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The blockade of cyclopiazonic acid-induced store-operated Ca^{2+} entry pathway by YC-1 in neutrophils

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Abstract

In the presence of external Ca^{2+}, pretreatment of neutrophils with 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) inhibited the cyclopiazonic acid (CPA)-induced $[Ca^{2+}]_i$ elevation in a concentration- but not a time-dependent manner, while YC-1 had no effect on the Ca^{2+} signals in a Ca^{2+}-free medium. YC-1 failed to inhibit ATP- and interleukin-8 (IL-8)-induced $[Ca^{2+}]_i$ changes. Addition of YC-1 after cell activation strongly inhibited the CPA-induced $[Ca^{2+}]_i$ changes. In a classical Ca^{2+} readdition protocol, a similar extent inhibition of Ca^{2+} spike by YC-1 introduced either prior to or after CPA stimulation was obtained. In rat neutrophils, mRNA for endothelial differentiation gene (edg)1, edg5, edg6 and edg8, the putative targets for sphingosine 1-phosphate (S1P), could be detected. However, S1P was found to have little effect on Ca^{2+} signals. YC-1 did not inhibit but enhanced the sphingosine-induced $[Ca^{2+}]_i$ changes. Inhibition by YC-1 of CPA-induced $[Ca^{2+}]_i$ changes was not prevented by 7-nitroindazole and *N*-(3-aminomethyl)benzylacetamidine (1400W), two nitric oxide synthase (NOS) inhibitors, by aristolochic acid, a phospholipase A_2 inhibitor, or by suspension in a Na^+-deprived medium. YC-1 did not affect the mitochondrial membrane potential. Moreover, YC-1 did not alter $[Ca^{2+}]_i$ changes in response to ionomycin after CPA and formyl-Met-Leu-Phe (fMLP) stimulation in a Ca^{2+}-free medium. YC-1 had no effect on the basal $[Ca^{2+}]_i$ level, the pharmacologically isolated plasma membrane Ca^{2+}-ATPase activity, and Ba^{2+} entry into CPA-activated cells. YC-1 alone resulted in the accumulation of actin filaments in neutrophils, while significantly reduced the intensity of actin filament staining in the subsequent activation with CPA. These results indicate that YC-1 inhibited CPA-activated store-operated Ca^{2+} entry (SOCE) probably through the direct blockade of channel activation and/or the disruption of the integrity of the actin cytoskeleton necessary for supporting Ca^{2+} entry pathway in neutrophils.

Keywords: YC-1; Store-operated Ca^{2+} entry; Intracellular free-Ca^{2+}; Cyclopiazonic acid; Actin filament; Neutrophils

1. Introduction

Neutrophils are central effectors of the innate immune response to inflammation and host defense through an array of microbicidal mechanisms, including chemotaxis, phagocytosis, exocytosis and generation of reactive oxygen species. One of the early characteristic cellular reactions of neutrophils in response to extracellular signaling molecules is an increase in $[Ca^{2+}]_i$. Many cellular functions of neutrophils, such as migration, exocytosis and respiratory burst, are regulated by the Ca^{2+} signals [1,2].

Abbreviations: AA, arachidonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CPA, cyclopiazonic acid; edg, endothelial differentiation gene; ER, endoplasmic reticulum; fMLP, formyl-Met-Leu-Phe; GPCR, G protein-coupled receptor; HBSS, Hanks' balanced salt solution; IL-8, interleukin-8; IP_3, D-myo-inositol 1,4,5-trisphosphate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; NO, nitric oxide; PMCA, plasma membrane Ca^{2+}-ATPase; ROCE, receptor-operated Ca^{2+} entry; S1P, sphingosine 1-phosphate; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; sGC, soluble guanylyl cyclase; SOCE, store-operated Ca^{2+} entry; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole

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Mechanisms relevant for increase in $[Ca^{2+}]_i$ include release of Ca^{2+} from internal stores as well as a Ca^{2+} influx through the plasma membrane. It is well established that the stimulation of GPCR induces the Ca^{2+} signal via activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. IP_3 triggers rapid Ca^{2+} release from internal Ca^{2+} stores by activating IP_3 receptors and a consequent transient increase in $[Ca^{2+}]_i$ as the initial phase, which is followed by sustained $[Ca^{2+}]_i$ changes [3]. The mechanism that regulates the Ca^{2+} influx across the plasma membrane, accounting for the sustained increase in $[Ca^{2+}]_i$, remains unclear. ROCE might occur by several different mechanisms [4]. In non-excitable cells, including neutrophils, depletion of the intracellular Ca^{2+} stores induces entry of Ca^{2+} across the plasma membrane, referred to as SOCE (or capacitative Ca^{2+} entry) [5]. This Ca^{2+} entry has been proposed to replenish depleted intracellular storage compartments and serves to prolong the initial agonist-induced Ca^{2+} signal, although the actual channel responsible for ROCE or SOCE has not been identified. A transient receptor potential family of ion channels that display characteristics similar to the SOCE pathway has been reported [6]. Several hypotheses have been considered for the mechanism of SOCE. Recently, a secretion-like coupling model based on a physical and reversible trafficking of portions of the ER toward the plasma membrane has been proposed [7]. However, little is known about the cellular signals governing SOCE or the Ca^{2+} channels mediating this particular form of Ca^{2+} entry. A major problem in studying SOCE has been the lack of selective inhibitors of such channels, which has thus limited the pharmacology approaches. Study of SOCE might be even more difficult in neutrophils because their short life span makes the current molecular biology approaches impracticable.

YC-1, a NO-independent sGC activator, was first identified as an inhibitor of platelet aggregation [8]. Subsequent study noted that YC-1 not only activated sGC but also affected cGMP metabolism by the inhibition of phosphodiesterase activity [9,10]. Despite clear evidence that many biological actions of YC-1 occur through a cGMP-dependent mechanism, growing evidence supports the cGMP-independent signaling pathway in YC-1 action, including the axonoprotective action in the optic nerve [11], suppression of the hypoxic accumulation of HIF-1α in Hep3B cells [12], enhancement of lipopolysaccharide/interferon-γ-induced TNF-α formation in rat alveolar NR 8383 macrophages [13], and the antiproliferation effect in human umbilical vein endothelial cells [14]. Our previous reports revealed that YC-1 inhibits the formyl peptide-induced respiratory burst via cGMP-dependent and -independent signaling mechanisms [10], while it also inhibits $[Ca^{2+}]_i$ changes through a cGMP-independent mechanism in neutrophils [15]. The aim of this study was to further characterize the effect of YC-1 on Ca^{2+} signaling in rat neutrophils.

2. Materials and methods

2.1. Materials

Dextran T-500 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Hanks' balanced salt solution was obtained from Invitrogen. Fluo-3/AM, fura-2/AM, and fluorescein phalloidin were purchased from Molecular Probes. CPA, S1P, 7-nitroindazole, *N*-(3-aminomethyl)benzylacetamidine (1400W), JC-1, ionomycin, and oligomycin A were obtained from Calbiochem-Novabiochem. Sphingosine was obtained from Biomol Research. YC-1 (>99%) was synthesized as described previously [16]. All other reagents and chemicals were purchased from Sigma–Aldrich. The final volume of dimethyl sulfoxide (DMSO) in the reaction mixture was ≤0.5% (v/v).

2.2. Preparation of rat neutrophils

Neutrophils were isolated from Sprague–Dawley rats as described previously [17]. Briefly, fresh whole blood was obtained from the abdominal aorta and immediately mixed with EDTA. The neutrophils were purified by dextran sedimentation followed by centrifugation through Ficoll-Paque and hypotonic lysis of erythrocytes. Neutrophils were suspended in HBSS containing 10 mM HEPES (pH 7.4) and 4 mM $NaHCO_3$, and kept in an ice bath before use.

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

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fluo-3/AM for 45 min at 37 °C. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL. In some experiments, cells were suspended in Na^+-deprived HEPES buffer (124 mM *N*-methyl-D-glucamine, 4 mM KCl, 0.64 mM K_2HPO_4, 0.66 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 5.56 mM dextrose, and 15.2 mM $KHCO_3$) or in a Ca^{2+}-free medium containing 0.1 mM EDTA. Fluorescence changes were monitored with a fluorescence spectrophotometer at 535 nm with excitation at 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. The values F_{max} and F_{min} were obtained at the end of experiments by the sequential addition of 0.33% Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM.

2.4. Measurement of Ba^{2+} influx

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fura-2/AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL [17]. The entry of Ba^{2+} into the stimulated cells was measured in a Ca^{2+}-free medium followed by addition of 3 mM Ba^{2+} to

the medium. Fluorescence changes were monitored at 510 nm with excitation at 340 and 380 nm in a ratio mode.

2.5. Measurement of mitochondrial membrane potential

Neutrophils (5×10^7 cells/mL) were loaded at room temperature with 5 μM JC-1 for 10 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL. Fluorescence changes were monitored with a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) alternatively at 528 nm, with excitation at 485, and at 633 nm with excitation at 575 in a ratio mode [18]. JC-1 fluorescence has two emission peaks, with red fluorescence of J-aggregates indicating hyperpolarized mitochondria and green fluorescence (JC-1 monomers) due to low mitochondria membrane potential.

2.6. RT-PCR and electrophoresis of products

Total RNA was prepared and the PCR amplification was performed as previously described [19]. Six pairs of the following primers were synthesized: forward 5′-CTTCAGCCTCCTTGCTATCG-3′ and reverse 5′-GCAGGCAATGAAGACACTCA-3′ for the 409-bp rat edg 1; forward 5′-TCAGGGAGGGCAGTATGTTC-3′ and reverse 5′-CTGACTCTTGAAGAGGATGG-3′ for the 503-bp rat edg3; forward 5′-TTCTGGTGCTAATCGCAGTG-3′ and reverse 5′-GAGCAGAGAGTTGAGGGTGG-3′ for the 695-bp rat edg5; forward 5′-GTGCTCAACTCAGCCATCAA-3′ and reverse 5′-CTGCCAAACATTCATCATGG-3′ for the 418-bp rat edg6 [20]; forward 5′-ATCCCTTGGTGCTGAAGTTG-3′ and reverse 5′-AAGAGCACAGCCAGGTTCTC-3′ for the 356-bp rat edg8 (NM_021775); forward 5′-TATGACAACTCCCTCAAGAT-3′ and reverse 5′-AGATCCACAACGGATACATT-3′ for the 317-bp rat GAPDH (as an internal standard). For the first strand cDNA synthesis, 5 μg of total RNA was used with oligo$(dT)_{15}$. PCR amplification was performed with initial heating for 3 min at 94 °C, followed by 28 cycles of 45 s denaturation at 94 °C, annealing for 30 s at 58–68 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were resolved using a 2% agarose gel, and the sequences of these products were confirmed using a ABI 3730 sequencer (Applied Biosystems) with BigDye Terminator Cycle Sequencing kit.

2.7. Confocal microscopy

Neutrophils were fixed for 10 min with 3% paraformaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.5)) at room temperature, and then plated onto poly-L-lysine-coated coverslips. Cells were then thoroughly rinsed twice and permeabilized with 0.2% saponin. After rinsing, cells were incubated with 1% BSA followed by incubation with fluorescein phalloidin for 10 min. Cells were then washed twice and mounted with 50% glycerol. Fluorescence images were viewed with an oil objective (100×, 1.40 NA) using an upright Leica confocal laser scanning microscope system (TCSNT). F-actin was identified by its green emission at LP515 nm with excitation at 488 nm. To study the distribution and localization of intracellular fluorescence, cells were scanned; increments in the Z plane were 0.2 μm with confocal images being required throughout the central portion of cells. The fluorescence density of F-actin of total 150 cells for each test was quantified using Multi Gauge (Fuji) software and expressed as quantum level (QL) per cell.

2.8. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance; $P < 0.05$ was considered significant. Data are expressed as means ± S.D.

3. Results and discussion

3.1. Effect of YC-1 on $[Ca^{2+}]_i$ changes in response to CPA

Our previous study demonstrated that YC-1 inhibited formyl peptide-induced $[Ca^{2+}]_i$ changes in neutrophils [15]. In the presence of external Ca^{2+}, exposure of neutrophils to CPA, a specific inhibitor of the SERCA, evoked slow but long-lasting $[Ca^{2+}]_i$ changes independently of receptor activation. It is conceivable that the inhibition of Ca^{2+} uptake into ER by CPA allows the stores to be emptied, which results in the activation of SOCE. Treatment of cells with 10–50 μM YC-1 at 1 min prior to CPA stimulation showed a concentration-dependent reduction of $[Ca^{2+}]_i$ (37.2 ± 7.8, 74.1 ± 6.2, and 92.6 ± 5.7% inhibition of maximal $[Ca^{2+}]_i$, respectively; $P < 0.05$ for 10 μM YC-1, $P < 0.01$ for 30 and 50 μM YC-1 treatment) (Fig. 1A) with an IC_{50} value of about 15 μM. Inhibition of $[Ca^{2+}]_i$ changes in cells pretreated with YC-1 for 10 min (87.1 ± 6.4% inhibition of maximal $[Ca^{2+}]_i$) (Fig. 1B) occurred to the same extent as that for 1 min prior to CPA stimulation ($P > 0.05$). The lack of time-dependence clearly confirms the previous postulation that YC-1 inhibited the Ca^{2+} signal independent of cGMP [15].

In the absence of external Ca^{2+}, CPA induced a small and gradual increase in $[Ca^{2+}]_i$, reached a maximal level at 1 min, then progressively declined. The source of the Ca^{2+} leak, which gives rise to Ca^{2+} mobilization in response to CPA, is IP_3-dependent Ca^{2+} pools, which are caused by resting IP_3 levels [21]. Unlike 2-APB (100 μM), a cell-permeant antagonist of IP_3 receptors, which abolished the CPA-induced Ca^{2+} signals (data not shown), YC-1 (50 μM) had no inhibitory effects on CPA-induced responses (Fig. 1C). The results indicate that YC-1 did

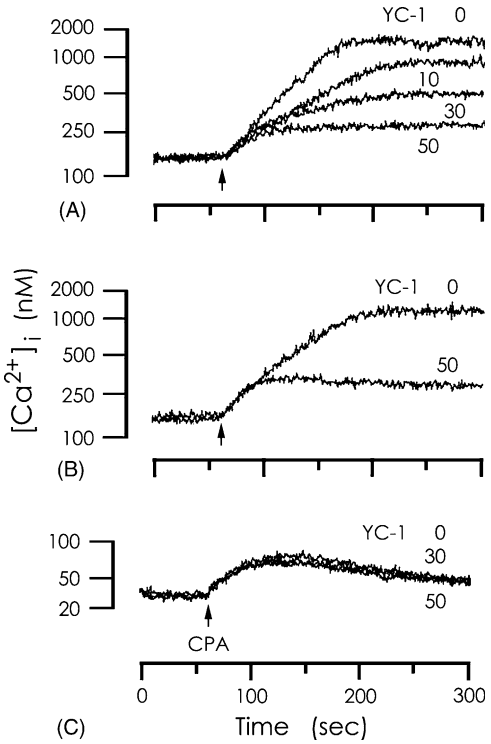

Fig. 1. Effects of YC-1 on CPA-stimulated $[Ca^{2+}]_i$ changes. Neutrophils were incubated with (A) DMSO or 10–50 μM YC-1 for 1 min, or with (B) DMSO or 50 μM YC-1 for 10 min at 37 °C in a Ca^{2+} (1 mM)-containing medium before stimulation with 5 μM CPA. (C) Neutrophils were incubated with DMSO or 30–50 μM YC-1 for 1 min in a Ca^{2+}-free medium before stimulation with 10 μM CPA. The traces shown are representative of three to four separate experiments.

not affect the phospholipase C/IP_3 signal, and that the inhibition of SOCE pathway by YC-1 not via accelerating SERCA-dependent refilling of Ca^{2+} stores.

3.2. Effect of YC-1 on $[Ca^{2+}]_i$ changes in response to ATP and IL-8

In the presence of external Ca^{2+}, ATP stimulation induced $[Ca^{2+}]_i$ changes in neutrophils. So far, seven mammalian P2X receptor proteins ($P2X_{1–7}$) and six P2Y receptors ($P2Y_{1, 2, 4, 6, 11, 12}$) have been identified [22]. P2X receptors are ATP-gated ion channels. In contrast, activation of P2Y receptors, coupled to G proteins, results in mobilization of IP_3-sensitive Ca^{2+} store. Transcripts of $P2X_5$, $P2X_7$, and all P2Y receptors, except for the $P2Y_6$ receptor, are found in blood leukocytes [23]. In the present study, application of a selective P2X purinergic agonist, 2′,3′-(4-benzoyl)-benzoyl-ATP, which is more potent than ATP at the $P2X_7$ receptor [24], had only a slight effect on $[Ca^{2+}]_i$ changes as compared to that mediated by ATP (Fig. 2A), indicating that ATP stimulated $[Ca^{2+}]_i$ changes via P2Y receptors in rat neutrophils. Addition of ATP in a Ca^{2+}-free medium induced transient and small $[Ca^{2+}]_i$ changes, and subsequent addition of Ca^{2+} resulted in robust Ca^{2+} entry into cells (Fig. 2B). Thus, exposure to ATP in the presence of external Ca^{2+} results in Ca^{2+} release and SOCE, which overlap in time.

The IL-8 receptor protein is highly expressed in human neutrophils [25]. Application of IL-8 in the presence of external Ca^{2+} induced an initial fast component followed by a smaller and more persistent one in rat neutrophils (Fig. 2D). Moreover, transient and small $[Ca^{2+}]_i$ changes in response to IL-8 were observed in a Ca^{2+}-free medium, and a subsequent entry of Ca^{2+} immediately followed the addition of Ca^{2+} (Fig. 2E). Thus, activation of cell surface IL-8 receptors induces $[Ca^{2+}]_i$ changes consisting of an initial external Ca^{2+}-independent fast component followed by a SOCE phase. The finding that YC-1 had no effect on ATP- and IL-8-induced responses (Fig. 2C and D) makes it unlikely that the YC-1 inhibition of external Ca^{2+} entry into neutrophils was attributable to its action as a non-selective Ca^{2+} channel blocker. Moreover, these results reveal that YC-1 had no appreciable effect on the Ca^{2+} signal caused by ligands other than formyl peptide for GPCRs [15]. Several studies have demonstrated that ROCE might occur by several different mechanisms besides the SOCE mechanism [4], and more than one class of SOC channel is present in leukemic HL60 cells [26]. To date, the molecular identity of any ROC and SOC channels in leukocyte remains elusive. Patterson et al. [27] have proposed that significant differences exist between the receptor-induced activation of SOCE and SOCE activation resulting from depletion of stores through a nonphysiological SERCA pump blockade. The finding that YC-1 inhibited fMLP- [15] and CPA-induced Ca^{2+} entry allowed us to speculate the existence of common signal steps or common target site in channels of these two stimuli-activated SOCE, which were affected by YC-1. It has been reported that both SERCA blocker- and fMLP-stimulated entry of Ca^{2+} are sensitive to SK&F96365, Gd^{3+}, calyculin A, okadaic acid, and pertussis toxin in human neutrophils [28].

3.3. Effect of YC-1 on Ca^{2+} entry

Exposure to SERCA blocker elevates $[Ca^{2+}]_i$ in the presence of external Ca^{2+}, which is the consequence of the activation of SOCE. Subsequent addition of YC-1 when the increase in $[Ca^{2+}]_i$ by CPA had reached a steady state immediately suppressed the Ca^{2+} signal in a concentration-dependent manner (88.2 ± 4.8% inhibition of maximal $[Ca^{2+}]_i$ at 50 μM YC-1; $P < 0.01$) (Fig. 3A) with a very similar IC_{50} value to that shown in Fig. 1A. The results reaffirm the inhibition by YC-1 of CPA-induced SOCE in neutrophils. After stimulation with CPA in a Ca^{2+}-free medium, the subsequent addition of Ca^{2+} to the medium elicited an instantaneous Ca^{2+} spike in cells, representing the overshoot response of the SOC channel. Application of YC-1 prior to addition of CPA or Ca^{2+} significantly reduced the Ca^{2+} spike (76.3 ± 4.8 and 83.6 ± 6.1% inhibition of maximal $[Ca^{2+}]_i$, respectively; both

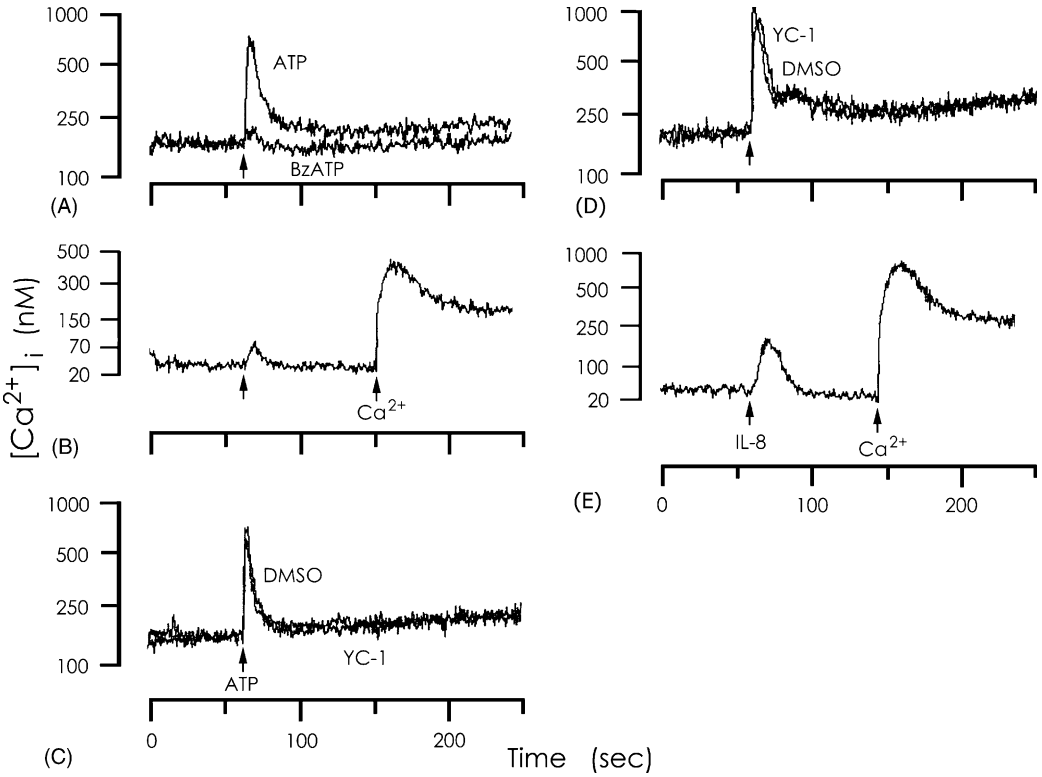

Fig. 2. Effects of YC-1 on $[Ca^{2+}]_i$ changes in response to ATP and IL-8. (A) Neutrophils were stimulated with 100 μM ATP or 300 μM 2′,3′-(4-benzoyl)-benzoyl-ATP (BzATP) in a Ca^{2+} (1 mM)-containing medium. (B) Neutrophils were stimulated with ATP (first arrow) in a Ca^{2+}-free medium followed by addition of 1 mM Ca^{2+}. Neutrophils were incubated with DMSO or 50 μM YC-1 for 1 min at 37 °C in a Ca^{2+}-containing medium before stimulation with (C) ATP or (D) 100 nM IL-8. (E) Neutrophils were stimulated with IL-8 in a Ca^{2+}-free medium followed by addition of Ca^{2+}. The traces shown are representative of three to four separate experiments.

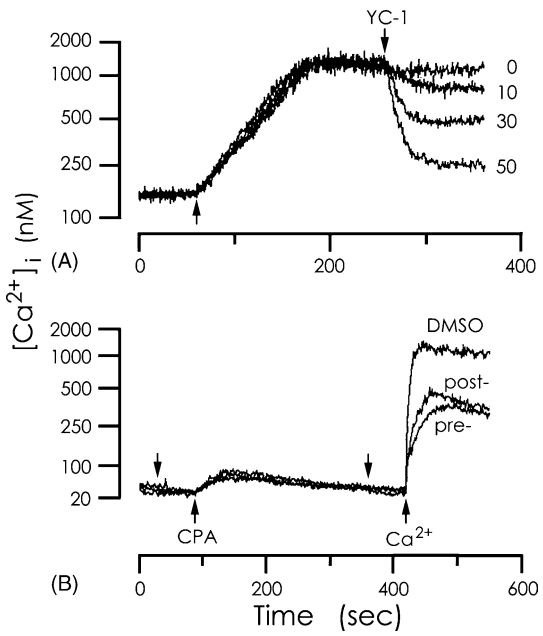

Fig. 3. Effect of YC-1 on CPA-induced Ca^{2+} entry. (A) Neutrophils were stimulated with 5 μM CPA (first arrow) in a Ca^{2+} (1 mM)-containing medium followed by addition of DMSO or 10–50 μM YC-1. (B) Neutrophils were stimulated with 10 μM CPA in a Ca^{2+}-free medium followed by supplement with 1 mM Ca^{2+}, and 50 μM YC-1 was introduced (downward arrow) at 1 min prior to the addition of either CPA (pre-) or Ca^{2+} (post-). The traces shown are representative of three to four separate experiments.

$P < 0.01$) (Fig. 3B). The lack of difference between the inhibitions of Ca^{2+} spike by YC-1 introduced prior to or after CPA stimulation ($P > 0.05$) is consistent with the results shown in Figs. 1A and 3A. Based on the secretion-like coupling mechanism of SOCE, it is likely that YC-1 effectively modified Ca^{2+} entry in the initiation and maintenance of the coupling process.

3.4. Expression of edg receptors and the effect of YC-1 on $[Ca^{2+}]_i$ changes in response to sphingosine

Lipid mediators derived from membrane sphingolipids, such as sphingosine and S1P, have been characterized as important intracellular messengers. However, their physiological functions have remained obscure. A recent report demonstrated that depletion of internal Ca^{2+} store by SERCA blocker induces S1P synthesis and proposed that S1P is a calcium influx factor mediating SOCE [29]. We therefore determined the implication of S1P in YC-1 inhibition of SOCE. The Ca^{2+}-mobilizing effect of S1P is often mediated through GPCR. Edg receptors are GPCR for S1P. Recently, eight different edg receptors have been cloned, of which edg1, edg3, edg5, edg6, and edg8 receptors are the putative targets for S1P [22]. To determine the S1P receptors expressed in rat neutrophils, RT-PCR was performed with primers designed to amplify

the cDNA of edg receptor isoforms. Of the five putative S1P receptors, four pairs of primers, namely those complementary to edg1, edg5, edg6, and edg8, produced a single PCR product band of the expected size for each product in agarose gel electrophoresis (Fig. 4A). Clear bands were detected for edg1, edg5, and edg6 receptors, but less extent for the edg8 receptor was detected. Comparison of sequences obtained with the GenBank database (data not shown) demonstrated 100, 100, 99, and 100% identity between PCR products and the published rat edg1, edg5, edg6, and edg8, respectively. There was no detectable edg3 expression in rat neutrophils. RT-PCR showed the presence of mRNA. Whether the protein is expressed to any significant extent is not known yet because the antibodies for specific edg receptor isoforms are not available. The expression of multiple S1P receptors in rat neutrophils suggests that S1P might play a role in the physiological function. However, neutrophils show only slight $[Ca^{2+}]_i$ changes in response to 100 μM S1P (8.2 ± 3.5% of maximal $[Ca^{2+}]_i$ caused by 0.3 μM fMLP) (Fig. 4B). It is plausible that the S1P signal is not associated with $[Ca^{2+}]_i$ changes in rat neutrophils and, therefore, unlikely to be involved in YC-1 inhibition of SOCE.

It has been reported that sphingosine induces a biphasic increase in $[Ca^{2+}]_i$ with an initial transient peak followed by a sustained increase in differentiated neutrophil-like HL60 cells [30]. The sustained sphingosine-induced Ca^{2+} influx does not appear through the SOC channel. In the presence of external Ca^{2+}, sphingosine evoked a monophasic response with a sustainned $[Ca^{2+}]_i$ increase in a concentration-dependent manner in rat neutrophils (Fig. 4C). Interestingly, YC-1 failed to inhibit but instead enhanced sphingosine-induced $[Ca^{2+}]_i$ changes (225.3 ± 12.3 nM for control versus 397.2 ± 15.1 nM at the plateau phase; $P < 0.05$) (Fig. 4D). Our current data cannot clearly explain this phenomenon. Nevertheless, the present data indicate that YC-1 had no inhibitory effect on the non-SOCE pathway, and sphingosine might not play a role in the inhibition of the Ca^{2+} entry by YC-1.

3.5. Effects of 7-nitroindazole, 1400W, aristolochic acid, and Na^+-deprivation on the inhibition by YC-1

NO has been proposed to control Ca^{2+} transport via cGMP-dependent and -independent pathways. NO suppressed SOCE activated by SERCA blocker in HEK293 cells [18] through cGMP-independent inhibition of mitochondrial function, thus causing the disruption of local Ca^{2+} handling by mitochondria and the promotion of Ca^{2+}-mediated feedback inhibition of the SOCE pathway. A functional neuronal nitric oxide synthase (nNOS) system was found, while no evidence exists for inducible nitric oxide synthase (iNOS) in normal rat neutrophils [31]. However, iNOS expression is upregulated in neutrophils after administration of endotoxin. The results indicate that 7-nitroindazole, a relatively selective inhibitor of nNOS, and 1400W, a potent and selective inhibitor of iNOS, failed to attenuate the inhibition of CPA-induced Ca^{2+} entry by YC-1 (Fig. 5A–C), thus obviating the role for NO generation. It has been clearly demonstrated that YC-1 activates sGC without releasing NO [32].

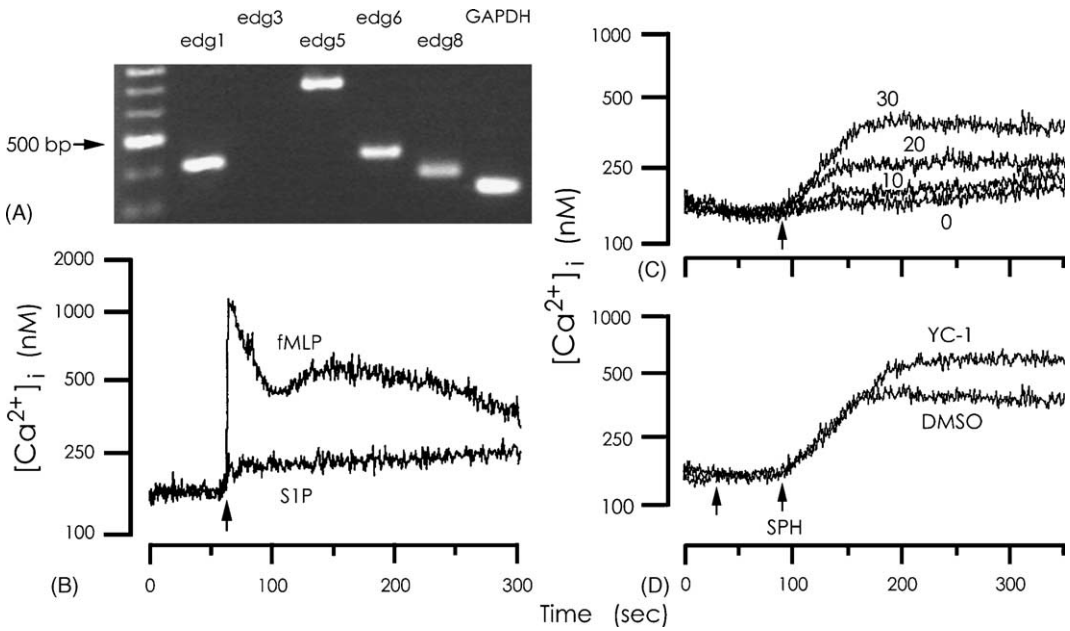

Fig. 4. Expression of edg isoforms mRNA and the effect of YC-1 on sphingosine-stimulated $[Ca^{2+}]_i$ changes. (A) The agarose gel electrophoresis of RT-PCR products of edg isoforms and GAPDH. Result presented is representative of three independent experiments with similar results. Neutrophils were stimulated with (B) 0.3 μM fMLP or 100 μM sphingosine 1-phosphate (S1P), or (C) DMSO or 10–30 μM sphingosine (SPH) in a Ca^{2+} (1 mM)-containing medium. (D) Neutrophils were incubated with DMSO or 50 μM YC-1 for 1 min at 37 °C in a Ca^{2+}-containing medium before stimulation with 30 μM sphingosine. The traces shown are representative of three to four separate experiments.

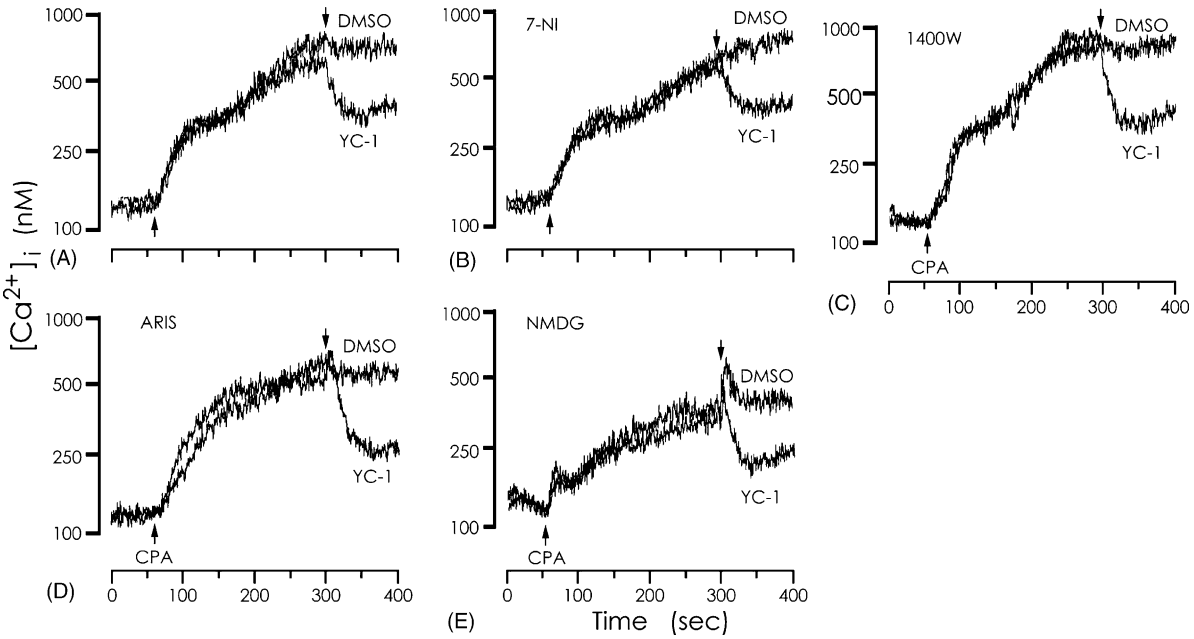

Fig. 5. Effects of 7-nitroindazole, 1400W, aristolochic acid, and Na^+-deprivation on YC-1 inhibition of CPA-induced Ca^{2+} entry. Neutrophils were incubated with (A) DMSO, (B) 250 μM 7-nitroindazole (7-NI), (C) 100 μM 1400W for 10 min, or (D) 50 μM aristolochic acid (ARIS) for 1 min at 37 °C in a Ca^{2+} (1 mM)-containing medium before stimulation (upward arrow) with 5 μM CPA followed by addition (downward arrow) of DMSO or 30 μM YC-1. In some experiments, neutrophils were stimulated with CPA followed by addition of DMSO or YC-1 in a Na^+-deprived medium (NMDG). The traces shown are representative of three to four separate experiments.

Growing evidence indicates that AA stimulates the non-SOCE, but inhibits the SOCE pathway. AA inhibited the SERCA blocker-induced SOCE pathway in human neutrophils [33] and by action via NO in mouse parotid acini [34]. It is well documented that activation of phospholipase A_2 hydrolyses arachidonyl phospholipid into AA and lysophospholipid. Pretreatment of cells with aristolochic acid, a phospholipase A_2 inhibitor, did not prevent the inhibition of CPA-induced Ca^{2+} entry by YC-1 (Fig. 5D), which suggests that the AA generation is probably not linked to the YC-1 inhibition. It is believed that the plasmalemmal Na^+–Ca^{2+} exchanger plays a role in the removal of Ca^{2+} from the cytosol. There is no indication that YC-1 acts via promoting Na^+–Ca^{2+} exchange activity on the plasma membrane, because inhibition of Ca^{2+} entry by YC-1 was obtained in normal, as well as in Na^+-deprived medium to prevent the occurrence of Na^+–Ca^{2+} exchange (Fig. 5E).

3.6. Effect of YC-1 on mitochondria potential and Ca^{2+} release from internal stores

Mitochondria are well known participants in the regulation of $[Ca^{2+}]_i$ homeostasis, capable of modulating cytosolic Ca^{2+} signals. It is commonly assumed that human neutrophils possess few, if any, functional mitochondria based on electron microscope data. Fluorescent indicators of mitochondrial function revealed that neutrophils possess a complex mitochondrial network that extends through the cytoplasm [35]. Functional mitochondria appear to maintain SOCE by effective sequestration of subplasmalemmal Ca^{2+} and by the consequent attenuation of Ca^{2+}-induced inactivation of SOC channels [36]. Mitochondrial Ca^{2+} uptake is driven by the membrane potential that is maintained by extrusion of H^+. Thus, hyperpolarization increases the driving force for mitochondrial Ca^{2+} uptake. CCCP, an uncoupler of mitochondria function, dissipates mitochondrial H^+ gradients and inactivates SOCE channels [37]. To test whether YC-1 affects mitochondrial function in neutrophils, we studied the effect of YC-1 on mitochondrial membrane potential using JC-1 as a reporter dye. JC-1 fluorescence was reduced by CCCP in a concentration-dependent manner. Because the fluorescence remained relatively unchanged by YC-1 (Fig. 6A), it excluded the role of mitochondria in YC-1 inhibition of Ca^{2+} entry.

In the absence of external Ca^{2+}, application of ionomycin, the Ca^{2+} ionophore, induced the increase in $[Ca^{2+}]_i$ as a consequence of Ca^{2+} release from internal stores (Fig. 6B). Besides ER and mitochondria, various distinct cellular organelles, including Golgi apparatus, nucleus, and lysosomes, can act as Ca^{2+} stores. Davies and Hallett [38] have demonstrated the existence of two distinct Ca^{2+} storage locations in neutrophils, in which Ca^{2+} storage site deep within the neutrophil released by SERCA blocker and fMLP. Addition of fMLP together with CPA in a Ca^{2+}-free medium induced small $[Ca^{2+}]_i$ changes. However, subsequent $[Ca^{2+}]_i$ changes, which followed the addition of ionomycin, were significantly reduced ($52.7 \pm 6.3\%$ inhibition of maximal $[Ca^{2+}]_i$; $P < 0.01$) (Fig. 6C). Stimulation of GPCRs can release the Ca^{2+} stored in the ER

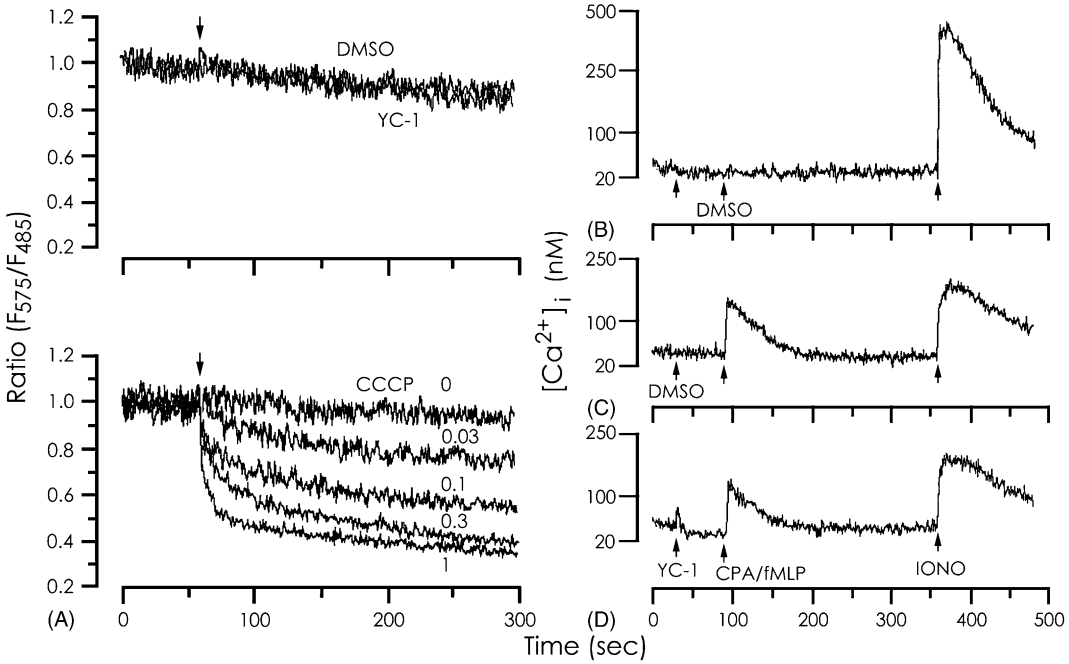

Fig. 6. Effects of YC-1 on mitochondrial membrane potential and ionomycin-induced internal Ca^{2+} release. (A) DMSO, 50 μM YC-1, or 0.03-1 μM CCCP was added (downward arrow) to JC-1-loaded neutrophils at 37 °C in a Ca^{2+} (1 mM)-containing medium. Fluorescence changes were measured in a ratio mode. Fluo-3-loaded neutrophils were incubated with DMSO or 50 μM YC-1 for 1 min before addition of (B) DMSO or (C, D) 10 μM CPA plus 0.3 μM fMLP in a Ca^{2+}–free medium, then subsequent stimulation with 1 μM ionomycin (IONO). The traces shown are representative of three to four separate experiments.

and Golgi apparatus [39] by activation of the IP_3 receptors in these organelles. Therefore, ER and Golgi store emptying may account, at least in part, for the reduction of $[Ca^{2+}]_i$ changes in response to the subsequent addition of ionomycin. Accumulation of Ca^{2+} in mitochondria is readily released by Ca^{2+} ionophore [40]. The result that YC-1 failed to modify the $[Ca^{2+}]_i$ changes in response to the subsequent addition of ionomycin (Fig. 6D) further strengthened the notion that mitochondria, perhaps including other Ca^{2+} stores, play no role in the inhibition of Ca^{2+} entry by YC-1.

3.7. Effect of YC-1 on membrane Ca^{2+}-ATPase activity and Ba^{2+} influx

It is conceivable that the $[Ca^{2+}]_i$ increase caused by activation of the channels is followed by activation of the SERCA in the ER and of the Ca^{2+}-ATPase in the plasma membrane. These contribute equally to removal of cytosolic free Ca^{2+}, and stabilizing $[Ca^{2+}]_i$ at a plateau determined by the relative activities of the Ca^{2+} pump and Ca^{2+} channels. In the presence of EDTA, YC-1 had no effect on the resting $[Ca^{2+}]_i$ (Fig. 7A). In addition, when cells were activated with CPA in the presence of EDTA, subsequent addition of YC-1 after $[Ca^{2+}]_i$ return to the basal level failed to reduce the $[Ca^{2+}]_i$ level (Fig. 7B). These results preclude the roles of Ca^{2+} uptake into mitochondria and ER in the inhibition of the Ca^{2+} signal by YC-1. Therefore, Ca^{2+} efflux across the plasma membrane through PMCA may be the other Ca^{2+} clearance pathway targeted by YC-1. To address the effect of YC-1 on PMCA, we attempted to isolate the PMCA activity pharmacologically. Neutrophils were stimulated with fMLP/CPA to maximally empty the internal Ca^{2+} store, inhibit SERCA, and to induce Ca^{2+} entry in a Na^+-deprived medium containing 1 μM CCCP to prevent mitochondrial Ca^{2+} uptake and

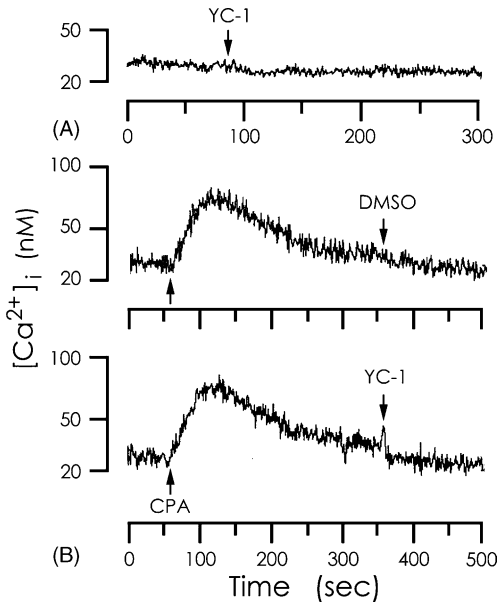

Fig. 7. Effect of YC-1 on the basal levels of $[Ca^{2+}]_i$. (A) Addition of 50 μM YC-1 to neutrophil suspensions in a Ca^{2+}-free medium. (B, C) Neutrophils were stimulated with 10 μM CPA followed by addition of DMSO or YC-1 in a Ca^{2+}-free medium containing 0.1 mM EDTA.

1 μM oligomycin A to inhibit the mitochondrial ATP synthase and prevent ATP consumption. This treatment caused a large increase in $[Ca^{2+}]_i$ (Fig. 8A). Subsequent removal of external Ca^{2+} with 1 mM EDTA evoked a slow decline in $[Ca^{2+}]_i$ possibly due to Ca^{2+} clearance by PMCA. Under these conditions, the Ca^{2+} clearance rate was relatively consistent between experiments from YC-1 and vehicle treatment (Fig. 8B). Subsequent YC-1 addition, in the absence of EDTA, retained reduction of the Ca^{2+} signal in cells (Fig. 8C). These findings suggest that YC-1 did not affect PMCA activity.

Certain Ca^{2+} influx pathways in neutrophils are permeable for Mn^{2+} [41]. Mn^{2+} is not a substrate for the Ca^{2+} pump and hence is a surrogate of Ca^{2+} influx. Our previous report indicated that YC-1 (50 μM) nearly abolished the CPA-induced Mn^{2+} influx into neutrophils [15]. Like Mn^{2+}, Ba^{2+} is not pumped by Ca^{2+}-ATPase either into internal stores or out of the cell [42] and hence can be used as a surrogate for Ca^{2+} to trace channel activity. CPA was added in a Ca^{2+}-free medium with subsequent 3 mM Ba^{2+} addition resulting in an increase in Ba^{2+} entry into cells. Surprisingly, YC-1 did not modify Ba^{2+} influx (Fig. 8D). Although Ba^{2+} can be used to separate effects on Ca^{2+} pump activity from the regulation of channel activity, the difference in the YC-1 effect between the entry of Mn^{2+} and Ba^{2+} makes it unlikely that YC-1-inhibited Ca^{2+} entry can be attributed to promotion of Ca^{2+}-mediated autoregulation. It has been proposed that Ca^{2+} and Ba^{2+} enter neutrophils through different pathways, and that certain Mn^{2+}-permeable cation entry pathways are impermeable for Ba^{2+} [41]. The Ca^{2+}/Mn^{2+} entry pathway opened by the SERCA blocker in human neutrophils is the same that activates on emptying the Ca^{2+} stores [43]. It is plausible that YC-1 blocked the Ca^{2+}/Mn^{2+} but not the Ca^{2+}/Ba^{2+}-permeable cation entry pathways in neutrophils.

3.8. Effect of YC-1 on intracellular F-actin distribution and content

Based on the secretion-like coupling model, Ca^{2+} store-depletion leads to facilitation of the coupling between portions of the ER and the plasma membrane. This process is dependent on actin filament reorganization. Unstimulated neutrophils show a diffuse cytoplasmic distribution of F-actin. Upon activation, neutrophils showed a transformation of G-actin to F-actin and a shift of F-actin, which appeared to be associated with the cell outline [44]. This actin remodeling consists of an initial net depolymerization, whereas stabilization of the cortical actin network preventing the constitutive interaction between the ER and plasma membrane. This is followed by a net increase in the actin filament content, whereas disruptions of the actin network inhibiting the maintenance of coupling between proteins therein [45]. Thus, the actin cytoskeleton has been shown to play a dual role in the initiation and maintenances of SOCE.

In this study, neutrophils were fixed and stained with fluorescein phalloidin, a fluorescent probe for F-actin, and examined by confocal laser microscopy. In the resting cells, actin filaments were organized in a thin cortical layer located beneath the membrane and had a diffuse cytoplasmic distribution (Fig. 9A). Cells treated with YC-1

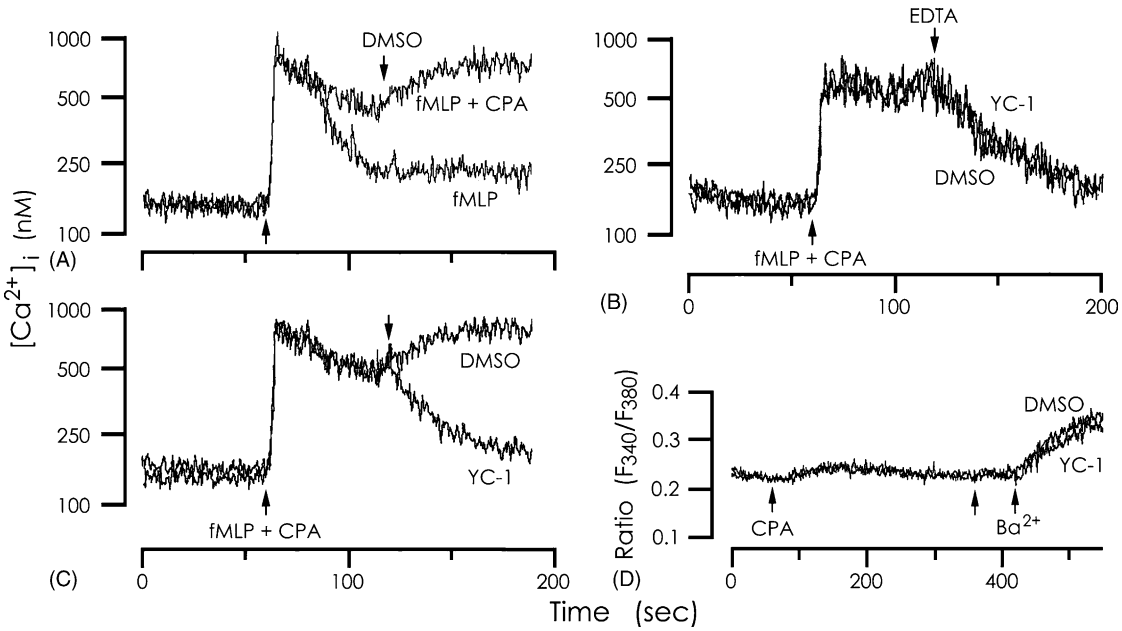

Fig. 8. Effects of YC-1 on PMCA activity and Ba^{2+} influx. Neutrophils were stimulated (A) with 0.3 μM fMLP alone or with 0.3 μM fMLP plus 10 μM CPA followed by addition of DMSO, or stimulated with fMLP plus CPA followed by addition (downward arrow) of DMSO or 50 μM YC-1 either (C) alone or (B) together with 1 mM EDTA in a Ca^{2+} (0.3 mM)-containing Na^+-deprived medium supplemented with 1 μM oligomycin A and 1 μM CCCP. (D) Fura-2-loaded neutrophils were stimulated with 10 μM CPA in a Ca^{2+}-free medium followed by supplement with 3 mM Ba^{2+}, and DMSO or YC-1 was introduced at 1 min prior to the addition of Ba^{2+}. Fluorescence changes were measured in a ratio mode. The traces shown are representative of three to four separate experiments.

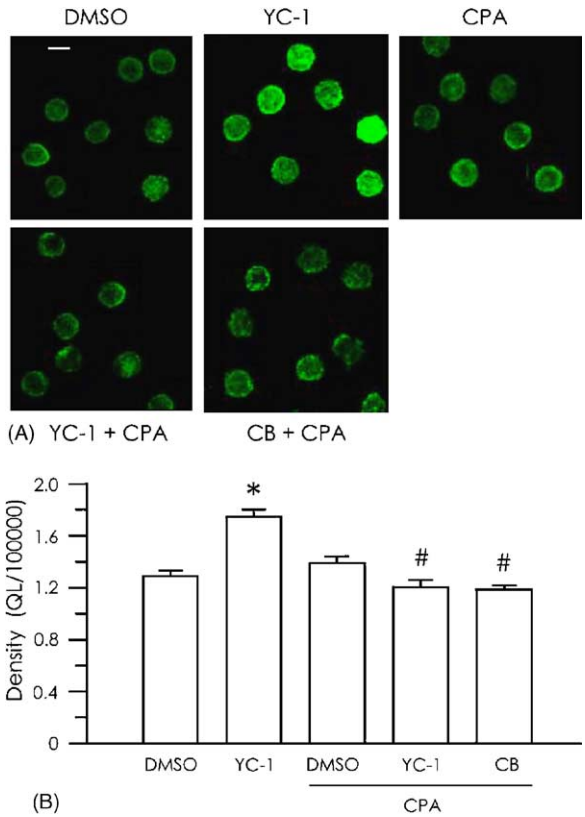

Fig. 9. Effects of YC-1 on the distribution and the content of cellular F-actin. (A) Addition of DMSO, 30 μM YC-1, or 10 μM CPA to neutrophil suspensions for 3 min, or pretreatment of cells with YC-1 or 5 μg/mL of cytochalasin B (CB) for 1 min before CPA stimulation for 3 min in a Ca^{2+} (1 mM)-containing medium. After fixation, cells were then stained with fluorescein phalloidin. Confocal images are representative of three separate experiments. Scale bar (upper left panel) is 10 μm. (B) The fluorescence density was quantified and expressed as quantum level (QL). Values are means ± S.D. of three independent experiments. $^{*}P < 0.01$, as compared with the DMSO only-treated group (first column). $^{\#}P < 0.01$, as compared with the DMSO-pretreated followed by CPA stimulation group (third column).

showed a similar morphology, but there was an increased intensity of actin filaments, which diffused cytoplasmic distribution. After exposure to CPA, the actin filaments became condensed and were denser than those observed in control cells. Treatment with YC-1 followed by CPA challenge did not change the morphology; interestingly, it significantly reduced the actin filaments accumulated in cells (Fig. 9B). Pretreatment of cells with cytochalasin B, a membrane-permeant inhibitor of actin polymerization, the cortical F-actin band appeared discontinuous and wider with some aggregate or patches at the cell cortex and in the cell interior. YC-1 inhibited the CPA-induced Ca^{2+} entry probably by depolymerization and reorganization of actin filaments, in which maintenance of the integrity of the actin cytoskeleton is necessary for the CPA activation of Ca^{2+}/Mn^{2+}-permeable cation entry pathways in neutrophils.

Actin filament reorganization is modulated by small G protein of the Rho family and tyrosine kinase [46]. The involvement of tyrosine kinase in SOCE is entirely mediated by the actin cytoskeleton, rather than through phosphorylation of a Ca^{2+} entry channel [47]. Our previous report indicated that YC-1 inhibited the fMLP-induced protein tyrosine phosphorylation in rat neutrophils [15]. It is plausible that this inhibitory effect involved in the blockade of Ca^{2+} entry through SOCE pathway. However, a recent report indicated that the inhibition of protein tyrosine phosphorylation after initiation of SOCE did not reverse SERCA blocker-induced Ca^{2+} entry in human platelets [47]. This observation was inconsistent with the result that YC-1 inhibited Ca^{2+} entry after CPA activation as shown in Fig. 3. The molecular mechanisms implicated in the YC-1 inhibition of Ca^{2+} entry remain to be clarified. It has been reported that rearrangement of the actin cytoskeleton regulates integrin-mediated adhesion of activated neutrophils, as well as their migration and mechanical properties [48]. Therefore, it would be of interest to study whether YC-1 inhibits the neutrophil migration. This awaits further investigation.

In conclusion, our findings demonstrate that the inhibition by YC-1 of the CPA-activated Ca^{2+} entry pathway is not attributed to the sphingosine/S1P, NO, and AA signals, to the influence on mitochondrial function, or to the promotion of SERCA and PMCA activity, but is probably attributable instead to the direct blockade of channel activation and/or the disruption of integrity of the actin cytoskeleton necessary for supporting the SOCE pathway in neutrophils.

Acknowledgments

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