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W-7 primes or inhibits the fMLP-stimulated respiratory burst in human neutrophil by concentration-dependent dual expression of the formyl peptide receptors on cell surface

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Abstract

It was investigated why the fMLP-stimulated respiratory burst in human neutrophils was enhanced by *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a considered calmodulin antagonist, at lower concentration but inhibited at higher concentration. Flow cytometric analysis on binding of the receptor to the fluorescence-labeled formyl peptide and the polymerization of actin in cells showed that the drug inhibited actin polymerization and promoted expression of the fMLP receptors on cell membrane at lower concentration, while promoted the actin polymerization and depressed the receptor expression at higher concentration. As intracellular Ca^{2+} ($[Ca^{2+}]_i$) is elevated, polymerization of actin decreases and the receptor expression increases. At normal physiological and two moderately high intracellular calcium levels, the dual effect of W-7 became less significant as $[Ca^{2+}]_i$ was elevated indicating that the dual effect is calcium-dependent. Under two extreme conditions that the intracellular calcium was either depleted or highly elevated, the dual effect disappeared but only an inhibitory effect on actin polymerization was observed. Colchicine and taxol study showed that disruption or stabilization of microtubules had no effect on formyl peptide receptor expression. The results suggest that W-7 primes the fMLP stimulation by direct action on actin leading to breakdown of microfilaments and more expression of formyl peptide receptors, and inhibits the stimulation by indirect action on actin through inactivation of some Ca^{2+}-dependent proteins resulting in assembly of actin into microfilaments. Which action is favorable depends on the drug concentration.

Keywords: W-7; Neutrophil; Respiratory burst; Formyl peptide receptor; Actin polymerization; Calmodulin

1. Introduction

The main function of neutrophils is to kill invading bacteria and the killing largely depends on the agonist induced respiratory burst [1]. The agonist induced signal transduction involves its binding to receptor on cell surface firstly, and then G protein-mediated activation of a phosphatidylinositol-specific phospholipase C, which results in the generation of two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces a transient rise of intracellular free Ca^{2+} while DAG remains associated with the membrane and

Abbreviations: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; fMLP, *N*-formylmethionylleucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; DiC8, 1,2-dioctanoyl-*sn*-glycerol; fNLPNTL, formyl-Nle-Leu-Phe-Nle-Tyr-Lys

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participates in the activation of protein kinase C (PKC), which results in the assembly and activation of NADPH oxidase leading to the respiratory burst [2].

In the year 1978, Hidaka and his co-workers found that W-7 could inhibit the interaction between actin and myosin [3]. Later he found that the inhibitory effect of W-7 was through its binding to calmodulin [4]. From that time, W-7 became a useful tool for studying the role of calmodulin in many cell functions such as cell proliferation and cell cycle. It was also widely used to study the function of calmodulin and many calmodulin-dependent proteins in neutrophil [5–8]. In earlier reports, W-7 was shown to depress the fMLP-stimulated respiratory burst and the depression was attributed to its inhibitory effect on PKC translocation and activation [9–11]. However, the study by Perianin and his co-workers showed that W-7 could inhibit the fMLP-stimulated respiratory burst only at high concentrations (>20 μM), while low concentrations of W-7 (5–10 μM) actually enhanced the respiratory burst [12]. Meanwhile, they found that either at low or high concentration only an inhibitory effect of W-7 was observed if the respiratory burst was stimulated by PMA, a direct activator of PKC, or A23187, a calcium ionophore. These findings suggested that the priming effect of W-7 at low concentration may occur at the steps of signal transduction upstream from PKC activation or even mobilization of intracellular calcium. Although the authors mentioned that the major modifications that led to the priming might take place between receptor stimulation and activation of phospholipase C which generate two second messengers [12], the mechanism of the priming effect of W-7 at low concentration on the neutrophil respiratory burst is still not clear. Logically, the change from priming to inhibition only due to the increase of W-7 concentration implies that there might be a dual action underlying the effect of the drug on the fMLP-stimulated respiratory burst. In addition, the possible role of intracellular calcium and calmodulin should be taken into account, since W-7 has been considered a calmodulin antagonist. The present investigation was designed to clarify the mechanisms behind the peculiar concentration-dependent effect of W-7.

2. Materials and methods

2.1. Reagents

W-7, fMLP, thapsigargin (TG), cytochalasin B, lysopalmitoylphosphatidylcholine, Histopaque 1077 and dextran T-500 were from Sigma. Formyl-Nle-Leu-Phe-Nle-Tyr-Lys Bodipy FL, Bodipy FL phallacidin and BAPTA-AM were from Molecular Probes. 1,2-Dioctanoyl-*sn*-glycerol (DiC8) was from Calbiochem. Colchicine and taxol were from the Institute of Pharmacology, Chinese Academy of Medical Sciences. Other chemicals were all of analytical grade. Stock solutions of W-7 (10 mM), fMLP (10 mM), DiC8 (1 mM) were prepared in dimethyl sulfoxide (DMSO) and further diluted with Hanks' balanced salt solution (HBSS). The final concentration of DMSO never exceed 0.1%.

2.2. Cell preparation

Human neutrophils were isolated from heparinized donor blood according to the following procedure: blood was mixed with 4.5% dextran in 0.9% NaCl solution for 45 min and the leukocyte-rich plasma was layered on top of Histopaque-1077. The neutrophils were obtained as pellet after centrifugation at $500\times g$ for 15 min. Contaminating erythrocytes were removed by hypotonic lysis. The cells were finally washed twice with saline and suspended in HBSS.

2.3. Measurement of respiratory burst in neutrophils

The respiratory burst of neutrophils was monitored as concomitant chemiluminescence resulted from the reaction of luminol with the superoxide anion (O_2^-) and H_2O_2 generated by cells [13]. Each of 2 ml neutrophil suspension (10^6 cells/ml) containing 1 μM luminol was filled in two identical quartz cuvettes placed in the rotatable sample holder of a laboratory-made photon counter and measured at 37°C. W-7 was incubated with cell suspension 5 min before stimulation and the cell suspension containing the same amount of DMSO was used as control. The respiratory burst of the neutrophils under studied condition and the respiratory burst of the cells used as control were recorded simultaneously. Thus, the

errors caused by the time-dependent variation in cell viability, kinetics and intensity of respiratory burst were minimized to a great extent.

2.4. Measurement of the formyl peptide binding to its receptors

Flow cytometry was used to measure the ligand binding according to the method described by Prossnitz et al. [14]. In brief, cells (10^6/ml, 0.5 ml) were incubated with fNLPNTL Bodipy FL (100 nM) on ice for at least 15 min. Then, 10 000 cells per sample were analyzed on Becton Dickson Flow cytometer (FACS 420).

2.5. Measurement of F-actin content

The flow cytometric measurement of F-actin was referred to [15]. In brief, cells (10^6/ml, 1 ml) were incubated with or without W-7 and then fixed for 15 min in 3.7% formaldehyde. The cells were then permeabilized with lysopalmitoylphosphatidylcholine (50 μg/ml) to increase the membrane permeability and simultaneously stained with 5 units of Bodipy FL phallacidin for 15 min at room temperature. Ten thousand cells per sample were analyzed.

3. Results

3.1. Effects of W-7 on the fMLP- and DAG-stimulated respiratory burst

The respiratory burst stimulated by fMLP in neutrophils treated with W-7 of concentration from 5 to 25 μM for 10 min was measured. The kinetic patterns of three typical respiratory bursts are shown in Fig. 1. It clearly shows that W-7 at lower concentration (5 μM) significantly enhances the respiratory burst, but inhibits the burst at higher concentration (20 μM). The maximal rates of the respiratory burst in the neutrophils treated with various concentrations of W-7 relative to the rate of the burst in the untreated cells are plotted in Fig. 2. It can be seen that below approx. 12 μM the respiratory burst was enhanced by W-7, but was inhibited when W-7 concentration exceeded this value.

To know whether the priming effect of low concentration W-7 on fMLP-stimulated respiratory burst is due to its action to modify the signaling events occurring at a step downstream from calcium mobilization, a synthetic DAG, DiC8 [16], was used to stimulate the respiratory burst in the neutrophils whose intracellular free Ca^{2+} had been substantially raised by pretreatment with 500 nM TG for 10 min [17]. The DiC8-stimulated respiratory bursts in those cells were measured in the presence of various concentrations of W-7. The results are also plotted in Fig. 2. It shows clearly that the respiratory burst stimulated by the synthetic DAG was inhibited by W-7 either at lower or higher concentration. In contrast with fMLP stimulation, no priming effect of W-7 was observed. However, in both cases some similarity in the concentration dependence of the effect of W-7 may be noticed. When concentration of W-7 exceeds approx. 12 μM, the priming effect begins to disappear and the inhibitory effect becomes dominant for fMLP-stimulated respiratory burst, while the inhibitory effect becomes more obvious for DAG stimulation.

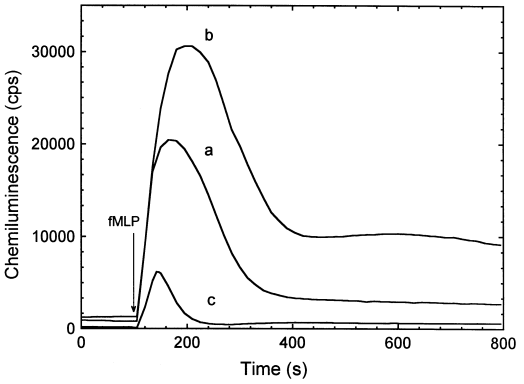

Fig. 1. fMLP (100 nM)-stimulated respiratory bursts in neutrophils (10^6 cells/ml) pretreated without W-7 (a), or with 5 μM W-7 (b) and 20 μM W-7 (c) for 10 min. The bursts were monitored as their concomitant chemiluminescence at 37°C.

3.2. Effect of W-7 on the binding of fMLP to its membrane receptor

If the priming effect of W-7 occurred at a step upstream from calcium mobilization, a possible regulation of W-7 on the binding of formyl peptide to its membrane receptor should be examined first. The capacity of fMLP receptor on cell membrane to bind fluorescence-labeled ligand, formyl-Nle-Leu-Phe-Nle-

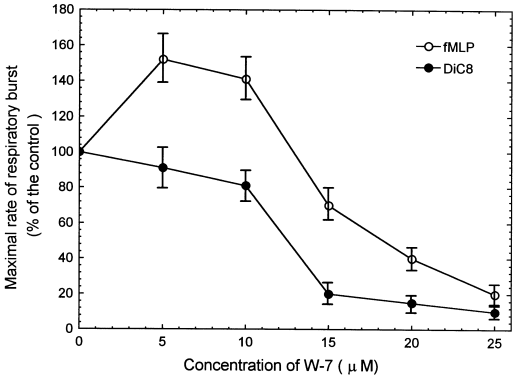

Fig. 2. The effect of various concentrations of W-7 on the respiratory bursts in neutrophils stimulated by fMLP and in the cells, which were pretreated with 500 nM thapsigargin for 10 min, stimulated by DiC8. The maximal rates of the respiratory bursts in the cells treated with different concentrations of W-7 were plotted as the values relative to that of the burst in the control neutrophils. Neutrophils (10^6/ml) were incubated at 37°C in the absence (control) or presence of W-7 for 10 min before 100 nM fMLP or DiC8 stimulation. Each datum is the mean of three independent measurements, and bars stand for S.D.

Tyr-Lys Bodipy FL, was analyzed by flow cytometry in the cells treated with or without W-7. The cell number distribution against fluorescence intensity, namely the numbers of the occupied fMLP receptor, in the cell populations treated with 5 μM and 20 μM W-7 as well as in untreated neutrophils are measured and shown in Fig. 3A. It turns out that the distribution in the cells treated with the lower concentration of W-7 was shifted to the right, while the distribution in the cells treated with the higher concentration of W-7 was moved to the left. Obviously, right shift means that more fMLP receptors were available on cell surface, while left shift reflects that less agonists were bound to the receptors. The flow cytometric analysis evidently indicates that at least as one step in signaling events W-7 affects the fMLP-stimulated respiratory burst by regulating the binding of the formyl peptide to its membrane receptors.

To know whether the capacity or ability of the agonist to bind to its receptor, or in other words the expression of the receptors, is related to cytoskeleton, cytochalasin B, a blocker of the assembly of microfilaments and reported enhancer of the fMLP-stimulated respiratory burst [18] was also used to see its effect on the expression of the membrane receptors. Fig. 3B shows the cell number distributions in the neutrophils untreated and treated with 1 μM cytochalasin B (CB) respectively. A right shift of the distribution was observed in CB-treated cells, suggesting a correlation between the number of the fMLP receptors capable of binding formyl peptide and the structure of cytoskeleton.

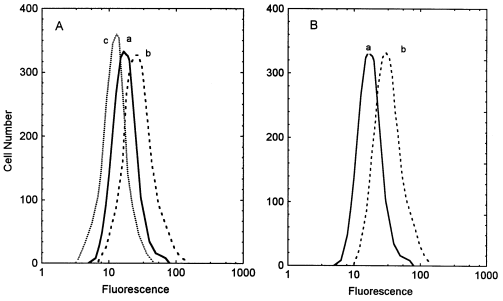

Fig. 3. Cell number distribution against the fluorescence from the membrane receptor-bound formyl-Nle-Leu-Phe-Nle-Tyr-Lys Bodipy FL. Neutrophils (10^6/ml, 0.5 ml) were incubated with the fluorescence probe (100 nM) on ice for at least 15 min, then 10^4 cells in each sample were analyzed by flow cytometry. (A) The cells were untreated (curve a) or treated with 5 μM W-7 (curve b) and 20 μM W-7 (curve c) for 10 min respectively. (B) The cells were untreated (curve a) or treated with 1 μM cytochalasin B (curve b) for 10 min. Data are representative of at least three independent experiments.

3.3. *Effect of W-7 on the actin polymerization in neutrophil*

To check whether W-7 has a similar effect as CB

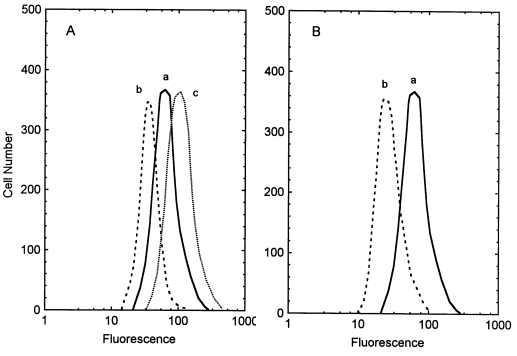

Fig. 4. Flow cytometric analysis of actin polymerization in neutrophils treated with W-7. Cells (10^6/ml, 0.5 ml) were loaded with Bodipy FL phallacidin (5 units), and 10^4 cells in each sample were analyzed. (A) Control cells (curve a), and cells incubated with 5 μM W-7 (curve b) and 20 μM W-7 (curve c) for 10 min. (B) Control cells (curve a), and cells treated with 1 μM cytochalasin B (curve b) for 10 min. Data are representative of at least three independent experiments.

on cytoskeleton, the fluorescence probe Bodipy FL phallacidin, whose fluorescence increases tremendously by binding to F-actin, was used to detect the effect of W-7 on the polymerization of actin in microfilament assembly. The cell number distributions against fluorescence intensity in the Bodipy FL phallacidin-loaded neutrophils, which had been incubated with W-7 at two extreme concentrations, were analyzed by flow cytometry. Meanwhile, the untreated cells were also analyzed as control. As shown in Fig. 4A, a left shift of the distribution relative to the control was found in the cells treated with the lower concentration (5 μM) of W-7, but a right shift was observed in the cells incubated with the higher concentration (20 μM) of W-7. The results indicate that W-7 inhibits the polymerization of actin at lower concentration, but promotes assembly of actin into microfilaments at higher concentration.

To further confirm the correlation between the enhancement of the expression of the fMLP receptors on cell surface and the depolymerization of actin, the effect of CB on polymerization of actin in neutrophils was also measured using the same technique. The cell number distribution against the fluorescence intensity of Bodipy FL phallacidin in CB-treated and untreated neutrophils is shown in Fig. 4B. It demonstrates that cytochalasin B, which can increase the formyl peptide binding to its receptor on membrane, also promotes the depolymerization of actin from microfilament.

3.4. The effect of microtubule disruptor (colchicine) and stabilizer (taxol) on the formyl peptide receptor expression and actin polymerization

To test whether microtubules also take part in formyl peptide receptor regulation, colchicine and taxol, which could disrupt or stabilize microtubules respectively, were used to test the role of microtubules in regulating the receptor expression and actin polymerization. The receptor binding ability and actin polymerization in the cells treated with cytochalasin (1 μM), colchicine (10 μM) and taxol (10 μM) were determined. The results, which are given in Table 1, clearly showed that cytochalasin obviously enhances the receptor binding (increasing it about 1-fold) but neither colchicine nor taxol had any effect on it. For the effect of these agents on actin polymerization, the results showed that cytochalasin strongly depolymerize the microfilament while the other two agents had no effects on actin polymerization.

Table 1
The effects of cytochalasin, colchicine and taxol on formyl peptide binding to its receptors and actin polymerization

Cytoskeleton agents	Peptide binding (% of control ± S.D.)	Actin polymerization (% of control ± S.D.)
Cytochalasin (1 μM)	200 ± 9	43 ± 4
Colchicine (10 μM)	99 ± 2	101 ± 2
Taxol (10 μM)	99 ± 2	100 ± 3

3.5. The intracellular calcium dependence in W-7 regulated polymerization of actin

Since the equilibrium between unassembled actin and the assembled actin is regulated by some calmodulin-dependent proteins, it is natural to consider the possible role of intracellular calcium ($[Ca^{2+}]_i$) in modifying the effect of W-7 on the polymerization of actin in cytoskeleton. In one case, BAPTA, the chelator for intracellular free Ca^{2+}, and EGTA, the chelator for extracellular Ca^{2+}, were used in combination to deplete intracellular calcium in neutrophils. In the other case, thapsigargin, a potent endomembrane Ca^{2+}-ATPase inhibitor, was used at different concentrations to elevate the intracellular free Ca^{2+} to various substantial levels [19].

The effects of W-7 on the polymerization of actin were measured by flow cytometry in the $[Ca^{2+}]_i$-free

Table 2
The regulation of intracellular free calcium on the effects of W-7 on polymerization of actin in human neutrophils treated with BAPTA or different concentrations of TG

Physiological conditions		Mean fluorescence		
		Control	W-7 (5 μM)	W-7 (20 μM)
BAPTA	(5 μM)	157.3	122.3	109.2
	Normal	111.9	84.9	146.9
TG	100 (nM)	93.3	78.0	112.4
	200 (nM)	76.4	68.3	81.1
	500 (nM)	59.7	55.3	49.6

Note: Actin polymerization is shown as the mean fluorescence of the special F-actin probe Bodipy phallacidin per cell. Data are one representative of three independent experiments.

and $[Ca^{2+}]_i$-rich cells. The results are summarized in Table 2. It was found that: (1) as $[Ca^{2+}]_i$ is elevated, less polymerization of actins was observed. This is even true in the cells treated with a low concentration of W-7 (see Table 2, columns 2, 3). (2) At normal and two moderately elevated $[Ca^{2+}]_i$ (TG 100, 200 nM), the low concentration of W-7 depolymerizes actins while the high concentration of W-7 polymerizes them. However, the dual effect becomes less significant as $[Ca^{2+}]_i$ is raised. (3) Under two extreme conditions (depleted $[Ca^{2+}]_i$ and highly elevated $[Ca^{2+}]_i$), both low and high concentrations of W-7 depolymerize actins. The higher the concentrations of W-7, the more obvious was the depolymerization of actins.

The effects of W-7 on formyl peptide binding to its receptors on cell membrane at different $[Ca^{2+}]_i$ were also measured. The results are summarized in Table 3. It was found that: (1) as $[Ca^{2+}]_i$ is elevated, more expression of the fMLP receptor was found, indicating a close correlation between actin polymerization and formyl peptide receptor expression on membrane. (2) At normal and two moderately elevated $[Ca^{2+}]_i$, the low concentration of W-7 promotes receptor expression while the high concentration of W-7 depresses it. However, the decreasing dual effect was also observed as $[Ca^{2+}]_i$ is raised. (3) Under two extreme conditions (depletion of intracellular calcium and highly elevated $[Ca^{2+}]_i$), both low and high concentrations of W-7 promote receptors expression.

Table 3
The regulation of intracellular free calcium on the effects of W-7 on the expression of formyl peptide receptors on the cell membrane of neutrophils treated with BAPTA or different concentrations of TG

Physiological conditions		Mean fluorescence		
		Control	W-7 (5 μM)	W-7 (20 μM)
BAPTA	(5 μM)	27.6	38.0	44.2
	Normal	40.6	56.1	29.6
TG	100 (nM)	49.1	59.1	39.2
	200 (nM)	58.9	60.4	55.2
	500 (nM)	69.3	75.8	80.0

Note: The expression of formyl peptide receptors is shown as the mean fluorescence of the fluorescent formyl peptide fNLPNTL Bodipy FL on the cell membrane. Data are one representative of three independent experiments.

By comparing Tables 2 and 3, a profound law can be noticed: depolymerization of actins is always correlated to more expression of the formyl peptide receptors and vice versa regardless of whether the depolymerization is caused by elevation of $[Ca^{2+}]_i$ or by W-7. Even the concentration-dependent dual effect of W-7 does not change this law.

4. Discussion

The purpose of this study is to explore the mechanisms by which the effect of W-7 on the fMLP-stimulated respiratory burst in human neutrophils is changed from priming at lower concentration to inhibition at higher concentration. In order to achieve this goal, we investigated at which steps the signaling events could be modified by W-7 to lead to priming or potentiating of the fMLP-stimulated cell response. To mimic the fMLP stimulation but restrict the signaling events at the step downstream from the intracellular calcium mobilization, TG was used to mobilize the cytosol-free Ca^{2+} and the permeable synthetic DAG, DiC8, was used to activate protein kinase C. As also reported by Perianin et al. in their experiments using PMA or A23187 to stimulate respiratory burst [12], only an inhibitory effect was observed whatever the concentration of W-7 used to treat neutrophils (see Fig. 1). The result suggests that the priming effect of W-7 is unlikely due to its action on the signaling events occurring downstream from the calcium mobilization in cytoplasm. It may also confirm the previous report that W-7 depresses the respiratory burst by direct or indirect (through binding to calmodulin) inhibition of PKC translocation and activation [9].

Upstream from the intracellular calcium mobilization, the binding of the agonist to its receptor on the cell surface may be the most likely step in the signal transduction pathway at which the priming of respiratory burst could occur. The results on the binding of the fluorescence-labeled ligand to its membrane receptor (see Fig. 3) revealed that treatment of the cells with the lower concentration of W-7 enhanced expression of the receptors on the cell surface, while treatment with the higher concentration of W-7 resulted in less expression of the receptors. Since more receptors bound by agonist induce more intensive

respiratory burst [20], it may be concluded that at least as one mechanism the enhancement of the fMLP-stimulated respiratory burst in neutrophils by the lower concentration of W-7 is the consequence of more expression of fMLP receptors on the cell membrane, and the inhibition of the burst by the higher concentration of W-7 is due to less expression of the receptors besides inhibition of protein kinase C. However, how does W-7 make such a dual effect? Experiments were carried out to elucidate the dual effect of W-7 in two aspects: (1) How is cytoskeleton involved in the regulation of the receptor expression and how does the regulation depend on the concentration of W-7? (2) How does the intracellular calcium level regulate the receptor expression and the behavior of W-7 at different concentrations?

Some previous studies, using cytochalasin B or its derivative, dihydrocytochalasin B, as the cytoskeleton-perturbing agent, have established that cytoskeleton regulates the expression of fMLP receptor on the cell surface through two mechanisms in human granulocytes [18,21]. First, after receptor occupancy the formyl peptides-receptor complex may be converted into a slowly dissociating or high affinity form that can be endocytosed into cytoplasm, which results in decrease of receptor expression on the cell membrane. Second, microfilament controls the receptor expression by regulating the traffic of latent or internal receptors to the cell surface. The studies with cytochalasin B or its derivative, dihydrocytochalasin, revealed that both mechanisms are related to the interaction between receptor and cytoskeleton. Loosening of the interaction by inhibiting microfilament assembly or promoting the breakdown of existing microfilaments results in less endocytosis of the formyl peptide-bound receptors and favoring the flow of the internal receptors from the latent pool to the cell surface. Tightening of the interaction results in just the opposite effect. Based on the above knowledge, the effect of W-7 on the cytoskeleton, namely the actin polymerization, in neutrophils was investigated in comparison with the effect of cytochalasin B. It was found that lower concentration W-7 inhibited the actin polymerization and enhanced the receptor expression, but higher concentration W-7 promoted the actin polymerization and depressed the receptor expression (see Fig. 3AFig. 4A). Meanwhile, the comparative measurements showed that cytochalasin B inhibited actin polymerization (see Fig. 4B) and increased the capacity of the receptors to bind formyl peptide (see Fig. 3B). The comparison indicates that the priming effect of W-7 at low concentration on the fMLP-stimulated respiratory burst may be realized through a similar mechanism as that of cytochalasin B. In order to know whether microtubule has any relevance to the fMLP receptor expression, colchicine, a disruptor of microtubules, and taxol, a microtubule stabilizer, were used to see their effect on receptor expression. The results obtained (see Table 1) showed that both colchicine and taxol affected neither the binding of the formyl peptide to receptors nor the actin polymerization, which further proves that microfilaments but not microtubules control the formyl peptide receptor expression and that the priming effect of W-7 is through its action on microfilaments.

Actin molecules exist inside cells both in an unassembled pool and in assembled microfilaments; both forms of the actin set up an equilibrium:

$$\text{actin molecules} \rightleftharpoons \text{microfilaments}$$

More than 60 known proteins bind to actin and adjust the actin-microfilament equilibrium in the direction of unassembled subunits. The activities of actin-binding proteins are readily reversible, allowing cells to push the actin-microfilament equilibrium quickly in either direction. At least in one mechanism, an actin-binding protein links to unassembled actin molecules and blocks their polymerization into microfilaments. Many actin-binding proteins are inactive at a low Ca^{2+} concentration and become active only when intracellular Ca^{2+} is elevated. The effect of Ca^{2+} may be direct, through direct binding of Ca^{2+} to the protein, or indirect, through binding of Ca^{2+} to the calmodulin [22]. For this reason, the intracellular calcium dependence in W-7 regulated polymerization of actin was investigated in this study. The actin polymerization and fMLP receptor expression were measured at five different intracellular calcium levels (normal, depleted and three elevated $[Ca^{2+}]_i$) in the absence or presence of W-7. The results summarized in Tables 2 and 3 clearly showed that (1) in the cells without treatment of W-7, the actin polymerization decreased and the receptor expression increased as intracellular free calcium increased (see the second columns in both tables). This indicates

not only a close correlation between depolymerization of actins and increasing expression of the receptors, but also the role of intracellular calcium in adjusting the actin-microfilament equilibrium in the direction of unassembled subunits. (2) In the cells treated with lower concentration W-7 (5 μM), less polymerization of actins and more expression of the receptor were always observed at various intracellular calcium levels in comparison with controls. It should be noticed that when intracellular free calcium is depleted, most of the Ca^{2+}-dependent actin-binding proteins are inactivated while W-7 still depolymerizes actins in a dose-dependent manner. This implies that W-7 may break microfilaments down by direct action on them. (3) Except for the two extreme cases where intracellular calcium was either depleted or highly elevated by 500 nM TG, at normal or moderately elevated $[Ca^{2+}]_i$, in contrast to the lower concentration the higher concentration W-7 promoted the actin polymerization but the dual effect became less obvious as $[Ca^{2+}]_i$ increased. This indicates that there might be another action of W-7 on some calcium-dependent proteins. The action adjusts the actin-microfilament equilibrium to the direction of assembling microfilament. The second action of W-7 is indirect and becomes dominant at a higher concentration. It should be expected that when those proteins are fully activated by the highest dose of TG or fully inactivated in calcium-depleted cells, no indirect action but only a direct action of W-7 should be observed. Such expected results were actually observed under the two extreme intracellular calcium conditions where higher concentration W-7 resulted in even less actin polymerization and even more receptor expression. Although the calcium-dependent proteins responsible for the indirect action of W-7 have not been identified so far, calmodulin may be a most likely candidate since it has been considered as the main target of W-7. Although W-7 is not absolutely specific because of some experimental evidence that it could strongly inhibit PKC [9–11,23], to some extent inhibit cAMP-dependent protein kinase, casein kinases I and II, and in rod outer segment activate PLC [24,25], most of the literature reported that the inhibitory effects of W-7 on the activity of many calmodulin-dependent proteins, such as myosin light chain kinase (MLCK), cyclic nucleotide phosphodiesterase, Ca^{2+}, Mg^{2+}-ATPase, phospholipase C, phospholipase D and phospholipase A_2, were through its binding to calmodulin [26–30]. So it might be reasonable to consider calmodulin a likely calcium-dependent protein responsible for the indirect action of W-7 on actin polymerization.

In summary, the present study clearly demonstrates that the ternary complex of formyl peptide, receptor and cytoskeleton plays an important role in the stimulation of respiratory burst. Depolymerization of actin results in more expression of formyl peptide receptors on the cell membrane. Low concentration W-7 enhances the fMLP-stimulated respiratory burst by decreasing the actin polymerization and increasing the receptor expression, while high concentration W-7 inhibits the respiratory burst both by depressing formyl peptide receptor expression and inhibiting PKC activity. This dual effect of W-7 is due to a direct action on actins leading to breakdown of microfilaments, and an indirect action on actins through inactivation of some Ca^{2+}-dependent proteins resulting in the assembly of actins into microfilaments. Which action is favorable depends on the drug concentration.

Acknowledgements

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References

[1] B.M. Babior, Oxygen-dependent microbial killing by phagocytes, New Engl. J. Med. 298 (1978) 659–668.
[2] M. Thelen, B. Dewaldand, M. Baggiolini, Neutrophil signal transduction and activation of the respiratory burst, Physiol. Rev. 73 (1993) 797–821.
[3] H. Hidaka, M. Asano, S. Iwadare, I. Matsumoto, T. Totsuka, N. Aoki, A novel vascular relaxing agent, N-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide which affects vascular smooth muscle actomyosin, J. Pharmacol. Exp. Ther. 207 (1978) 8–15.
[4] H. Hidaka, T. Yamaki, M. Naka, T. Tanaka, H. Hayashi, R. Kobayashi, Calcium-regulated modulator protein inter-

acting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase, Mol. Pharmacol. 17 (1980) 66–72.
[5] G. Marone, S. Poto, M. Columbo, R. Giugliano, A. Genovese, M. Condorelli, Possible role of calmodulin in the control of lysosomal enzyme release from human polymorphonuclear leukocytes, J. Pharmacol. Exp. Ther. 231 (1984) 678–684.
[6] R.J. Smith, D.E. Epps, J.M. Justen, L.M. Sam, M.A. Wynalda, F.A. Fitzpatrick, F.S. Yein, Human neutrophil activation with interleukin-1. A role for intracellular calcium and arachidonic acid lipoxygenation, Biochem. Pharmacol. 36 (1987) 3851–3858.
[7] K. Takahashi, K. Tago, H. Okano, Y. Ohya, T. Katada, Y. Kanaho, Augmentation by calmodulin of ADP ribosylation factor-stimulated phospholipase D activity in permeabilized rabbit peritoneal neutrophils, J. Immunol. 156 (1996) 129–1234.
[8] E. Capuozzo, D. Verginelli, C. Crifo, C. Salerno, Effects of calmodulin antagonists on calcium pump and cytosolic calcium level in human neutrophils, Biochim. Biophys. Acta 1357 (1997) 124–127.
[9] C.D. Wright, M.D. Hoffman, The protein kinase C inhibitors H-7 and H-9 fail to inhibit human neutrophil activation, Biochem. Biophys. Res. Commun. 135 (1986) 749–755.
[10] R. Seifert, C. Schachtele, Studies with protein kinase C inhibitors presently available cannot elucidate the role of protein kinase C in the activation of NADPH oxidase, Biochem. Biophys. Res. Commun. 152 (1988) 585–592.
[11] M. Ito, F. Tanabe, A. Sato, E. Ishida, Y. Takami, S. Shigeta, Possible involvement of microfilaments in protein kinase C translocation, Biochem. Biophys. Res. Commun. 160 (1989) 1344–1349.
[12] A. Periannin, E. Pedruzzi, J. Hakim, W-7, a calmodulin antagonist, primes the stimulation of human neutrophil respiratory burst by formyl peptides and platelet-activating factor, FEBS Lett. 342 (1994) 135–138.
[13] R.C. Allen, Chemiluminescence from eukaryotic and prokaryotic cells: reducing potential and oxygen requirements, Photochem. Photobiol. 30 (1979) 157–163.
[14] E.R. Prossnitz, O. Quehenberger, C.G. Cochrane, R.D. Ye, Signal transducing properties of the N-formyl peptide receptor expressed in undifferentiated HL60 cells, J. Immunol. 151 (1993) 5704–5715.
[15] M.U. Ehrengruber, T.D. Coates, D.A. Deranleau, Shape oscillations: a fundamental response of human neutrophils stimulated by chemotactic peptides?, FEBS Lett. 359 (1995) 229–232.
[16] C.C. Cox, R.W. Dougherty, B.R. Ganong, R.M. Bell, J.E. Niedel, R. Snyderman, Differential stimulation of the respiratory burst and lysosomal enzyme secretion in human polymorphonuclear leukocytes by synthetic diacylglycerols, J. Immunol. 136 (1986) 4611–4616.
[17] O. Thastrup, B. Foder, O. Scharff, The calcium mobilizing and tumor promoting agent, Thapsigargin elevates the platelet cytoplasmic free calcium concentration to a higher steady state level. A possible mechanism of action for the tumor promotion, Biochem. Biophys. Res. Commun. 142 (1987) 654–660.
[18] A.J. Jesaitis, J.O. Tolley, R.G. Painter, L.A. Sklar, C.G. Cochrane, Membrane-cytoskeleton interactions and the regulation of chemotactic peptide-induced activation of human granulocytes: the effects of dihydrocytochalasin B, J. Cell. Biochem. 27 (1985) 241–253.
[19] T.H. Hu, L. Bei, Z.M. Qian, X. Shen, Intracellular free calcium regulates the onset of the respiratory burst of human neutrophils activated by phorbol myristate acetate, Cell. Signal. 11 (1999) 355–360.
[20] L.A. Sklar, P.A. Hyslop, Z.G. Oades, G.M. Omann, A.J. Jesaitis, R.G. Painter, C.G. Cochrane, Signal transduction and ligand-receptor dynamics in the human neutrophil. Transient responses and occupancy-response relations at the formyl peptide receptor, J. Biol. Chem. 260 (1985) 11461–11467.
[21] A.J. Jesaitis, J.O. Tolley, R.A. Allen, Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes, J. Biol. Chem. 261 (1986) 13662–13669.
[22] D.G. Drubin, Actin and actin-binding proteins in yeast, Cell Motil. Cytoskeleton 15 (1990) 7–11.
[23] C.D. Wright, M.D. Hoffman, Comparison of the roles of calmodulin and protein kinase C in activation of the human neutrophil respiratory burst, Biochem. Biophys. Res. Commun. 142 (1987) 53–62.
[24] M. Inagaki, S. Kawamoto, H. Itoh, M. Saitoh, M. Hagiwara, J. Takahashi, H. Hidaka, Naphthalenesulfonamides as calmodulin antagonists and protein kinase inhibitors, Mol. Pharmacol. 29 (1986) 577–581.
[25] B.D. Gehm, R.M. Pinke, S. Laquerre, J.G. Chafouleas, D.A. Schultz, D.J. Pepperl, D.G. McConnell, Activation of bovine rod outer segment phosphatidylinositol-4,5-bisphosphate phospholipase C by calmodulin antagonists does not depend on calmodulin, Biochemistry 30 (1991) 11302–11306.
[26] M. Nishikawa, T. Tanaka, H. Hidaka, Ca^{2+}-calmodulin-dependent phosphorylation and platelet secretion, Nature 287 (1980) 863–865.
[27] H. Hidaka, Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii, T. Nagata, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation, Proc. Natl. Acad. Sci. USA 78 (1981) 4354–4357.
[28] J.E. Smolen, G. Weissmann, The effect of various stimuli and calcium antagonists on the fluorescence response of chlorotetracycline-loaded human neutrophils, Biochim. Biophys. Acta 720 (1982) 172–180.
[29] P.A. Craven, F.R. DeRubertis, Ca^{2+}-calmodulin-dependent release of arachidonic acid for renal medullary prostaglandin synthesis. Evidence for involvement of phospholipases A_2 and C, J. Biol. Chem. 258 (1983) 4814–4823.
[30] Y. Kanaho, A. Nishida, Y. Nozawa, Calcium rather than protein kinase C is the major factor to activate phospholipase D in fMLP-stimulated rabbit peritoneal neutrophils. Possible involvement of calmodulin/myosin L chain kinase pathway, J. Immunol. 149 (1992) 622–628.