fluo-4/AM under an inverted laser scanning confocal microscope (Olympus, FV1000-IX71, Japan). Cells were loaded with 2 μM Fluo-4/AM at 37 °C for 30 min in the dark in Hanks' buffered salt solution (pH 7.4). Ca^{2+}-free buffer solution was prepared by omitting $CaCl_2$ and adding 0.3 mM EGTA. In certain experiments, neutrophils were maintained in Ca^{2+}-free buffer for 30 min before Ca^{2+} image recording. The green fluorescence of Fluo-4 was excited by a 10-mW multi-tune argon laser at 488 nm, and the emitted fluorescence was recorded through a 525-nm channel. For imaging using Fluo-4, Ca^{2+} changes are presented as ΔF/F0 ratios after background subtraction, where F0 was the baseline of fluorescence signal intensity calculated by averaging the results for 10 frames before stimulus application and ΔF was the change of fluorescence signal intensity after F0 was subtracted.

2.4. Reversible electroporation

The neutrophil suspension was transferred to an electroporation chamber containing 2.5 μg/ml anti-STIM1 antibody, and the antibodies were transjected as described previously [12,13]. Reversible electropermeabilization was performed at 4 kV/cm at a setting of 25 μF capacitance and was achieved by seven pulses using a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). Following electroporation, cells were incubated with anti-STIM1 antibody for an additional 60 min at 37 °C and resuspended in HBSS prior to the experiments. This procedure was shown to have no effect on cell function as described previously [14,15].

2.5. Immunoprecipitation and western blotting

The antibody electrotransjection efficacy was determined by immunoprecipitation and western blotting, as described previously [15]. Briefly, aliquots of neutrophil lysates (previously electrotransjected with anti-STIM1 antibodies) were incubated with 25 μl of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and separated proteins were transferred onto nitrocellulose membranes. After blocking residual protein binding sites, immunodetection of STIM1 was achieved using anti-STIM1 antibody.

2.6. Cell polarity and chemotaxis in Zigmond chamber

Neutrophil polarization was quantified in Zigmond chambers (Neuro probe, MD), as described previously [16]. A total of 1×10^6 neutrophils were allowed to adhere to coverslips for 5 min at room temperature. The coverslips were then immediately inverted onto the chamber, which was assembled loosely. One channel of the chamber was filled with HBSS (vehicle) and the other with 100 nM fMLP. Digital images of the cells were taken after exposure to the chemoattractant for 15 min, using an inverted microscope with a 20× objective (Olympus IX-71). Polarized cells were quantified by counting the total number of cells, the cells that were polarized in any direction, and the cells polarized towards the direction of the gradient [17]. Cells with a leading edge and a trailing tail were scored as polarized. Analysis was performed for at least three experiments, with no fewer than 150 cells in five random fields counted for each set of conditions. The mean and SEM of each sample was used for statistical analysis.

2.7. PBD-GST pull-down assay

Freshly isolated neutrophils (1×10^7/ml) were treated as described above. Cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C, then 5% of each supernatant was removed for detection of total (both GTP- and GDP-bound) Rho-GTPases. The remaining supernatants were incubated with 20 μg PAK-GST Protein Beads (Cytoskeleton, Denver) for 1 h at 4 °C. The beads were eluted in 1 × sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue dye in 50 mM Tris–HCl, pH 6.8). Active Rac2 or Cdc42 were detected by western blot analysis using anti-Rac2 or anti-Cdc42 antibodies.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Results were expressed as mean ± SEM. Data were analyzed by *t*-tests or one-way analysis of variance. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. fMLP triggers both ROCE-mediated and SOCE-mediated calcium entry in neutrophils

The mechanisms responsible for Ca^{2+} store-depletion and influx in neutrophils were studied by treating human neutrophils isolated from the blood of healthy donors with fMLP under Ca^{2+}-free conditions (in the presence of 0.3 mM EGTA). SOCE can be inhibited by low concentrations of Gd^{3+}[18], and we therefore tested the effects of Gd^{3+} on Ca^{2+} influx in human neutrophils after the depletion of Ca^{2+} stores by fMLP. Only a very small response was detected in the absence of fMLP stimulation (Fig. 1A). A low $GdCl_3$ concentration (2 μM) had little effect on the Ca^{2+} release induced by fMLP, but it partially inhibited the subsequent Ca^{2+} influx by approximately 80% following the addition of $CaCl_2$ (Fig. 1A and B). STIM1 is a known ER Ca^{2+}-binding protein and a primary regulator of SOCE [19,20]. We therefore tested if STIM1 blockade could inhibit fMLP-induced Ca^{2+} influx by electrotransjection with an anti-STIM1 neutralizing antibody specifically recognizing the N-terminus of STIM1, which includes the Ca^{2+}-binding EF-hand region [21]. Similarly, we observed that the anti-STIM1 antibody had no effect on fMLP-induced Ca^{2+} store release, and it was unable to entirely abolish the Ca^{2+} influx (Fig. 1C and D). To confirm the specificity of the electrotransjection, non-permeabilized and permeabilized cells were compared by immunoprecipitation. As shown in Fig. 1E, STIM1 was clearly detected in electropermeabilized cells, whereas much lower levels were detected in non-permeabilized cells. We therefore conclude that, in addition to SOCE,

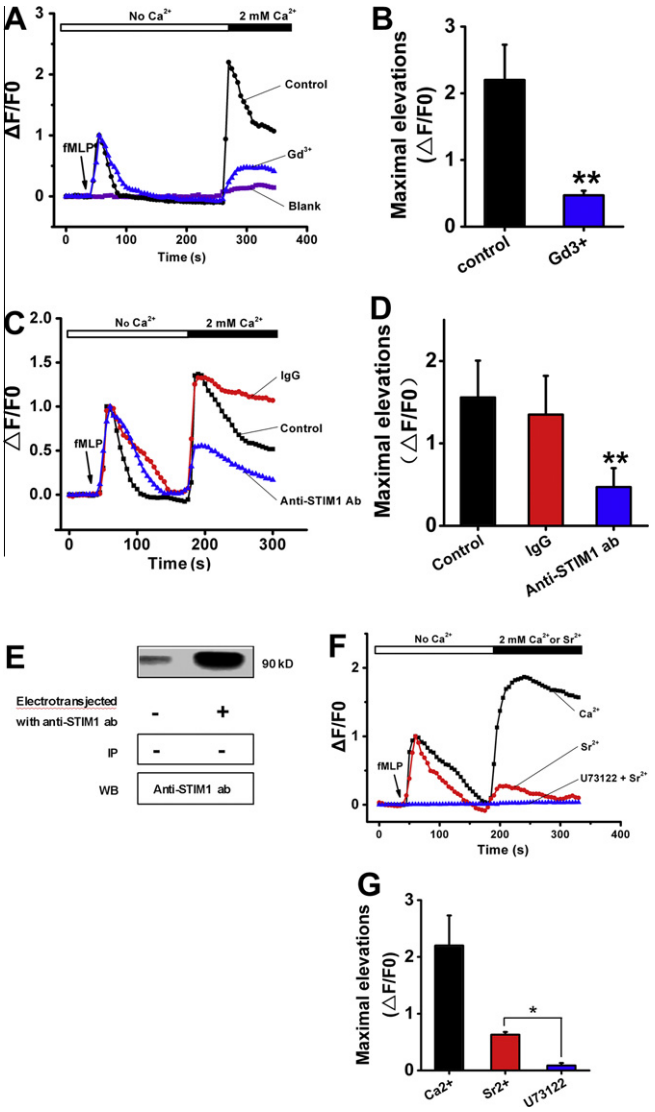

Fig. 1. fMLP induces Both ROCE-mediated and SOCE-mediated calcium entry in neutrophils. Cytoplasmic Ca^{2+} was assessed in Fluo-4/AM-loaded human neutrophils. Ca^{2+} (2 mM) was added to the extracellular medium as indicated and the influx of divalent cations was monitored by laser scanning confocal microscopy. Each point shows the mean of at least eight observations. Corresponding bar charts show the maximal elevation of the curve in Fluo-4/AM imaging after addition of Ca^{2+}. (A and B) Cells were incubated with or without 2 μM Gd^{3+} after stimulation with 100 nM fMLP for 1 min. (C and D) Cells were stimulated with 100 nM fMLP for 1 min after treatment with 1:200 anti-STIM1 antibody (2.5 μg/ml) or rabbit IgG. (E) Both resting and electropermeabilized neutrophils were incubated in the presence of 2.5 μg/ml anti-STIM1 antibody for 60 min and whole-cell lysates were immunoprecipitated in the absence of antibodies but with the addition of protein A-agarose, and immunoprecipitated proteins were analyzed by western blotting using anti-STIM1 antibody. (F and G) 2 mM Ca^{2+} or Sr^{2+} was added to the extracellular medium as indicated after stimulation by fMLP for 1 min and the influx of divalent cations was monitored. These results are representative of three independent experiments. Bars are mean ± SEM. $^{**}P < 0.01$.

fMLP triggered a non-SOCE mechanism that also participated in the fMLP-stimulated Ca^{2+} influx.

ROCE, but not SOCE, is known to be permeable to Sr^{2+}, and Sr^{2+} entry is cell surface-receptor operated in human neutrophils [6]. We therefore investigated the ability of fMLP to induce Sr^{2+} influx in human neutrophils. As shown in (Fig. 1F and G). Sr^{2+} influx in response to fMLP stimulation produced an obvious fluorescence ratio (ΔF/F0) that was approximately 25% of the fMLP-induced Ca^{2+} influx. These data suggest ROCE-mediated Ca^{2+} influx may be included in the residual Ca^{2+} influx. In addition, it is known that all fMLP-induced Ca^{2+} entry is mediated by PLC, and inhibition of PLC should thus completely eliminate the effect of fMLP on Ca^{2+} entry. To support of this hypothesis, we observed that the entire Ca^{2+} influx, including the Gd^{3+}-sensitive Ca^{2+} entry (SOCE) and the residual Ca^{2+} influx, possibly via Sr^{2+} (ROCE), could be completely inhibited by the PLC inhibitor U73122 in human platelets and neutrophils (Fig. 1F and G).

3.2. fMLP-induced neutrophil polarization requires both SOCE and ROCE pathways

When neutrophils were challenged with fMLP, their morphology became polarized with a leading edge (pseudopod) and a tail (uropod). As expected, inhibition of PLC by U73122 diminished the ability of fMLP stimulation to induce neutrophil polarization (Fig. 2A and B). These results indicate that Ca^{2+} influx is needed for neutrophil polarization. To confirm the role of SOCE in fMLP-induced neutrophil polarization, primary human neutrophils were pretreated with or without Gd^{3+} for 20 min prior to the addition of fMLP. In the absence of Gd^{3+}, approximately 80% of neutrophils exhibited a spread and polarized morphology after fMLP stimulation. However, only about 5% of neutrophils showed a similar polarized morphology in the presence of Gd^{3+} (Fig. 2C and D). To further confirm this finding, anti-STIM1 neutralizing antibody introduced into the cells by electroinjection reduced fMLP-induced neutrophil polarization to about 6% (Fig. 2E and F). These results indicate that SOCE is indispensable for fMLP-induced neutrophil polarization.

We also investigated the effects of the ROCE pathway on fMLP-induced neutrophil polarization. We proposed that ROCE uses the PLC > DAG > PKC activation pathway to promote Ca^{2+} influx and neutrophil polarization [3,9,10]. We therefore tested the effects of the specific PKC inhibitor calphostin C on fMLP-stimulated neutrophil polarization. Calphostin C had no effect on fMLP-induced Ca^{2+} store release (the first peaks of fluorescence signal intensity curves were similar), but it almost completely blocked Sr^{2+} influx (Fig. 3A and B). The PKC pathway thus appeared to be involved in Ca^{2+} entry via non-store-independent influx, but not via Ca^{2+}-store release, consistent with previous reports [22]. Moreover, in the presence of calphostin C, the ratio of fMLP-induced neutrophil polarization was reduced from 75% to only 13% (Fig. 3C and D). These data suggest that ROCE also plays a crucial role in fMLP-induced neutrophil polarization.

Finally, to further establish the importance of both ROCE and SOCE in mediating fMLP-induced neutrophil polarization, we tested the effects of fMLP on activation of their downstream effectors of cell polarization, Rac2 and Cdc42. The fMLP-triggered Rac2 and Cdc42 activities were significantly inhibited when neutrophils were pretreated with either Gd^{3+} or the PKC inhibitor calphostin C (Fig. 4). However, when the cells were pretreated with the PLC inhibitor U73122, which can completely eliminate the effect of fMLP on Ca^{2+} entry, no further inhibition of Rac2 or Cdc42 activity was observed (Fig. 4). These data suggest that both SOCE and ROCE mediate fMLP-stimulated Rac2 and Cdc42 activation. Overall, the above results confirm that the two types of calcium entry, ROCE and SOCE, are both required for neutrophil polarization in response to fMLP stimulation.

4. Discussion

The present study investigated the contributions of two routes of Ca^{2+} influx to human neutrophil polarization in response to key inflammatory agents. fMLP-induced Ca^{2+} entry was shown to be controlled by at least two signaling mechanisms, SOCE and ROCE. More importantly, both SOCE and ROCE contributed to

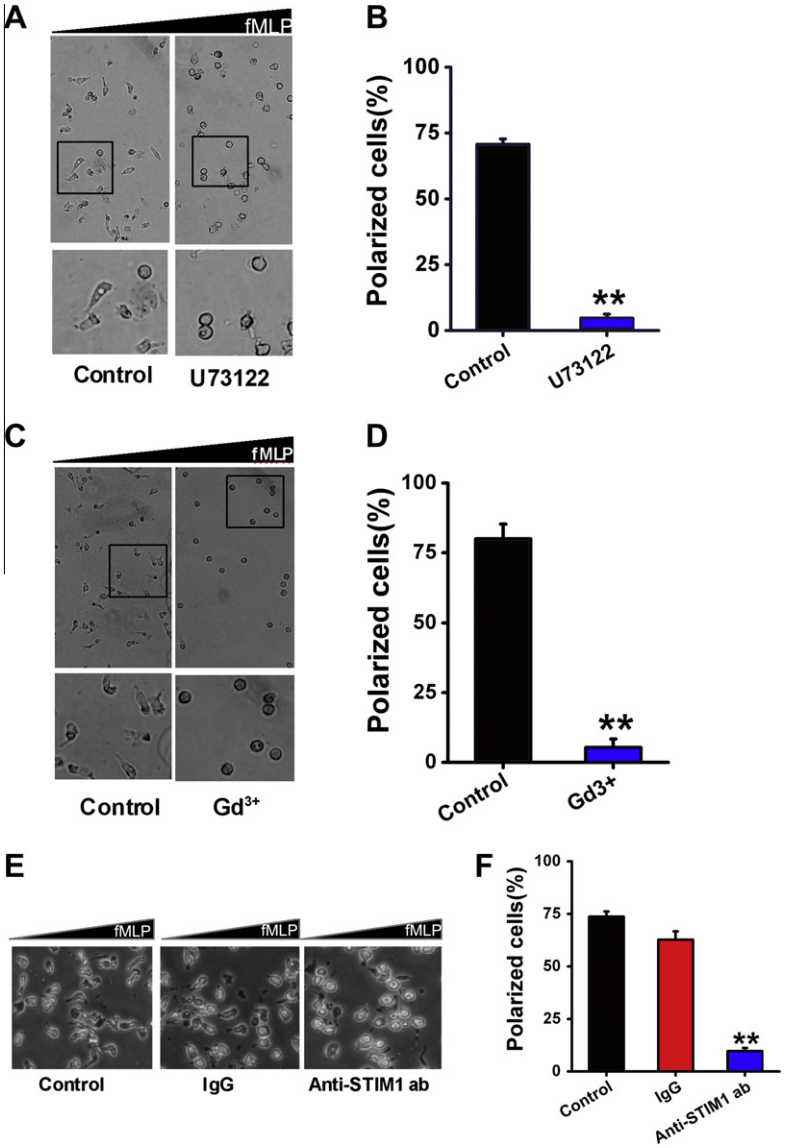

Fig. 2. Involvement of SOCE in fMLP-induced neutrophil polarization. Purified human neutrophils were placed in Zigmond chambers containing control buffer in the right groove and 100 nM fMLP in the left groove. Time-lapse microscopy was used to record the cell morphology on the bridge at 30-s intervals for 15 min. The proportion of neutrophils polarized after 15 min exposure in fMLP in a Zigmond chamber was analyzed. Neutrophils were preincubated with (A and B) 20 μM U73122, the PLC inhibitor, or (C and D) Gd^{3+} for 20 min. (E and F) Electropermeabilized neutrophils were incubated in the presence of 2.5 μg/ml IgG or 2.5 μg/ml anti-STIM1 antibody for 60 min. Corresponding bar charts show the proportion of polarized neutrophils. Similar results were obtained from three independent experiments. Bars are mean ± SEM. $^{**}P < 0.01$.

fMLP-induced neutrophil polarization, which plays a critical role in inflammatory responses and diseases. We also found that both Ca^{2+} influx pathways were required for fMLP-induced Rac2 and Cdc42 activation. These results strongly suggest that SOCE and ROCE work together to control neutrophil polarization.

Although rises in Ca^{2+} are known to be vital for the polarization and activation of neutrophils in response to pro-inflammatory agonists, the source of the intracellular Ca^{2+} during these processes remains debatable. Over the past decade, the molecular components of SOCE have begun to be identified and characterized [5,23]; however, there has been a lack of definitive evidence regarding whether or not SOCE could fully account for the sustained phase of Ca^{2+} influx following neutrophil stimulation by inflammatory mediators. Indeed, accumulating evidence in many cell types has implied the existence of a non-store operated or non-capacitative Ca^{2+} entry pathway (ROCE), in addition to SOCE's contribution. The role of receptor-operated cation channels is particularly well established in smooth muscle cells [7], but a few studies have reported that receptor-operated influx pathways may also be important in neutrophil activation [6,8]. The current results confirmed that the fMLP-induced Ca^{2+} influx was composed of not only SOCE but also ROCE, and could thus be only partially blocked by SOCE inhibitors, Gd^{3+} or STIM1 antibody. In addition, the membrane permeability for Sr^{2+} entry was increased, suggesting the presence of ROCE in fMLP-induced neutrophil activation.

Intracellular Ca^{2+} mobilization is known to play an important role in the control of cell polarization. Because two routes of Ca^{2+} influx appear to be activated following fMLP stimulation, we propose that both routes are required for fMLP-induced neutrophil polarization; indeed, fMLP-induced cell polarization was not observed if either SOCE or ROCE was inhibited. These findings suggest that different Ca^{2+} channel openings and inhomogeneous Ca^{2+} concentration distributions may cause neutrophil polarization. Cell polarization is defined by the development of morphological changes to create pseudopod and retraction of uropod. Pseudopdia and uropodia are regulated through Rac- and Rho-mediated

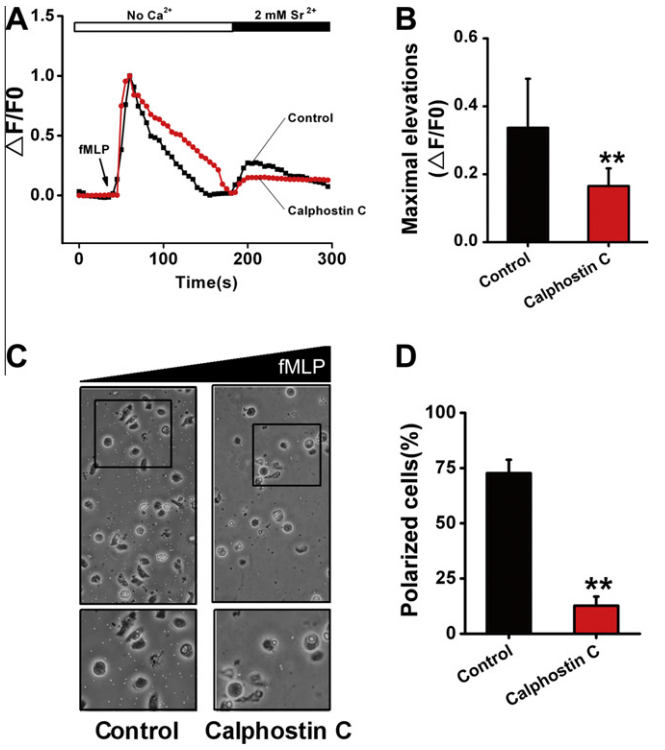

Fig. 3. ROCE inhibition abrogated fMLP-induced neutrophil polarization. (A and B) Neutrophils were incubated with or without 1 μM calphostin C, a PKC blocker, for 20 min before fMLP stimulation and then 2 mM Sr^{2+} was added as indicated. Corresponding bar charts show the maximal increase of the curve in Fluo-4/AM imaging after addition of Ca^{2+} or Sr^{2+}. (C) Neutrophils were preincubated with 1 μM calphostin C for 20 min before fMLP stimulation in a Zigmond chamber. (D) The proportion of neutrophils polarized after 15-min exposure to fMLP in a Zigmond chamber was analyzed. Bars are mean ± SEM. $^{**}P < 0.01$.

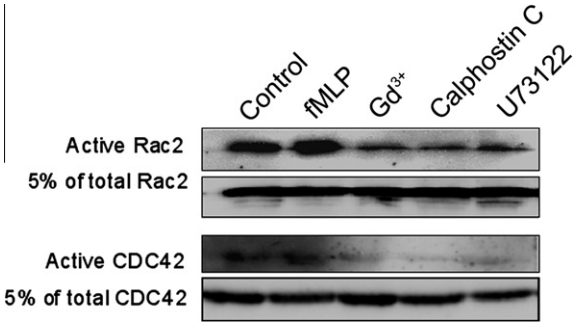

Fig. 4. Either SOCE or ROCE inhibitor can eliminate fMLP-induced Rac2 and Cdc42 activation. Rac2 and Cdc42 activation were assessed by PBD-GST pull-down assays. Representative results from three different experiments are shown.

signaling, respectively [2]. Members of the small Rho-GTPase family, including Rac2 and Cdc42, localize to the leading edge [24]. Accordingly, Rac2 and Cdc42 are essential for polarization and directional migration. We therefore also used these GTPases as markers to determine the involvement of Rho-GTPase family members in SOCE and ROCE signaling during fMLP-induced neutrophil polarization. However, the mechanisms whereby different Ca^{2+}-signaling routes communicate with Rho-GTPase family members, leading to subsequent cell polarization, remain unclear.

Fluo-4 is more suitable than other Ca^{2+} indicators for argon ion laser generator of confocal microscope because the fluorescence yield with Fluo-4 is higher than Fluo-3 or Fura-2. Therefore, Fluo-4/AM was chosen as the Ca^{2+} indicator in our study. Anti-STIM1 antibody is usually be used to reduce the function of STIM1 since RNA inference cannot be performed in neutrophils [21]. In this study, we investigated the role of STIM1 in fMLP-induced Ca^{2+} entry by electrotransjection with anti-STIM1 antibody. STIM-knockout animals will help to confirm it in the future study.

In summary, the results of the current study provide new insights into the source of intracellular Ca^{2+} influx in the control of fMLP-induced neutrophil polarization. fMLP triggers not only SOCE, but also ROCE, in human primary neutrophils. Each of these two routes of Ca^{2+} influx appears to play a critical role in regulating downstream small GTPase activity, leading to neutrophil polarization. However, further studies are needed to determine the exact mechanisms by which SOCE and ROCE signaling control neutrophil polarization, and to gain a full understanding of the relationship between the spatiotemporal Ca^{2+} distribution and neutrophil polarization. New insights into the mechanisms of Ca^{2+} influx regulating the migration of neutrophils could enable us specifically to modify neutrophil activation and recruitment to the site of infection, which could lead to novel therapeutic strategies for counteracting neutrophil-induced tissue damage.

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