

Original Paper

Store-operated Ca^{2+} Entry (SOCE) Plays a Role in the Polarization of Neutrophil-like HL-60 Cells by Regulating the Activation of Akt, Src, and Rho Family GTPases

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Key Words

Store-operated Ca^{2+} entry • Differentiated HL-60 cells • Akt • Src • Rho GTPases

Abstract

Neutrophil polarization is a basic activity involved in the innate immune response, and it may be initiated by extracellular Ca^{2+} entry, a process primarily mediated through store-operated Ca^{2+} entry (SOCE). Yet, the mechanisms by which SOCE participates in cell polarization remain unclear. We hypothesized that Akt- and Src-dependent pathways, traditionally linked to neutrophil polarization, may interact with SOCE in this event. In this study, SKF96365 and 2-APB, inhibitors of SOCE as proved by their inhibition on Mn^{2+} influx, were observed to inhibit the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced influx of Ca^{2+} , the activation of Akt, Src, Rac1, Rac2, and Cdc42, and the polarization of differentiated HL-60 (dHL-60) cells. Downregulation of stromal interaction molecule 1 (STIM1), a Ca^{2+} sensor identified to induce SOCE, by siRNA led to decreases in the following indexes: Ca^{2+} entry, activation of Akt, Src, Rac2 (rather than Rac1) and Cdc42, and fMLP-induced polarization. This study suggests that SOCE might be the predominant form of Ca^{2+} entry involved in the regulation of cell polarization, and it may act through the Akt/Src/Rac pathways, as modeled in dHL-60 cells. It also suggests that STIM1 is a key modulator of cell polarization, potentially serving as a target for the designation of anti-immune deficiency therapies.

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Introduction

Neutrophil polarization is a crucial constituent of the innate immune response during bacterial infection [1, 2]. Defects in the mobility and polarization of neutrophils in response to inflammatory stimuli have been reported in humans, such as lazy leucocyte syndrome (LLS) [3], Shwachman-Diamond syndrome [4], and dysgammaglobulinemia type I [5]. However, the mechanisms for these defects are poorly understood. Intracellular (cytoplasmic) calcium has generally been linked to cell movements, including cell polarization and chemotaxis. The concentration of free Ca^{2+} in the cytoplasm ($[\text{Ca}^{2+}]_i$) is supposed to play a key role in the regulation of neutrophil chemotaxis and migration [6, 7]. Levels of this Ca^{2+} signal are probably regulated through at least two mechanisms: (1) the state of filling of Ca^{2+} stores in the endoplasmic reticulum (ER), termed store-operated Ca^{2+} entry (SOCE) [8], and (2) receptor occupation, which is named receptor-operated Ca^{2+} entry (ROCE) [9, 10]. The molecular components of ROCE are still unknown, and efforts made on establishing pharmacological approaches for modulating ROCE have not come to a solution. SOCE, however, has been characterized more completely. SOCE was first described as the predominant mechanism for Ca^{2+} influx in non-excitable cells, and so far two major molecular components of SOCE have been identified: (1) stromal interaction molecule 1 (STIM1), which serves as a Ca^{2+} sensor that oligomerizes and translocates to ER/PM (plasma membrane) junctional domains to aggregate into puncta in response to Ca^{2+} store depletion [11, 12], and (2) Orai1 (also named CRACM1), which is localized diffusely in the plasma membrane of resting cells and is recruited by STIM1 into the puncta through an interaction with its C-terminal region [13]. These research findings established knowledge about the characterizations of SOCE is favorable for further study on its roles in various cell functions. For example, recent studies have indicated that SOCE is involved in cell polarization, migration and metastasis, by regulating a variety of cytosolic Ca^{2+} signals [11, 14, 15]. However, the exact mechanisms by which SOCE exerts these effects remain unknown. In the present study, differentiated HL-60 (dHL-60) cells, as a validated model system for the analysis of neutrophil polarization [16], were used to investigate the mechanisms whereby SOCE regulates neutrophil polarization.

Stimuli such as formyl-methionyl-leucyl-phenylalanine (fMLP) and IL-8 are known to induce actin polymerization and cell migration *via* G protein-coupled receptors. A number of downstream signaling systems are activated by these stimuli in neutrophils, including Rho family proteins, phospholipase C, phosphatidylinositol 3-kinase (PI3K) and its lipid products, MAP kinases, and tyrosine kinases [17]. As the most classical and widely studied pathway, the PI3K-dependent pathway (PI3K/Akt pathway or Akt-dependent pathway) is known to play a pivotal role in the sensing and polarization of cells toward a chemoattractant signal [1, 18]. The phosphorylation of Akt is generally considered to be an index of the activation of PI3K and PI3K-dependent pathway [19, 20], and it occurred in response to chemoattractant factors that induce cell polarization, such as fMLP [21]. However, recent studies have identified a PI3K-independent pathway that acts in parallel with the PI3K-dependent pathway, and these two pathways work together to activate Rac and Cdc42 and thereby regulate cell polarization and migration [22]. This PI3K-independent pathway has been recently identified as a Src-dependent pathway [23, 24]. Nonetheless, whether the activation of Akt and Src is of any relevance to SOCE and Ca^{2+} influx have poorly been understood.

Given the importance of PI3K-dependent and -independent pathways, as well as Rho family GTPases, in cell polarization, we hypothesized that SOCE regulates these signal transduction pathways and thus affects cell polarization. In the present study, two pharmacological agents, i.e., SKF96365 (a Ca^{2+} -entry blocker [25]) and 2-aminoethoxydiphenyl borate (2-APB) (an IP_3 receptor inhibitor [26]), both of which have been applied extensively as putative SOCE inhibitors, were used to investigate the role of SOCE in chemoattractant-induced polarization of dHL-60 cells. Additionally, for the purpose of isolating the activity of unidirectional SOCE from intracellular Ca^{2+} release and Ca^{2+} extrusion, a Mn^{2+} quenching assay is commonly used. The transportation of Mn^{2+} is supposed to be via some Ca^{2+} selective

channels (e.g., SOCE) which are associated with the plasma membrane for unidirectional influx of Mn^{2+} , while the Ca^{2+} pump and extrusion channels are not involved in the transportation of Mn^{2+} [27, 28]. Therefore, entry of Mn^{2+} to the cells and the influence of the putative SOCE inhibitors have been investigated in the present study, for overcoming the limitation of the specificity of the inhibitors for SOCE. Furthermore, since STIM1 is able to mediate the effects of SOCE and may participate in fMLP-evoked SOCE in dHL-60 cells [29], we used small interfering RNA (siRNA) to knockdown STIM1 expression, for further confirmation of the role of SOCE in the polarization of dHL-60 cells. In addition, the interaction of SOCE with other cell polarization-related molecules, such as Akt, Src, and GTPases, was also investigated.

Materials and Methods

Reagents and antibodies

The chemical compounds formyl-Met-Leu-Phe (fMLP), dimethylsulphoxide (DMSO), SKF96365, 2-Aminoethoxydiphenyl Borate (2-APB), thapsigargin (TG) and $CaCl_2$ were purchased from Sigma Chemical Company. Fluo-4 AM was purchased from Invitrogen. Goat polyclonal anti-AKT antibody and rabbit polyclonal anti-phospho-Akt (Ser473), anti-Cdc42 and anti-STIM1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit monoclonal anti-Src antibody and rabbit polyclonal anti-phospho-Src antibody from Cell Signaling. Goat polyclonal anti-Rac2 antibody was from GeneTex, Inc., mouse monoclonal anti-Rac1 antibody was from MILLIPORE.

Cell culture

HL-60 cells (provided by China Center for Type Culture Collection, Shanghai, China) were maintained in a RPMI 1640 cell culture medium (Invitrogen, Karlsruhe, Germany) supplemented with 10-12% fetal calf serum and L-glutamine (Invitrogen). The cells were grown in T-75 or T-25 flasks at 37°C in a humidified atmosphere containing 5% CO_2 . For differentiation, HL-60 cells (10^6 /ml) were treated with 1.3% DMSO for up to 6 days to induce a neutrophil-like phenotype (dHL-60 cells) [30]. Assessment of neutrophilic differentiation was performed on days 0, 4, and 6 by several criteria as followed: 1) morphological changes featured as nuclear segmentation, 2) CD11b expression as detected by flow cytometry, and 3) responsiveness to PAF, fMLP or TNF- α stimulated adhesion to fibrinogen-coated plates. dHL-60 cells that had been treated with DMSO for 4 days were used for the experiments, from which non-viable cells were removed by centrifugation (180 g, 5 min, at room temperature) and washing with 5 ml phosphate-buffered saline (PBS) (0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 , PH 7.2 \pm 0.1) for 3 times.

Neutrophil preparation

Human neutrophils were collected from healthy human volunteers and put into vacutainers anticoagulated with EDTA- Na_2 (Na_2 ethylenediaminetetraacetic acid). Neutrophils were freshly isolated from the whole blood using the density gradient technique [31]. More than 90% of the cells isolated were neutrophils. The viability, determined by trypan blue exclusion, was at least 95%. The isolated cells were kept on ice and used within 4 h.

Assessment of cell shape

dHL-60 cells (5×10^6 /ml) were incubated as indicated in the legend of Fig. 1. Cell morphology was determined with inverted microscopy using a OLYMPUS 1 \times 51 microscope with a 40 objective and digital images of the cells were taken and saved with PLYMPUS DP controller (DP71) and its software. The proportion of polarized cells following treatment of the cells with uniform concentration of fMLP for 5 min (a time point wherein the rate of cell polarization came to its peak level), was determined by observing at least 100 cells by inverted microscopy, and the cells were classified into three categories: spherical, non-polar and polar cells [32]. Three independent experiments were set up for each treatment, and the shape polarization was defined by a ratio of cell length to cell width as > 1.5 [15]. Apoptotic cells with blebs were not included in the above observation.

RNA interference assay

Double-stranded small interfering RNA (siRNA) targeting human STIM1 (as well as a non-silencing control RNA) were designed and obtained from Shanghai GenePharma Co., Ltd. Two siRNAs (100 nM) were used to test silencing efficiency and to mediate knockdown expression of STIM proteins. All experiments were performed with two siRNA duplexes for STIM1 (sense 1: 5'-GGCUCUGGAUACAGUGCUCU-3', antisense 1: 5'-GAG CAC UGU AUC CAG AGC CTT -3', sense 2: 5'-GAA GCU GCG CGA UGA GAU CTT -3', antisense 2: 5'-

GAU CUC AUC GCG CAG CUU CTT -3'). The scrambled siRNA (non-silencing sequence) (sense: 5'-UUC UCC GAA CGU GUC ACG UTT -3', antisense: 5'-ACG UGA CAC GUU CGG AGA ATT -3') was used as a negative control. All siRNA target sequences chosen in this study were screened by NCBI BLAST searches to predict whether a siRNA would have off-target effects and cause knockdown of a non-targeted gene with which it coincidentally has high homology. For electroporation, dHL-60 cells (5×10^6 /ml), which were differentiated with DMSO for 4 days, were washed twice with PBS lacking Ca^{2+} and Mg^{2+} and resuspended at a density of 2×10^7 cells/ml in ice-cold OPTI-MEM. A 400 μL aliquot of dHL-60 cells (8×10^6 cells) was mixed with 2 μL of 20 mM STIM1 siRNA or 20 mM non-silencing control sequence; the cell siRNA mixture was incubated for 10 min at room temperature, then transferred to an electroporation cuvette (0.4 cm electrode, Bio-Rad), and finally subjected to an electroporation pulse on ice at 295 V, 1180 μF , 500 Ω . Transferred cells were allowed to recover by staying on ice for 10 min and then once again transferred to 20 ml complete medium. The transfection efficiency was detected as about 85%. Approximately 36 h after transfection, the cells were processed for protein lysates, Ca^{2+} measurements and Zigmond assay.

Measurement of intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$)

dHL-60 cells, which were preincubated with SKF96365 and 2-APB at 37°C for 30 min, or which were transfected with STIM1 siRNA or control siRNA, were suspended at a concentration of 10^6 /ml in the modified Hanks' buffered salt solution containing 25 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 0.4 mM MgCl_2 , and 25 mM D-glucose (pH 7.4), and labeled with 3 μM Fluo-4 AM at 37°C for 30 min in darkness. Cells were then washed for three times with modified Hanks' buffer on ice and then resuspended in Ca^{2+} -free buffer solution (prepared by omitting CaCl_2 and adding 0.3 mM EGTA). Finally, 200 μL aliquots of cell suspension were added into 96 well plates (2×10^5 cells per well), and the fluorescence was recorded over time by using fluorescence spectrophotometer (Infinite M200, TECAN, Italy) with excitation wavelength of 495 nm and emission at 518 nm. For imaging with Fluo-4, $[\text{Ca}^{2+}]_i$ changes are presented as $\Delta F/F_0$ ratios after background subtraction, where ΔF was the change in fluorescence signal intensity and F_0 was the baseline as calculated by averaging the three independent experiments before stimulus application.

Measurement of Mn^{2+} influx

Due to the high affinity of Mn^{2+} to Fura-2 (a fluorescent agent), the Mn^{2+} influx was detected according to the intracellular quenching of Fura-2, as previously reported [33]. The fluorescence changes in Fura-2-loaded dHL-60 cells (with the cell density and fluorescence detection methods kept the same as described above) were monitored at 510 nm with excitation at 360 nm, wherein the fluorescence intensity declined as Mn^{2+} was added to the Ca^{2+} (1 mM)-containing medium. Diethylenetriamine pentaacetic acid (2 mM) was used at the end of the experiments for estimating the proportion of Fura-2 leakage-associated loss of intracellular Mn^{2+} , which was indicated as minimal (<5%).

Western blotting

For analysis of phosphorylation of Akt and Src or total Akt and Src, dHL-60 cells were either directly stimulated with fMLP at 37°C for the indicated durations, or at first pretreated with SOCE inhibitors (SKF96365 and 2-APB) of different concentrations or their vehicle DMSO at 37°C for 30 min and then followed by stimulating the cells with fMLP at 37°C for 1 min. dHL-60 cells transfected with STIM1 siRNA or control siRNA were stimulated directly with fMLP at 37°C for 1 min. The reaction was terminated by the addition of ice-cold PBS, then the reaction mixture underwent fast centrifugation at 15000 g for 0.5 min at 4°C. Cell pellets were suspended with (50 μL / 5×10^5 cells) lysis buffer (20 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM EDTA, 50 mM NaF, 10% glycerol, 1 mM Na_3VO_4 , 10 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ pepstatin, and 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 15000 g for 15 min at 4°C, the supernatants were collected as cell lysates, which were mixed with 5 \times Laemmli sample buffer and boiled for 5 min. The protein preparations were subjected to 8-10% SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked with 5% BSA in TBST (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 0.1% Tween 20) or PBST (0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 , pH 7.2-7.6, 0.15 NaCl, and 0.1% Tween 2) and probed with appropriate primary and secondary antibodies diluted in TBST or PBST. The bound antibodies were visualized using HRP Chemiluminescent Substrate Reagent Kit (Invitrogen).

Zigmond assay

dHL-60 cells were collected at the 4th day after being differentiated by DMSO. Non-transfected dHL-60 cells or neutrophils were pretreated with SOCE inhibitors (40 μM SKF96365 and 100 μM 2-APB) or inhibitor-free PBS for 30 min at 37°C. The pretreated or non-pretreated dHL-60 cells, neutrophils, or the dHL-60 cells transfected with STIM1 siRNA or control siRNA were allowed to attach to coverslip (22 \times 40 mm) at room temperature for 10 min. The cover slip was inverted onto a Zigmond chamber (Neuro Probe, USA) (as introduced by H. Zigmond [34]) and submitted to a 100 nM fMLP gradient. Digital images of the

cells were taken every 10 or 15 s, depending on the experiment, for a total of 30 min, using an OLYMPUS 1×51 Microscope (New York Microscope, Company). Images were processed for edge and contrast enhancement using photoshop software. An average of 100 cells was examined for each experiment, and three independent experiments were set up for each treatment. The percentage of cells that polarized, i.e. with a directionally oriented leading edge and trailing tail, was calculated as described previously [35].

GST Pull-down assay

dHL-60 cells in various treatments were collected and lysed in 400 µl cold lysis buffer. A total of 20 µl of each cell lysate at each experiment was examined on 12% SDS- PAGE and blotted with the indicated Rac and Cdc42 Ab to determine the levels of Rac1, Rac2 and Cdc42. To detect the activated Rac1, Rac2 and Cdc42, 400 µl of cell lysate (20 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM MgCl₂, 10 mM Na₂P₂O₇, 5 mM EDTA, 50 mM NaF, 10% glycerol, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 1mM PMSF) was used for incubation with 10 µg of PBD (GST-fusion protein containing the Rac/Cdc42 binding domain of PAK1) at 4°C for 1 h under shaking. The reaction was stopped and the reaction mixture was filtered by centrifugation at 10000 g (4°C). The filters were washed three times with 1 ml of wash buffer (50 mM Tris-HCl, PH 7.7, 5 mM MgCl₂), then mixed with 2×Laemmli sample buffer, subsequently boiled for 5 min and examined on 12% SDS- PAGE. The blot was first subjected to immunoblotting with an anti-Rac2 Ab, then the antibody stripped off, and the membrane re-blotted with anti-Rac1 and Cdc42 Abs; and this sequence was reversed for repeated experiments.

Statistical analysis

Data are shown as mean ± SD. Statistical analysis using SPSS (version 13.0) was performed by one-way analysis of variance and Bonferroni's multiple comparison test. Statistical significance was defined as $p < 0.05$.

Results

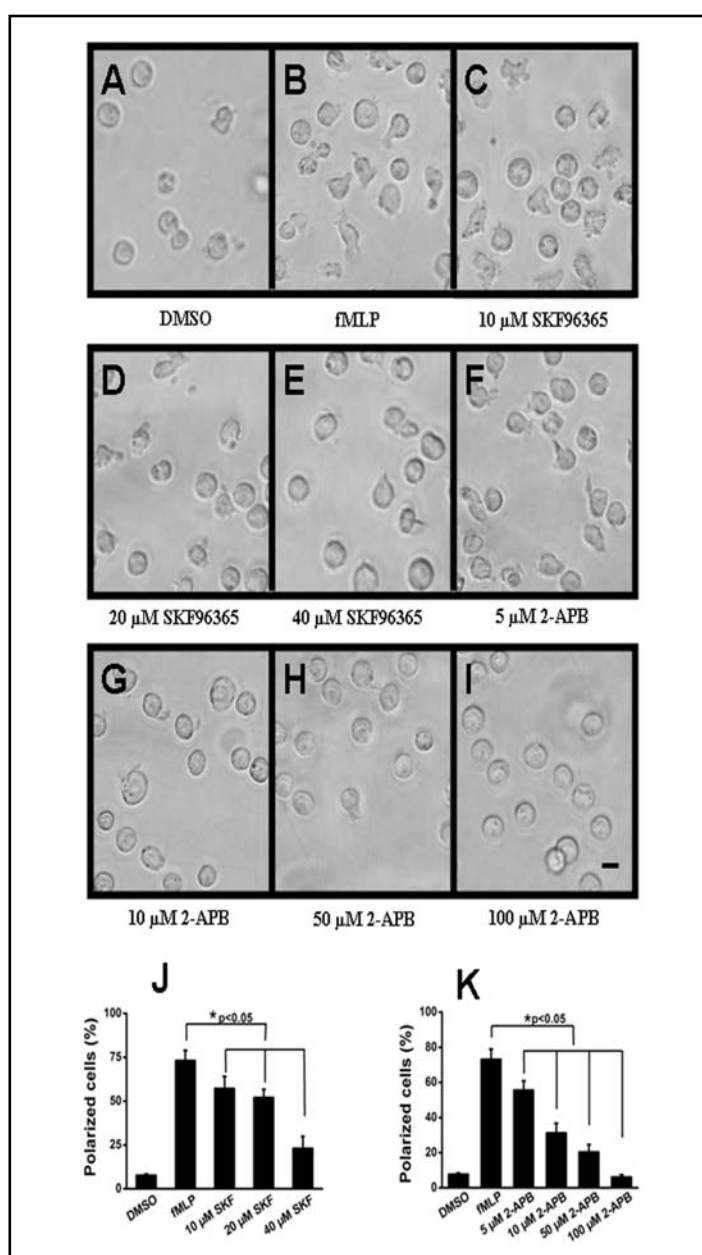
SKF96365 and 2-APB prohibited shape polarization of dHL-60 cells stimulated with uniform concentration of fMLP

Evidence exists that suppression of SOCE negatively regulates a variety of cellular responses to fMLP, including shape polarization in neutrophils [15]. In this study, we used two modulators, putatively inhibiting SOCE, i.e., SKF96365 and 2-APB, to examine whether that effect is also present in dHL-60 cells. The cells were exposed to SKF96365 at 10–40 µM or 2-APB at 5–100 µM prior to stimulation with fMLP at a uniform concentration (100 nM) to search for an optimal concentration of each inhibitor to inhibit shape polarization in dHL-60 cells. The results indicated that both SKF96365 and 2-APB attenuated fMLP-induced shape polarization in a concentration-dependent manner, and SKF96365 demonstrated the greatest inhibition of shape polarization at 40 µM, while 2-APB at 100 µM showed complete inhibition (Fig. 1).

SKF96365 and 2-APB downregulated fMLP-stimulated activation of Akt and Src

It has been reported that PI3K inhibitors, e.g., wortmannin and LY294002, reduced neutrophil polarization in response to fMLP [36, 37]; and a selective Src kinase inhibitor, i.e., 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine (pp2), has also been reported to inhibit fMLP-induced neutrophil migration [23]. These results suggest that both PI3K-dependent pathway and Src-dependent pathway may play important roles in neutrophil polarization. Thus, in the present study, the phosphorylation of Akt and Src subsequent to fMLP stimulation was measured to determine whether fMLP could activate these two signaling pathways in dHL-60 cells. Western blot analysis was performed by using antibodies against phospho-Akt, phospho-Src, total Akt, or total Src. Since the antibody against phospho-Akt detects phosphorylation of Akt at Ser 473, a single band was seen in the blot; in contrast, in the blot with antibody against total Akt (Akt1/2) which detects the serine/threonine kinase Akt family, two bands were seen. Although the antibody against phospho-Src detects phosphorylation of Src at tyrosine 416, it may cross-react with other members of phosphorylated Src family (Lyn, Fyn, Lck, Yes, and Hck), and as a result multiple

Fig. 1. Effects of SKF96365 and 2-APB on fMLP-induced cell polarization. dHL-60 cells were given 30-min pretreatment with: DMSO (A), DMSO (B), and 10, 20, and 40 μ M of SKF96365 (C, D, and E); which was followed by 5-min incubation with DMSO (A), or 100 nM of fMLP (B, C, D, E). The other set of experiments were carried out according to the above design with 30-min preincubation with 5, 10, 50, and 100 μ M of 2-APB (F, G, H, and I) in stead of the various concentrations of SKF96365. Scale bar: 10 μ M. The percentage of cells polarized in each panel was quantified as described under "Materials and Methods" and expressed as mean \pm SD from three independent experiments (J and K).



bands were seen in the blot. The antibody against total Src (36D10) detects the levels of the endogenous Src protein, and a single band was detected in the blot. As shown in Figs. 2A, B, following stimulation of dHL-60 cells with fMLP, Akt and Src were phosphorylated in a time-dependent manner, which was always paralleling to each other throughout the time course, with the reaction of both proteins peaked at 1 min, followed by a gradual decrease. These results suggested that PI3K-dependent and Src-dependent pathways might be activated simultaneously by fMLP in dHL-60 cells, and provided further support to the previous reports that the two pathways regulate cell polarization and chemotaxis in parallel [24].

As was hypothesized in this study, SKF96365 and 2-APB might inhibit shape polarization in dHL-60 cells (Fig. 1) by regulating these two signaling pathways. SKF96365 and 2-APB at the concentrations used for the above inhibition on fMLP-induced shape polarization were once again used to evaluate their effects on fMLP-induced activation of Akt and Src in dHL-60 cells. As shown in Fig. 2C, SKF96365 showed inhibitory effects on the activation of Akt and Src at 10 to 40 μ M, with major inhibition observed at 40 μ M. 2-APB demonstrated significant inhibition on the levels of both phospho-Src and phospho-Akt, with complete inhibition of

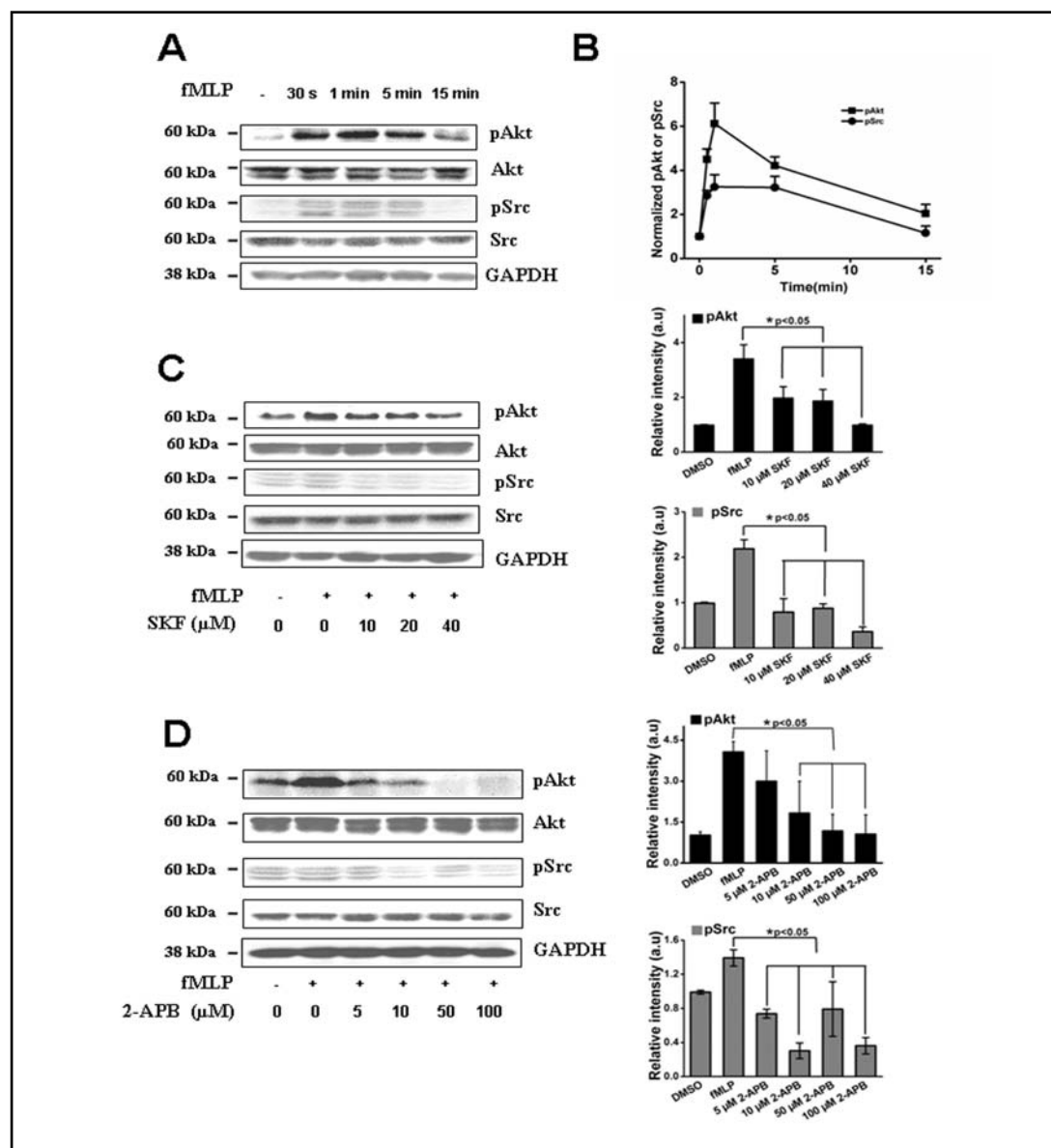


Fig. 2. Effects of SKF96365 and 2-APB on fMLP-induced activation of Akt and Src. dHL-60 cells were stimulated with 100 nM of fMLP at 37°C. Cell lysates were analyzed by SDS-PAGE followed by Western blot using antibodies against phosphorylated Akt (pAkt), Akt, phosphorylated Src (pSrc), and Src. Phosphorylation of Akt and Src of dHL-60 cells after being stimulated by fMLP for 30 s, 1 min, 5 min, and 15 min, was observed (A); the effects of SKF96365 (C) and 2-APB (D) on the levels of phosphorylation of Akt and Src after being exposed to fMLP for 1 min were also observed. Panel B shows the relative quantities of the activation of Akt and Src. Data are expressed as mean \pm SD from three independent experiments, * $p < 0.05$, by one-way ANOVA, compared with the group with fMLP stimulation in the absence of modulators.

them attained at 100 μ M (Fig. 2D). Therefore, 40 μ M SKF96365 and 100 μ M 2-APB were also used in the subsequent experiments for estimation of SOCE inhibition and related activities.

SKF96365 and 2-APB downregulated fMLP-stimulated activation of Rho GTPases

The dependence of cell polarization and chemotaxis on Rho family GTPases have been described extensively [38, 39], and tyrosine kinase- and PI3K-regulated activation of Rac1, Rac2, and Cdc42 has also been observed [40–42]. Thus, a GST pull-down assay was performed to evaluate the effects of the SKF96365 and 2-APB on the signaling molecules

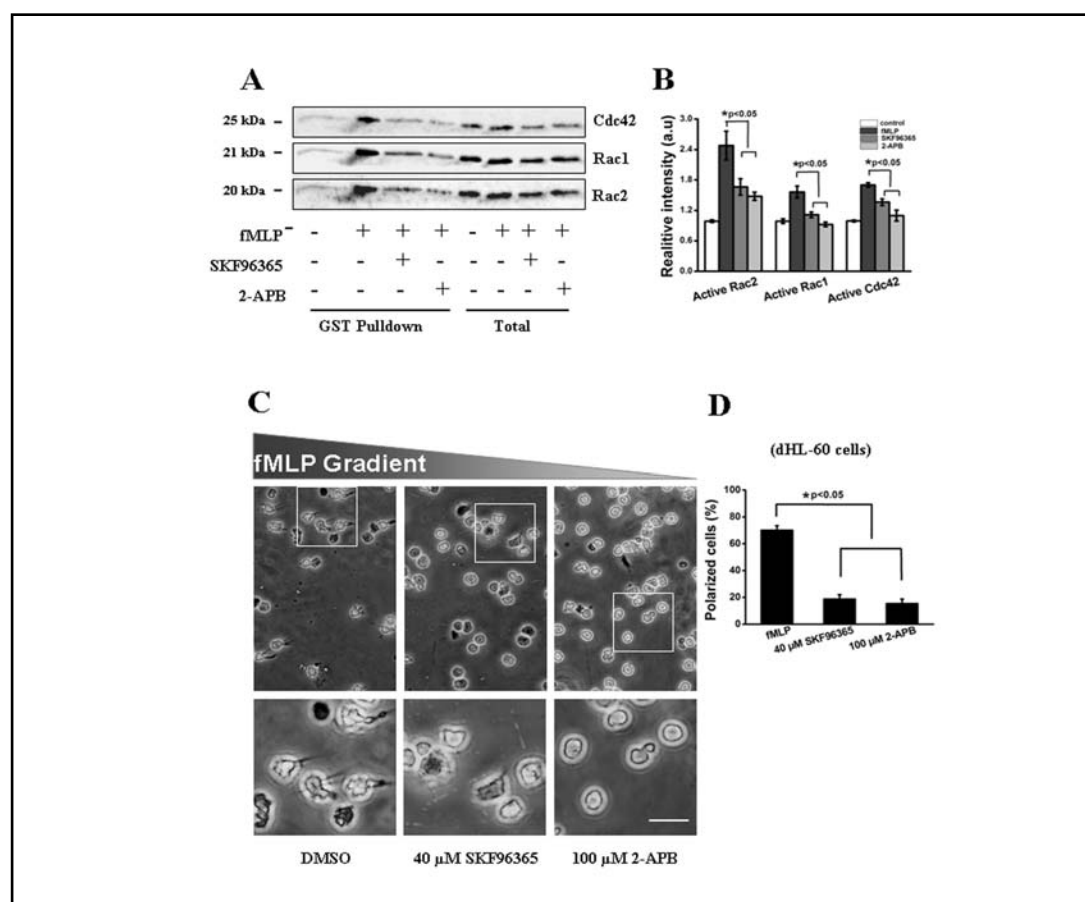


Fig. 3. Effects of SKF96365 and 2-APB on fMLP-induced activation of Rac2, Rac1, and Cdc42 and cell polarization toward an fMLP gradient. SKF96365 (40 μM) and 2-APB (100 μM) were observed to inhibit fMLP-induced activation of Rac2, Rac1, and Cdc42 (A), whose relative quantities are shown as mean ± SD from three independent experiments (B), as detected with the GST-PBD pull-down assay. Inhibition of SKF96365 and 2-APB on cell polarization toward an fMLP gradient was observed by using the Zigmond chamber, and cell images were acquired at 20× objective after the 30-min incubation with fMLP. The icon above the images indicates the direction of the fMLP gradient. The bottom panels show a higher magnification image of each boxed section in the top panels. Scale bar: 20 μm (C). The percentage of polarized cells was quantified by observing 100 cells in each experiment, data are means and SD (n=3) (D). *p < 0.05, compared with the fMLP group.

downstream to SOCE in fMLP-stimulated dHL-60 cells. As shown in Fig. 3A, the activation of Rac2, Rac1, and Cdc42 was enhanced markedly 1 min after stimulation with fMLP. fMLP (100 nM) produced an activation of Rac2 (~2.5-fold over that in the non-stimulated group) at a level slightly higher than that of Rac1 activation (only ~1.5-fold increase) or the level of Cdc42 activation (~1.7-fold increase). The levels of activated Rac2, Rac1, and Cdc42 were significantly reduced by pretreatment of the cells with either SKF96365 at 40 μM or 2-APB at 100 μM. In order to evaluate the effects of SKF96365 and 2-APB on cell polarization in response to an fMLP gradient (0–100 nM), the Zigmond chamber was used for microscopic analysis of polarized cells (Fig. 3C). In three independent experiments, 70% of the control cells were polarized in the direction of the fMLP gradient. Pretreatment with SKF96365 (40 μM) and 2-APB (100 μM) significantly reduced the percentage of polarized cells to 19% and 15%, respectively.

SKF96365 and 2-APB reduced Ca^{2+} and Mn^{2+} influx in dHL-60 cells

To make it clear whether the inhibitory effects of SKF96365 and 2-APB on cell polarization and signal transduction are due to changes in cytosolic Ca^{2+} influx, fluo-4-

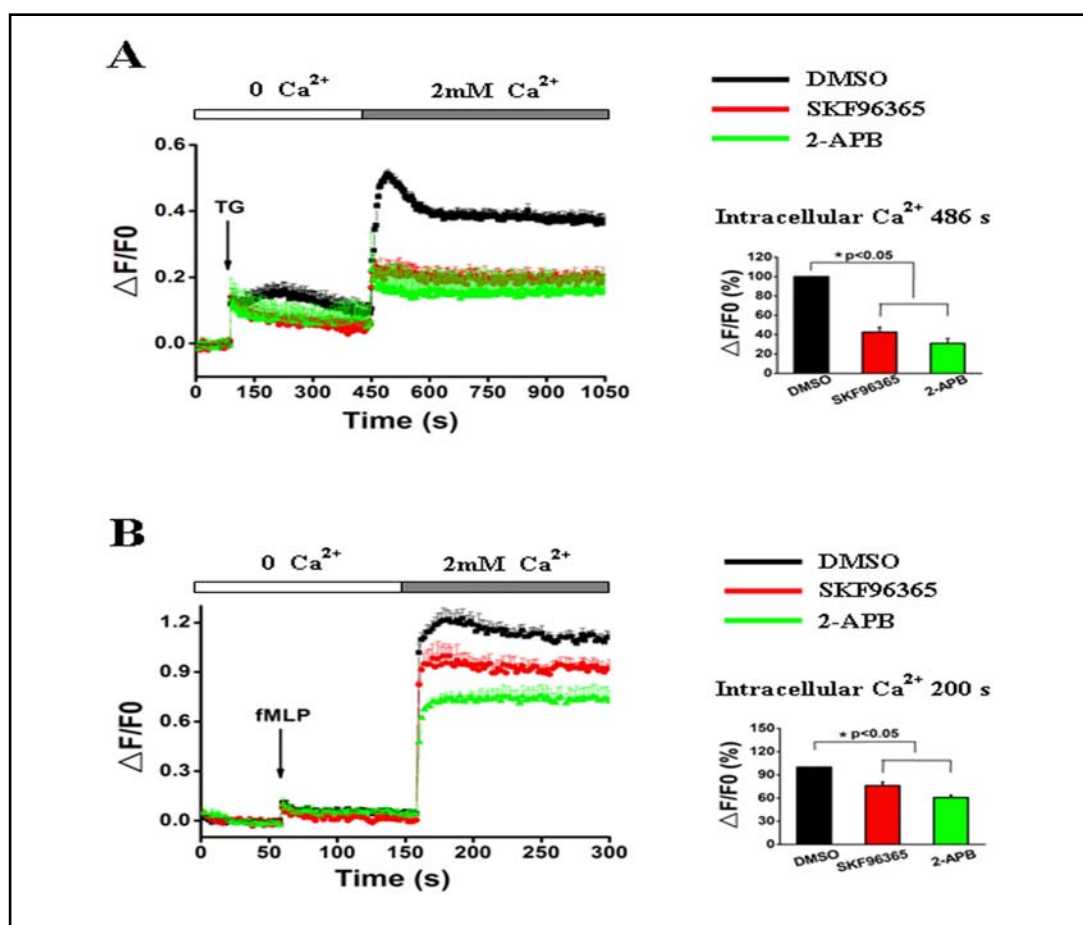
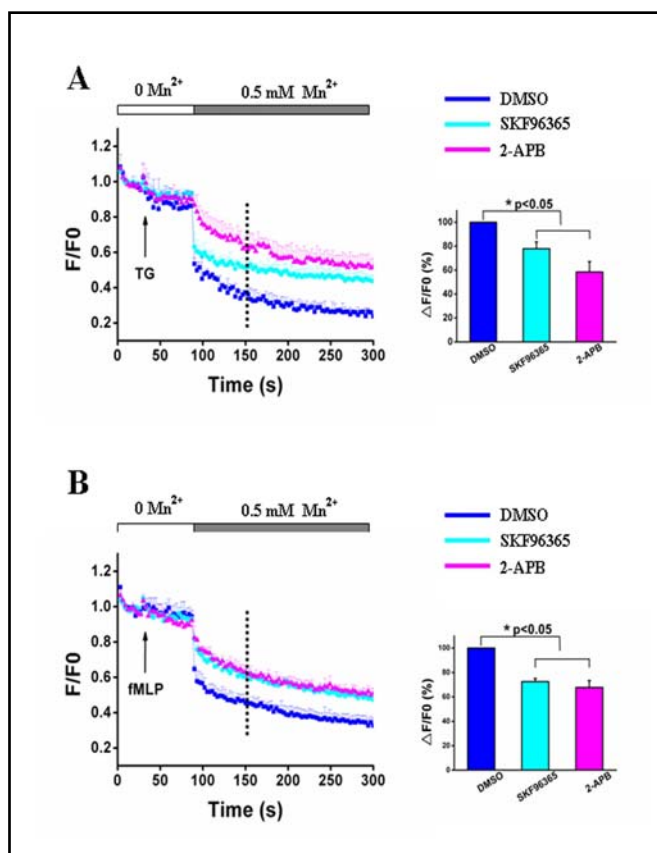


Fig. 4. Effects of SKF96365 and 2-APB on $[Ca^{2+}]_i$ in dHL-60 cells. dHL-60 cells were pretreated with 40 μ M SKF96365, 100 μ M 2-APB, or the vehicle (DMSO), then stimulated with thapsigargin (TG, 1 μ M), followed by the addition of 2 mM extracellular Ca^{2+} , and quantification of intracellular Ca^{2+} was done at 486 s during Ca^{2+} influx (A). The other set of experiments were started with pretreatment of the cells as described above, followed by stimulation with fMLP and the subsequent addition of 2 mM extracellular Ca^{2+} , then the $[Ca^{2+}]_i$ was quantified at 200 s during Ca^{2+} influx (B). The time courses in (A) and (B) represent three independent experiments. Data are means and SD from 4–5 (A) or 3 (B) experiments. * $p < 0.05$, compared with the DMSO control.

mediated Ca^{2+} imaging technique was applied to the experiments wherein a SERCA pump inhibitor thapsigargin (TG; an exceptionally effective tool for studying SOCE [26, 43]) was introduced. Fig. 4A showed that the basal Ca^{2+} stores were depleted with TG, and the level of cytoplasmic Ca^{2+} was restored by the supplementation of 2 mM Ca^{2+} to the medium, which might reveal SOCE. The basal level of cytoplasmic Ca^{2+} prior to the addition of fMLP or TG was standardized as $\Delta F/F_0 \sim 0$, and $\Delta F/F_0$ was used as a measure of the increased cytosolic Ca^{2+} influx. As shown in the right panel of Fig. 4A, the apparent Ca^{2+} influx was significantly inhibited by $\sim 60\%$ and $\sim 70\%$ by pretreatment of the cells with SKF96365 (40 μ M) and 2-APB (100 μ M), respectively, with the Ca^{2+} store depletion unaffected. As shown in Fig. 4B, fMLP elicited an initial small increase in cytosolic Ca^{2+} , in the presence or absence of SKF96365 and 2-APB. The re-addition of extracellular Ca^{2+} resulted in a rapid Ca^{2+} influx into the cells, and this increase in cytoplasmic Ca^{2+} was reduced by $\sim 23\%$ and $\sim 40\%$ in the cells pretreated with SKF96365 (40 μ M) and 2-APB (100 μ M), respectively, without affecting the Ca^{2+} store release.

It has been reported that activation of the store-operated entry mechanism may increase the rate of Mn^{2+} influx [44], which could be generally regarded as a probe for Ca^{2+} influx. Mn^{2+}

Fig. 5. Effects of SKF96365 and 2-APB on Mn^{2+} influx in dHL-60 cells. Fura-2-loaded cells were preincubated with SKF96365 (40 μ M), 2-APB (100 μ M), or the vehicle (DMSO), followed by the addition of 1 μ M TG (A) or 100 nM fMLP (B). The cells were subsequently exposed to Mn^{2+} (0.5 mM) in Ca^{2+} (1 mM)-containing medium, and the Mn^{2+} influx was indicated by the fluorescence quenching, the values of which at 150s were extracted from the curves in the left panels and reflected as bars in the right panels. The time courses in (A) and (B) represented three independent experiments. Data are means and SD from 3 experiments. * $p < 0.05$, compared with the DMSO control.



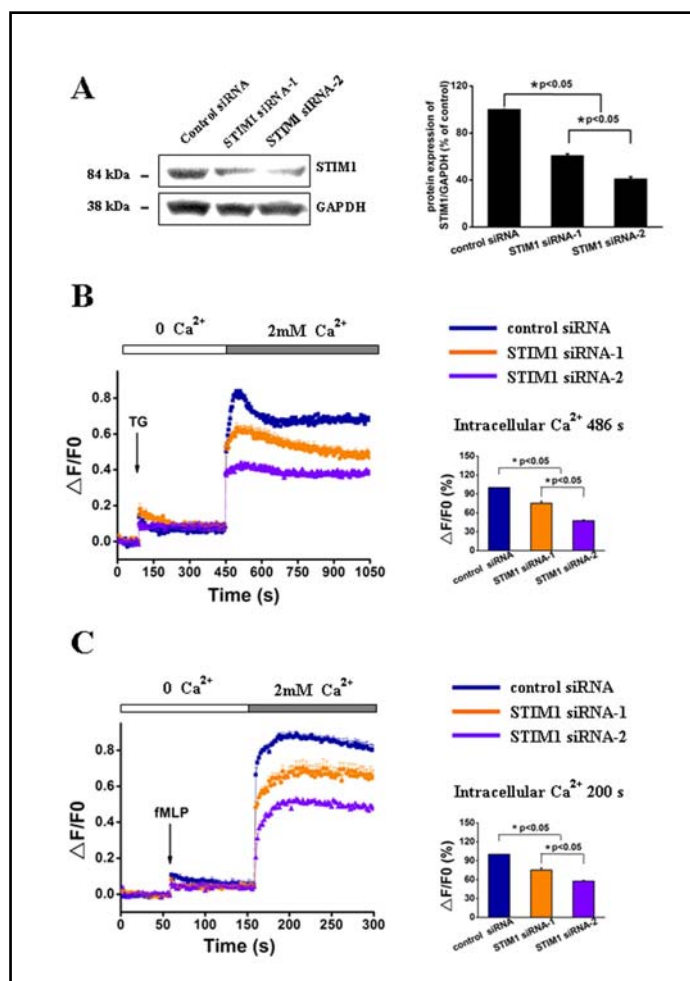
has been shown to permeate through the neutrophils via a Ca^{2+} influx pathway activated by fMLP [45]. In the present study, after depletion of intracellular Ca^{2+} stores by TG or fMLP, the addition of Mn^{2+} caused a significant decrease in fluorescence. The slope of fluorescence, which positively correlated to SOCE activity [46], decreased in SKF96365 and 2-APB treated dHL-60 cells, which indicated that the SOCE activity was inhibited by these two compounds, as represented by the change rates of fluorescence at 150s with SKF96365 and 2-APB (in comparison with the no modulator control) (Fig. 5). These results supported that SOCE was involved in TG- and fMLP-simulated Ca^{2+} influx.

STIM1 siRNA inhibited Ca^{2+} influx and cell polarization in dHL-60 cells

It has been reported that STIM1 activates SOCE in neutrophil-like cells [29]. Therefore, experiments using STIM1 siRNA were performed to further confirm its role as deduced from our study using inhibitors. Two independent sets of specific siRNA sequences were used to selectively target and suppress the endogenous STIM1 protein. As shown in Fig. 6A, the level of STIM1 protein was significantly reduced by both gene silencing sequences (STIM1 siRNA-1 and STIM1 siRNA-2). STIM1 siRNA-2 worked more effectively than STIM1 siRNA-1, and the level of STIM1 expression was reduced by ~60% and ~40%, respectively, as compared with that detected in cells treated with control siRNA.

The present study also determined the level of SOCE in dHL-60 cells transfected with STIM1 siRNA-1 or siRNA-2 and stimulated with TG. As shown in Fig. 6B, SOCE was inhibited by 25% and 53% with siRNA-1 and siRNA-2, respectively, with TG-induced Ca^{2+} release unaffected. To investigate the contribution of STIM1 in fMLP-induced Ca^{2+} influx, we performed an experiment modified from the one described in Fig. 4B with STIM1 siRNAs to replace SOCE inhibitors. It showed that STIM1 siRNA-1 and siRNA-2 inhibited the fMLP-induced elevation in $[Ca^{2+}]_i$ by 20~ % and 40~ %, respectively, without altering Ca^{2+} store release (Fig. 6C).

Fig. 6. Effects of STIM1 knockdown on $[Ca^{2+}]_i$ in dHL-60 cells. See the legend of Fig. 4. The cells were treated according to that described in Fig. 4, with the exception that STIM1 was knocked down by using siRNA rather than modulation of SOCE by the putative inhibitors. The levels of STIM1 were observed after transfection of the cells with STIM1 siRNA-1, -2 or the negative siRNA (A). The Ca^{2+} influx was observed in the transfected cells after stimulation with TG (B) and fMLP (C). Data on the right panels of (B) and (C) are means and SD (n=3). *p < 0.05, compared with the control siRNA group.



We next investigated the effect of STIM1 knockdown on cell polarization in response to the fMLP gradient. As shown in Fig. 7, knockdown of STIM1 by siRNAs resulted in a substantial suppression of cell polarization in the direction of the fMLP gradient, with the percentage of polarized cells in the presence of STIM1 siRNA-1, STIM1 siRNA-2 and control siRNA being 30, 18, and 65 %, respectively.

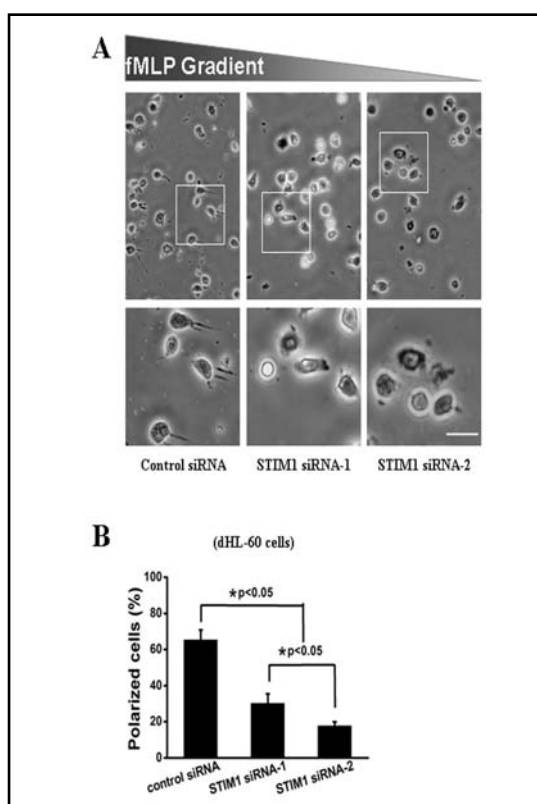
STIM1 siRNA inhibited activation of Akt, Src, Rac2, and Cdc42, but not Rac1, in dHL-60 cells

Our data clearly established that cell polarization was attenuated by STIM1 siRNA. We then explored the effects of STIM1 siRNA on the PI3K- and Src-dependent pathway as well as Rho GTPase in dHL-60 cells. As expected, Akt and Src were activated in dHL-60 cells transfected with the control siRNA in response to fMLP treatment for 1 min, and this was significantly reduced by knockdown of STIM1 with each specific siRNA (Fig. 8A). As shown in Fig. 8B, fMLP induced obvious activation of Rac2, Rac1 or Cdc42 in cells transfected with the control siRNA, a response similar to that observed in untransfected cells. STIM1 knockdown with the two specific siRNAs significantly inhibited fMLP-induced activation of Rac2 and Cdc42, while Rac1 activation was not reduced, but actually increased (Fig. 8B).

SKF96365 and 2-APB inhibited the polarization of human neutrophils in response to fMLP

Because the neutrophil is a terminally differentiated cell type, its genetic manipulations (e.g., siRNA transfection) would be difficult. To examine whether inhibition of SOCE affects the polarization of human neutrophils, experiments with the SOCE inhibitors were conducted

Fig. 7. Effects of STIM1 knockdown on cell polarization toward an fMLP gradient. See the legend of Fig. 3. dHL-60 cells transfected with control siRNA, STIM1 siRNA-1, and STIM1 siRNA-2 were fixed in a Zigmond chamber, and exposed to an fMLP gradient (0–100 nM). Scale bar: 20 μ m. Data are means and SD from three independent experiments. * $p < 0.05$, compared with the control siRNA group.



using the Zigmond chamber. The results revealed that fMLP induced 68 % of neutrophils to polarize in the direction of the fMLP gradient, and this effect was reduced by SKF96365 and 2-APB to 16 % and 13 %, respectively (Fig. 9). These results with human neutrophils were similar to those observed in dHL-60 cells, suggesting that the signaling pathway controlling the fMLP-induced polarization in dHL-60 cells *via* SOCE may well represent the responsiveness of human neutrophil to chemoattractant stimuli.

Discussion

Elevation of $[Ca^{2+}]_i$ is very crucial for a number of responses of the neutrophil to a variety of stimuli [47, 48]. Although the modulation of Ca^{2+} influx in neutrophils has been studied for many years, there still exist a lot of conflicting reports. Several recent studies have indicated that Ca^{2+} influx occurs through both SOCE and ROCE [9, 10], and more specifically there have been many reports that Ca^{2+} influx *via* SOCE plays a vital role in various cellular processes, such as cell polarization, survival, migration and metastasis of non-excitable cells following exposure to a variety of stimuli [14, 15, 49–51]. A clinical study by Hauser et al. have further suggested that human neutrophil dysfunction secondary to injury and inflammation involves an enhancement in SOCE [52]. Despite of the above findings on the neutrophil, the mechanisms by which SOCE mediate cell polarization and other functions remain unclear. The present study has revealed some specific molecules downstream to SOCE that may participate in the regulation of cell polarization.

SKF96365 and 2-APB demonstrated inhibition on calcium influx following stimulation of dHL-60 cells with TG, a SERCA pump inhibitor (supposed to activate SOCE); similarly, in the experiment wherein fMLP was used to induce calcium influx and cell polarization, SKF96365 and 2-APB exhibited inhibition on both processes, yet in general the specificities of SKF96365 and 2-APB for inhibiting SOCE are both limited [53, 54]. To make confirmation of the effects of the two compounds on SOCE-associated Ca^{2+} influx, we determined the

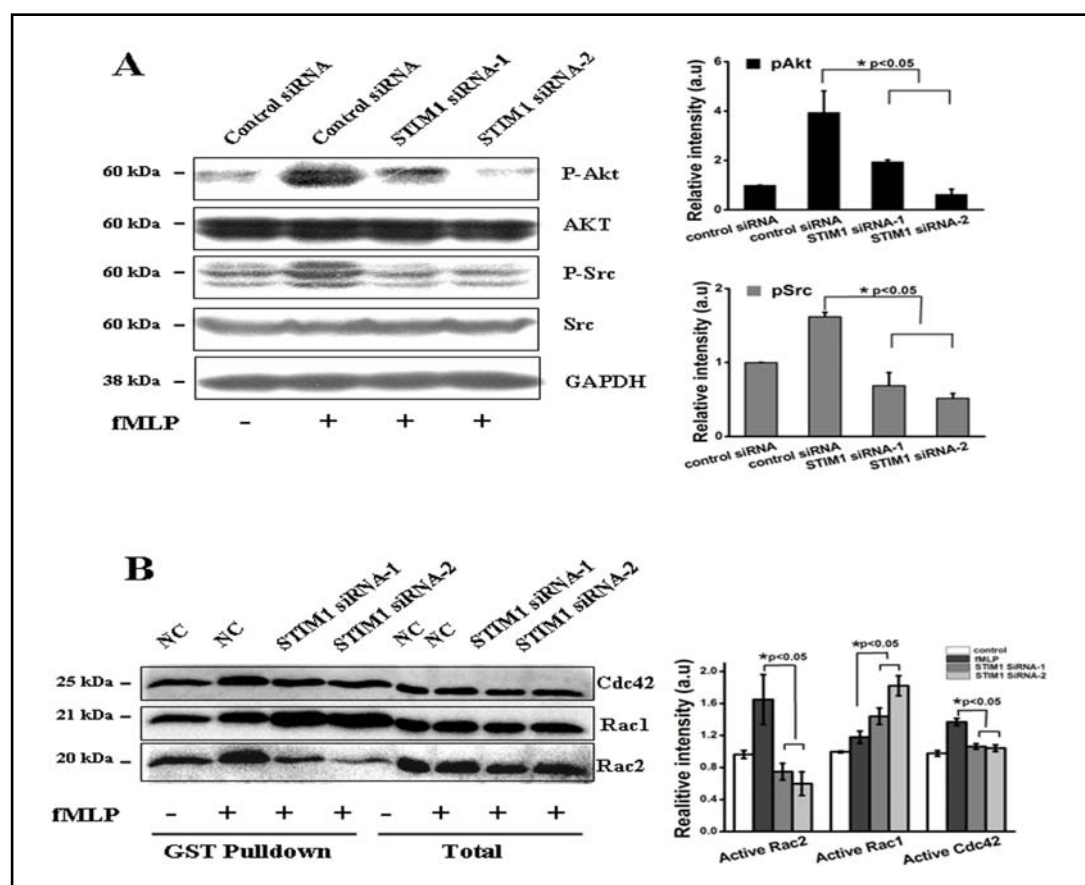
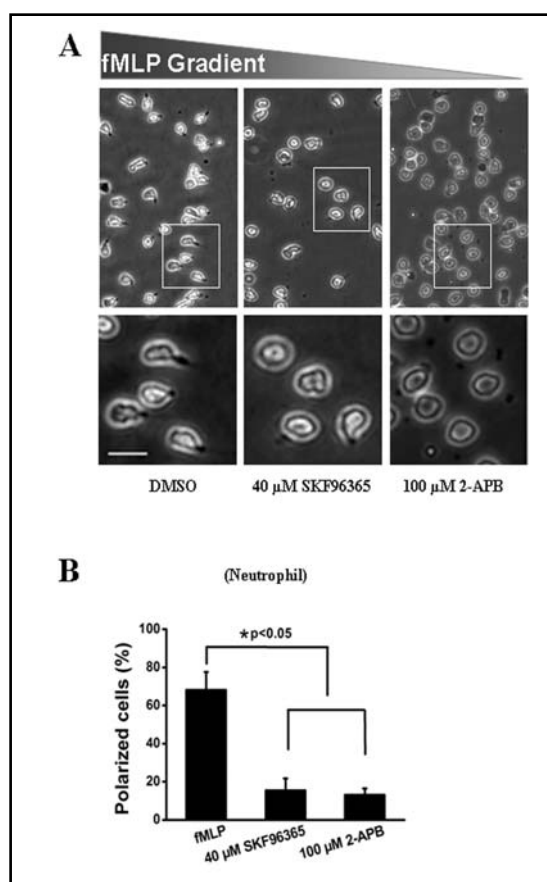


Fig. 8. Effects of STIM1 knockdown on the activation of Akt, Src, Rac2, Rac1, and Cdc42. dHL-60 cells were transfected with control siRNA, STIM1 siRNA-1, or STIM1 siRNA-2, then stimulated with fMLP (100 nM) for 1 min. The activation of Akt and Src was detected by Western blot (A). The activation of Rac2, Rac1, and Cdc42 was detected using the GST-PBD pull-down assay (B). The right part of each panel shows the relative quantities of activated Akt, Src, Rac2, Rac1 and Cdc42, expressed as means and SD (n=3). *p < 0.05, compared with the control siRNA group.

Mn²⁺ quenching and the influences by the compounds. Our observation of the inhibitory effects of SKF96365 and 2-APB on Mn²⁺ influx suggests that these two modulators do inhibit SOCE, though the possibility for them to inhibit other Ca²⁺-driven mechanisms could not be excluded in the present study. Thus, it has come to the conclusion that SOCE is involved in TG- and fMLP-induced Ca²⁺ influx. Modulation of STIM1, an important component of SOCE, by using siRNA resulted in the reduction of calcium influx as well as cell polarization. This provides further support to the conclusion that SOCE is involved in both processes, with calcium influx supposed to be the elicitor of cell polarization.

An early study revealed that SKF96365 had no effect on fMLP-stimulated activation of ERK or p38, even with the concentration extended up to 100 μ M. This suggests that fMLP-stimulated phosphorylation of ERK and p38 is not dependent on receptor-mediated Ca²⁺ entry or SOCE [55]; however, no reports have been made regarding the effect of SKF96365 on other fMLP-stimulated pathways *via* G protein-coupled receptors. In this study, both SKF96365 and 2-APB were observed to abolish fMLP-stimulated phosphorylation of Akt and Src at the same concentrations as for inhibition of cell shape polarization. Moreover, SKF96365 and 2-APB demonstrated inhibition on fMLP-induced activation of Rac2, Rac1 and Cdc42. These results suggest that fMLP-stimulated activation of Akt, Src and Rho GTPases is probably dependent on SOCE. In addition, the results from the Western blot analysis and the GST pull-down assay

Fig. 9. Effects of SOCE inhibitors on the polarization of human neutrophils toward an fMLP gradient. See the legend of Fig. 3 and 6. Human neutrophils were pretreated with 40 μ M of SKF96365, 100 μ M of 2-APB, or the vehicle (DMSO) for 30 min at 37°C, then fixed in a Zigmond chamber, and exposed to an fMLP gradient (0–100 nM). Scale bar: 20 μ m (A). The percentage of cells polarized along the direction of the gradient was expressed as means and SD (n=3). *p < 0.05, compared with the fMLP control.



showed that STIM1 knockdown abolished the fMLP-induced phosphorylation of Akt, Src, Rac2, and Cdc42. Together, these results have established that fMLP-stimulated activation of Akt, Src, and Rho GTPases are mediated by STIM1, which is an important constituent of SOCE. In addition to the indicated relevance of SOCE to cell polarization, this study is the first report on STIM1 involvement in the signaling pathways mediating chemoattractant-induced cell polarization.

Interestingly, in this study fMLP-induced activation of Rac1 was decreased by SKF96365 and 2-APB but increased by STIM1 knockdown; this suggests that Rac1 might be regulated by Ca^{2+} channels other than SOCE (i.e., ROCs or TRPM7 channel [56, 57]), which may be sensitive to SKF96365 and 2-APB, and a signaling web more complex than a pure SOCE system may exist in dHL-60 cells. Furthermore, how the influx of extracellular calcium leads to regulation of Akt, Src and Rho GTPases is still unclear, and further investigations are required for a more complete knowledge of the whole processes wherein calcium influx is engaged in cell polarization.

The inhibitory effects of both SOCE inhibitors and downregulation of STIM1 on the extracellular Ca^{2+} entry have evidenced that Ca^{2+} influx *via* SOCE is a critical step in the control of polarization of dHL-60 cells. Because PI3K-dependent and Src-dependent pathways have been identified as signaling pathways upstream to Rho GTPases in the regulation of cell polarization [24], and in the present study fMLP-activated cell polarization and signaling pathways were inhibited significantly by both SOCE inhibitors and STIM1 knockdown in dHL-60 cells, we propose a model whereby fMLP receptors elicit Ca^{2+} entry *via* SOCE initially, which activates PI3K-dependent and -independent pathways (i.e., phosphorylation of Akt and Src), followed by activation of Rac/Cdc42, and finally cell polarization is resulted. Our additional results with the inhibitors of SOCE in human neutrophils suggest that the proposed mechanisms might also be applicable to the human neutrophil.

In conclusion, we have provided evidences that SOCE may play an important role in fMLP-induced cell polarization and the activation of the Akt/Src/Rho GTPases pathways. This study also suggests potential molecular targets, such as STIM1, for therapeutic modulation (up- or down-regulation by pharmacological agents) of neutrophil chemotaxis towards either infectious agents or autoimmune challenges.

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