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Inhibition of extracellular Ca^{2+} entry by YC-1, an activator of soluble guanylyl cyclase, through a cyclic GMP-independent pathway in rat neutrophils

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Abstract

The effects of a soluble guanylyl cyclase (sGC) activator, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), on formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated $[Ca^{2+}]_i$ elevation in rat neutrophils were examined. YC-1 produced a concentration-dependent inhibition of $[Ca^{2+}]_i$ elevation. Pretreatment of neutrophils with YC-1 did not enhance its inhibitory effect. YC-1 also inhibited the $[Ca^{2+}]_i$ changes caused by ionomycin. In a biphasic model, measuring the $[Ca^{2+}]_i$ stimulation by fMLP in a Ca^{2+}-free medium followed by reintroduction of Ca^{2+}, YC-1 mainly affected Ca^{2+} influx. YC-1 also inhibited active and passive Mn^{2+} influx, and this inhibitory effect was not attenuated by the sGC inhibitor 6-anilino-5,8-quinolinequinone (LY83583). Sodium nitroprusside did not affect the fMLP-stimulated $[Ca^{2+}]_i$ changes. Pretreatment of neutrophils with the cyclic GMP-dependent protein kinase inhibitor 8-(4-chlorophenylthio) guanosine-3′,5′-monophosphorothioate, Rp-isomer (Rp-8-pCPT-cGMPS), LY83583, the protein phosphatase 2B inhibitor cyclosporin A, or the protein kinase inhibitor staurosporine did not attenuate the inhibition of $[Ca^{2+}]_i$ by YC-1. YC-1 inhibited the fMLP-stimulated protein tyrosine phosphorylation. These results indicate that cyclic GMP does not play an important role in the regulation of $[Ca^{2+}]_i$ in rat neutrophils. Inhibition of fMLP-stimulated $[Ca^{2+}]_i$ changes by YC-1 is mainly via the blockade of Ca^{2+} entry through the inhibition of tyrosine kinase activity, but not the stimulation of protein kinase C and protein phosphatase 2B.

Keywords: Neutrophil; YC-1; Intracellular free calcium; Cyclic GMP; Protein tyrosine phosphorylation

1. Introduction

sGC exists as a heterodimeric hemoprotein [1] and acts by increasing intracellular cyclic GMP levels to mediate numerous cellular processes [2]. To understand the involvement of the cyclic GMP signaling pathway in cellular processes, there is a need to selectively change cellular cyclic GMP levels. The types of sGC activators known thus far are NO, CO, and YC-1 [3-(5′-hydroxymethyl-2′-furyl]-1-benzyl indazole]. NO has emerged as a key cellular messenger for a number of biological functions. NO activates sGC by interacting with the heme moiety to form a five-coordinated complex by cleavage of the Fe—His bond [3]. However, NO has other cyclic GMP-independent effects, including nitrosylation and nitration of proteins, disruption of iron–sulfur centers, and oxidation of macromolecules via the formation of peroxynitrite [4,5]. The risk of generating the adverse effects of NO by NO donors can be avoided using NO-independent activators of sGC. Activation of sGC by CO occurs without cleavage of the Fe—His bond and by the formation of a six-coordinated complex [3]. However, CO is a very weak sGC activator in comparison with NO. Recently, a direct and NO-independent sGC activator, YC-1, was introduced [6]. YC-1 was shown to be an antithrombotic agent, inhibiting platelet aggregation and proliferation of vascular smooth muscle [7,8]. Unlike NO and

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Abbreviations: CO, carbon monoxide; CPA, cyclopiazonic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; IP_3, inositol 1,4,5-trisphosphate; NO, nitric oxide; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; Rp-8-pCPT-cGMPS, 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphorothioate, Rp-isomer; sGC, soluble guanylyl cyclase; and SNP, sodium nitroprusside.

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CO, YC-1 exerts an allosteric regulation but does not affect the heme spectrum of sGC [9]. In addition, a synergistic action was observed by the combination of YC-1 with NO or CO [10]. Ligands at this allosteric site may represent a novel class of drugs that exert beneficial effects by sensitizing sGC toward its physiological activator, NO or CO. YC-1 has since been widely used as an important research tool to characterize sGC and to probe for the involvement of cyclic GMP in various biological processes.

Neutrophils play a pivotal role in inflammatory reactions and constitute the first line of host defense. In response to a variety of soluble and particulate stimuli, activated neutrophils display chemotaxis, phagocytosis, degranulation, and superoxide anion generation [11]. In general, the increase in cellular cyclic AMP levels inhibits chemoattractant-induced responses [12]. Neutrophils possess sGC and PKG [13,14]. However, the physiological role of cyclic GMP is still poorly understood. It appears that cyclic AMP and cyclic GMP play different roles in neutrophil activation. Cyclic GMP has been implicated as a modulator of neutrophil migration [15], and it inhibits or potentiates exocytosis in neutrophils [16,17]. Ca^{2+} signaling has been thought to be important in many neutrophil processes [18]. In this study, we used YC-1 to elucidate the role of cyclic GMP in the regulation of $[Ca^{2+}]_i$ in fMLP-stimulated rat neutrophils. However, our findings indicated that the inhibition by YC-1 of $[Ca^{2+}]_i$ elevation occurs in a cyclic GMP-independent manner. Therefore, testing this new type of sGC activator on biological functions may also include the risk of observing the results of cyclic GMP-independent actions.

2. Materials and methods

2.1. Materials

Dextran T-500 and enhanced chemiluminescence reagent were purchased from Amersham Pharmacia Biotech. HBSS was purchased from Gibco Life Technologies. Fluo-3 AM, fura-2 AM, and diethylenetriamine pentaacetic acid were purchased from Molecular Probes Inc. LY83583 (6-anilino-5,8-quinolinequinone) was purchased from RBI Laboratories. Rp-8-pCPT-cGMPS was purchased from Biolog Life Sciences. Wortmannin was purchased from the Calbiochem-Novabiochem Co. Mouse monoclonal antibody to phosphotyrosine was purchased from BD Transduction Laboratories. YC-1 was synthesized as described previously [19]. BW755C [3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline] was provided by Wellcome Research Laboratories. All other chemicals were purchased from the Sigma Chemical Co.

2.2. Neutrophil isolation

Rat blood was collected from the abdominal aorta, and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and the hypotonic lysis of erythrocytes [20]. Purified neutrophils containing $> 95\%$ viable cells were resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice bath before use.

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

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fluo-3 AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL. Fluorescence was monitored with a fluorescence spectrophotometer (PTI, Deltascan 4000) 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 [21]. 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 [22].

2.4. Assessment of Mn^{2+} influx

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fura-2 AM at 37° for 15 min, then diluted 5-fold with HBSS, and incubated for an additional 15 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL. The entry of Mn^{2+} into cells was measured with the fura-2 fluorescence quenching technique. Fluorescence was monitored in the presence or absence of 1 mM Ca^{2+} and 10 μM CPA at 510 nm with excitation at 360 nm [23,24], and fluorescence intensity declined as Mn^{2+} was added. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of an experiment, indicating that $< 5\%$ of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2.

2.5. Immunoblotting analysis

Cells were preincubated with test drugs for the indicated time before stimulation. Reactions were quenched by the addition of a stopping solution (20% trichloroacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 7 μg/mL of leupeptin and pepstatin). Proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] and probed with anti-phosphotyrosine antibody [1:1000 (v/v) dilution in TBST buffer with 0.5% (w/v) non-fat dried milk]. Detection was made using an enhanced chemiluminescence reagent.

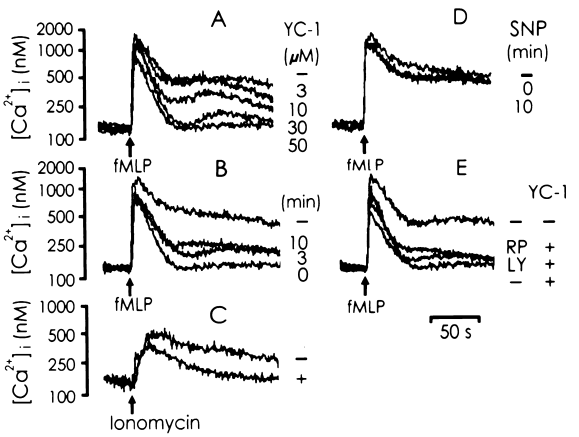

Fig. 1. Effect of YC-1 and SNP on the fMLP- or ionomycin-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils. Fluo-3-loaded cells in HBSS containing 1 mM Ca^{2+} were (A) stimulated with 0.3 μM fMLP in combination with the indicated concentrations of YC-1; preincubated with (B) 50 μM YC-1 or (D) 1 mM SNP for the indicated time periods at 37° before stimulation with 0.3 μM fMLP; (C) stimulated with 0.2 μM ionomycin in combination with or without 50 μM YC-1; and (E) preincubated with DMSO, 100 μM Rp-8-pCPT-cGMPS (RP) or 10 μM LY83583 (LY) at 37° for 10 min before stimulation with 0.3 μM fMLP in combination with or without 50 μM YC-1. The final volume of DMSO in the reaction mixture was 0.5%. The data presented are representative of three independent experiments with similar results.

3. Results and discussion

3.1. Effect of YC-1 on $[Ca^{2+}]_i$

Activation of neutrophils via the cell surface fMLP receptor causes a G protein-dependent activation of PLC that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP_3 [25], which, in turn, causes a transient increase of $[Ca^{2+}]_i$ due to Ca^{2+} release from the internal Ca^{2+} stores, followed by a sustained elevation of $[Ca^{2+}]_i$ due to Ca^{2+} entry from the extracellular medium [26]. The fMLP-stimulated elevation of $[Ca^{2+}]_i$ was inhibited by YC-1, added simultaneously with fMLP, in a concentration-dependent manner (Fig. 1A). Preincubation of neutrophils with YC-1 (50 μM) did not enhance the inhibitory effect (Fig. 1B). It has been reported that YC-1 (60 μM) suppresses IP_3 formation and the increase of $[Ca^{2+}]_i$ in activated platelets [7]. The inhibition of the late sustained phase but not the initial rapid transient phase of $[Ca^{2+}]_i$ elevation by YC-1 suggested that this compound probably did not affect significantly the PLC activity in rat neutrophils. YC-1 (50 μM) inhibited the ionomycin-stimulated $[Ca^{2+}]_i$ elevation (Fig. 1C), further implying the involvement of a PLC/IP_3-independent pathway because ionomycin complexes and transports Ca^{2+} in a one-to-one stoichiometry [27], and mobilization of intracellular Ca^{2+} by ionomycin is independent of IP_3 formation.

3.2. Role of cyclic GMP in the regulation of $[Ca^{2+}]_i$

YC-1 and an NO donor, SNP, increased cellular cyclic GMP levels in platelets in a concentration- and time-dependent manner [7]. The cyclic GMP system is an important component in the modulation of $[Ca^{2+}]_i$ by a mechanism involving activation of PKG in smooth muscle cells [28]. However, the role of cyclic GMP in the regulation of $[Ca^{2+}]_i$ in platelets is uncertain [29]. Neutrophils possess sGC, which is activated by SNP [30]. In fact, YC-1 (30 μM) increased the cellular cyclic GMP levels in rat neutrophils (1.47 ± 0.05 vs 0.37 ± 0.05 pmol/10^7 cells, treated vs control values), and this effect was greatly suppressed by an sGC inhibitor, LY83583 (0.46 ± 0.13 pmol/10^7 cells at 10 μM). The observation that neutrophils pretreated with SNP (1 mM) for various time periods did not show significant inhibition of $[Ca^{2+}]_i$ (Fig. 1D), together with a previous report that SNP and dibutyryl cyclic GMP, a cell-permeant analogue of cyclic GMP, had no effect on the rise in $[Ca^{2+}]_i$ induced by fMLP at a maximal concentration [31], argues against the critical role of cyclic GMP in the regulation of $[Ca^{2+}]_i$ in neutrophils. The cell-permeant PKG inhibitor Rp-8-pCPT-cGMPS antagonizes the YC-1-induced relaxation of aortic rings [32]. Pretreatment of neutrophils with 100 μM Rp-8-pCPT-cGMPS or 10 μM LY83583 did not attenuate the inhibition of fMLP-stimulated $[Ca^{2+}]_i$ elevation by YC-1 (Fig. 1E), precluding the involvement of a cyclic GMP/PKG signaling pathway in the regulation of $[Ca^{2+}]_i$. In agreement with this notion, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a specific inhibitor of sGC [33], at concentrations up to 60 μM failed to reverse the inhibition of YC-1 (data not shown). However, a recent report indicated that ODQ interferes with various heme protein-dependent processes and lacks specificity for sGC [34].

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

In an experiment to determine the effect of YC-1 on $[Ca^{2+}]_i$ in a Ca^{2+}-free medium, Ca^{2+} release from internal stores contributed to the observed fMLP-stimulated changes in $[Ca^{2+}]_i$. This was most likely mediated through the formation of IP_3. In contrast, the changes observed in $[Ca^{2+}]_i$ following the reintroduction of Ca^{2+} in the medium represented entry of extracellular Ca^{2+}. Neutrophils as non-excitable cells do not possess a voltage-dependent Ca^{2+} channel. Although the mechanism for the Ca^{2+} entry pathway is still obscure, the capacitative Ca^{2+} entry model (store-operated Ca^{2+}-entry pathway), in which depletion of the Ca^{2+} stores generates a signal that induces Ca^{2+} influx from the extracellular medium, seems applicable in neutrophils [26]. YC-1, added simultaneously with fMLP, slightly inhibited the $[Ca^{2+}]_i$; however, it concentration-dependently suppressed the changes in $[Ca^{2+}]_i$ caused by the subsequent addition of Ca^{2+} (Fig. 2A). The latter response was also confirmed when YC-1 was added simultaneously

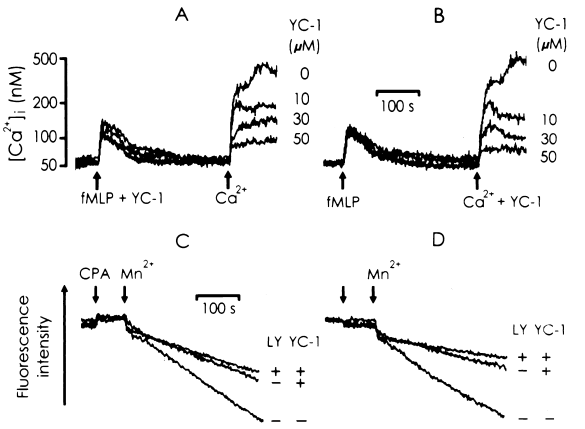

Fig. 2. Effect of YC-1 on fMLP-stimulated changes in $[Ca^{2+}]_i$ and on Mn^{2+} influx. Fluo-3-loaded cells in Ca^{2+}-free HBSS were (A) stimulated with 0.3 μM fMLP in combination with the indicated concentrations of YC-1 and subsequently added 0.5 mM Ca^{2+}, and (B) stimulated with 0.3 μM fMLP and subsequently added 0.5 mM Ca^{2+} in combination with the indicated concentrations of YC-1. Fura-2-loaded cells were suspended (C) in medium containing 1 mM Ca^{2+}, preincubated with DMSO or 20 μM LY83583 (LY) at 37° for 1 min followed by DMSO or 30 μM YC-1 for an additional 1 min, and subsequently exposed to 10 μM CPA and 0.5 mM Mn^{2+} as indicated; and (D) in Ca^{2+}-free medium, preincubated with DMSO or 20 μM LY83583 (LY) at 37° for 1 min followed by DMSO or 30 μM YC-1 (1st arrow), and subsequently exposed to 50 μM Mn^{2+} as indicated. The final volume of DMSO in the reaction mixture was 0.5%. The data presented are representative of three independent experiments with similar results.

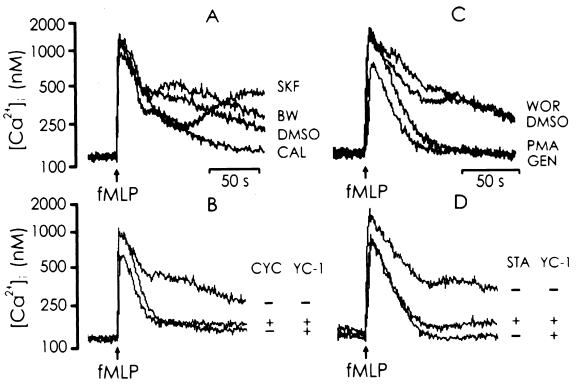

Fig. 3. Effect of several signaling step inhibitors on the inhibition by YC-1 of $[Ca^{2+}]_i$ elevation. Fluo-3-loaded cells in HBSS containing 1 mM Ca^{2+} were (A) preincubated with DMSO, 30 μM SKF525A (SKF), 30 μM BW755C (BW), or 0.1 μM calyculin A (CAL) for 3 min before stimulation with 0.3 μM fMLP; (B) preincubated with DMSO or 1 μM cyclosporin A (CYC) for 3 min before stimulation with 0.3 μM fMLP in combination with or without 50 μM YC-1; (C) preincubated with DMSO or 3 μM wortmannin (WOR) for 3 min before stimulation with 0.3 μM fMLP, or stimulated with 0.3 μM fMLP in combination with 0.1 μM PMA or 100 μM genistein (GEN); and (D) preincubated with DMSO or 150 nM staurosporine (STA) for 3 min before stimulation with 0.3 μM fMLP in combination with or without 50 μM YC-1. The final volume of DMSO in the reaction mixture was 0.5%. The data presented are representative of three independent experiments with similar results.

with the reintroduction of Ca^{2+} (Fig. 2B). These results suggest that the inhibition of fMLP-stimulated $[Ca^{2+}]_i$ elevation by YC-1 is mainly caused by the blockade of Ca^{2+} entry. This mode of action also explains the inhibition of the ionomycin-induced changes in $[Ca^{2+}]_i$ by YC-1 because low concentrations of ionomycin induce Ca^{2+} influx secondary to the ionophore-mediated emptying of the Ca^{2+} stores [35].

Mn^{2+}-mediated quenching of cytosolic fura-2 has proved to be a useful model system for investigating Ca^{2+} influx because Mn^{2+} traces only influx and it is not a substrate for the ATPase that pumps Ca^{2+} out of the cytosol. Mn^{2+} permeates through the Ca^{2+} influx pathway in neutrophils after depletion of Ca^{2+} stores [23] and subsequently quenches the fluorescence signal by its high-affinity binding with fura-2. CPA inhibits the Ca^{2+}-ATPase of intracellular Ca^{2+} stores and, consequently, activates Ca^{2+} and Mn^{2+} influx from the extracellular medium [23]. In a Ca^{2+}-free medium, the addition of Mn^{2+} induces a decline in the fluorescence signal, which is attributed to passive Mn^{2+} diffusion through the Ca^{2+} influx pathway in the resting fura-2-loaded neutrophils [24]. The finding that YC-1 (30 μM) inhibited both active and passive Mn^{2+} influx (Fig. 2, C and D) supported the evidence for a blockade of Ca^{2+} entry independent of stimulated Ca^{2+} efflux. In addition, LY83583 did not attenuate the inhibition of Mn^{2+} influx by YC-1 (Fig. 2, C and D), also providing evidence against the implication of a cyclic GMP signal.

3.4. Effect of Ca^{2+} influx modulators on $[Ca^{2+}]_i$

It has been reported that depletion of endothelial Ca^{2+} stores activates microsomal cytochrome P450, and its arachidonic acid metabolite is a second messenger for the activation of Ca^{2+} entry [36]. The cytochrome P450 inhibitor SKF525A (30 μM) could evoke a partial and transient inhibition of the fMLP-stimulated changes in $[Ca^{2+}]_i$; however, the dual cyclooxygenase/lipoxygenase inhibitor BW755C (30 μM) had a negligible effect (Fig. 3A), suggesting a plausible involvement of cytochrome P450 in the activation of the Ca^{2+} entry pathway in rat neutrophils. This finding conforms with a study in human neutrophils [37].

There is evidence that phosphorylation of certain proteins leads to regulation of Ca^{2+} entry through a store-operated Ca^{2+}-entry pathway. A protein phosphatase 1/2A inhibitor, calyculin A, diminished the Ca^{2+}-ATPase inhibitor-induced Ca^{2+} influx, whereas the inhibitor of protein phosphatase 2B, cyclosporin A, was a potentiator in human neutrophils [38]. The finding that calyculin A (0.1 μM) attenuated the fMLP-induced Ca^{2+} entry was consistent with a previous report in human neutrophils [39], suggesting the stimulation of protein phosphatase 1/2A during fMLP activation. However, protein phosphatase 2B probably did not play a crucial role in the fMLP-activated signal transduction pathway, as indicated by identical fMLP-stimulated changes in $[Ca^{2+}]_i$ in both the presence and absence of cyclosporin A (1 μM). In addition, YC-1 did not inhibit the fMLP-stimulated changes in $[Ca^{2+}]_i$ via activation of phosphatase 2B because cyclosporin A failed to overcome

the inhibitory effect of YC-1 (Fig. 3B). Whether protein phosphatase 1/2A is inhibited by YC-1 needs further investigation.

Phorbol ester acts through PKC-mediated phosphorylation and produces a sustained inhibition of Ca^{2+} entry in human neutrophils [39]. Moreover, the finding that the entry of Ca^{2+} via the capacitative entry mechanism is sensitive to the inhibition of tyrosine kinase has been reported in platelets [40]. The finding that both PMA and a tyrosine kinase inhibitor, genistein, attenuated the Ca^{2+} entry suggests that PKC and tyrosine kinase are also responsible for the regulation of Ca^{2+} entry in rat neutrophils (Fig. 3C). Since the protein kinase inhibitor staurosporine failed to overcome the inhibitory effect of YC-1 (Fig. 3D), the activation of PKC by YC-1 seems unlikely.

Ca^{2+} influx induced by Fcγ receptor cross-linking was inhibited by a phosphatidylinositol 3-kinase inhibitor, wortmannin [41]. However, a previous report indicating that the Ca^{2+} response evoked by fMLP is not sensitive to wortmannin [42] is in line with our observation (Fig. 3C). These results exclude the possibility of the inhibition of Ca^{2+} entry by YC-1 through phosphatidylinositol 3-kinase.

3.5. Effect of YC-1 on protein tyrosine phosphorylation

Growing evidence supports the hypothesis that activation of protein kinases phosphorylates either the store-operated Ca^{2+} entry pathway protein itself or the regulatory proteins, leading to the regulation of Ca^{2+} entry. Stimulation with fMLP resulted in an increase in tyrosine phosphorylation of several cellular proteins. This response was inhibited by 50 μM YC-1 and 100 μM genistein, affecting the phosphorylation of proteins of molecular mass 38–44, 50–62, 77–85, and 115 kDa (Fig. 4). Although little is known about specific substrates for tyrosine kinase in the regulation of Ca^{2+} entry, inhibition of tyrosine kinase is probably responsible for the inhibitory effect of YC-1.

In summary, our results demonstrated that the fMLP-stimulated elevation of $[Ca^{2+}]_i$ was inhibited by a sGC activator, YC-1, mainly via the blockade of Ca^{2+} entry. However, inhibition of Ca^{2+} entry by YC-1 might have had an additional component of action distal to its effect on sGC, because the cyclic GMP signal did not play a critical role in the regulation of fMLP-stimulated Ca^{2+} entry. Inhibition of Ca^{2+} entry by YC-1 was probably attributable to inhibition of tyrosine kinase, but not to inhibition of phosphatidylinositol 3-kinase or to stimulation of PKC or protein phosphatase 2B. Whether YC-1 inhibited protein phosphatase 1/2A and cytochrome P450 remains to be determined. Studies of the role of cyclic GMP in biological processes carried out with YC-1 should be evaluated carefully to determine whether the effects found are really attributable to cyclic GMP.

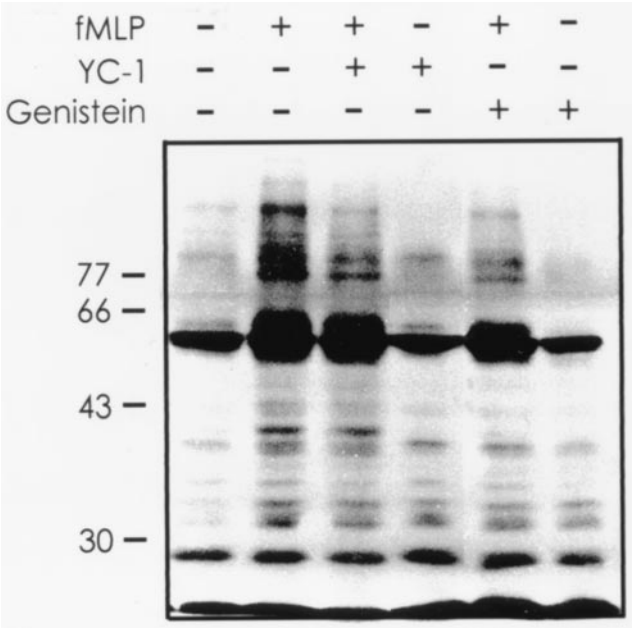

Fig. 4. Effect of YC-1 on protein tyrosine phosphorylation. Cells were preincubated at 37° with DMSO, 50 μM YC-1, or 100 μM genistein for 1 min, either before stimulation with 0.1 μM fMLP in combination with 5 μg/mL of cytochalasin B or without stimulation. One minute later, protein tyrosine phosphorylation was detected by immunoblot analysis using anti-phosphotyrosine antibodies. The final volume of DMSO in the reaction mixture was 0.5%. The data presented are representative of three independent experiments with similar results.

Acknowledgments

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