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W-7, a calmodulin antagonist, primes the stimulation of human neutrophil respiratory burst by formyl peptides and platelet-activating factor

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Abstract

Low concentrations of the calmodulin antagonist W-7 (1–10 μM) enhanced the respiratory burst (RB) of human polymorphonuclear leukocytes (PMN) stimulated by *N*-formyl-methionyl-leucyl-phenylalanine, whereas high drug concentrations (above 20 μM) depressed it. The maximal increase obtained with 5–10 μM W-7 affected both initial rate (50%) and total superoxide anion production (150%). W-7 also primed both parameters of the RB mediated by platelet-activating factor, although higher drug concentrations were required (15–50 μM). By contrast, W-7 depressed the RB induced by the calcium ionophore A23187 and by a protein kinase C activator, phorbol myristate acetate, with an IC_{50} of approximately 20 and 8 μM, respectively. These data show the enhancing effect of W-7 on chemoattractant-mediated RB and suggest that RB priming may involve calmodulin-dependent regulation of chemoattractant-mediated early signalling events.

Key words: Neutrophil; Respiratory burst; Calmodulin; W-7; Chemoattractant

1. Introduction

Stimulation of polymorphonuclear leucocytes (PMN) by soluble and particulate stimuli triggers rapid and massive production of superoxide anions, the so-called 'respiratory burst', which plays an important role in the killing of bacteria and in host tissue damage [1–3]. Stimulation of the PMN respiratory burst by chemoattractants such as formyl peptides (fMLP), platelet-activating factor (Paf), leukotriene B4 or C5a is partly dependent on mobilization of intracellular calcium [4–6]. Non-stimulatory concentrations of agonists [7,8] and some pharmacological agents [9,10] accelerate and potentiate the PMN respiratory burst induced by a second stimulus. This phenomenon, termed 'priming', may account for exaggerated physiological PMN responses. Priming and stimulation of PMN has been proposed to occur through different mechanisms [11].

Calmodulin, a ubiquitous low-molecular-weight protein, regulates the calcium-dependent activity of various enzymes [12,13]. The possibility that calmodulin regulates PMN functions is supported by reports indicating its presence in PMN [14] and by alterations of PMN functions by pharmacological inhibitors. Among these, W-7, a specific calmodulin antagonist [15], has been shown to depress the fMLP-mediated respiratory burst, aggregation, exocytosis, and directed locomotion [16–19], indicating a positive role for calmodulin in these PMN functions. In this report, we describe a novel property of W-7: its ability to sharply enhance stimulation of the PMN respiratory burst by two chemoattractants, fMLP and Paf, a finding which suggests a role for calmodulin in RB priming.

2. Materials and methods

2.1. Reagents

W-7 was from Calbiochem (Meudon, France), Dextran T-500 was from Pharmacia (Uppsala, Sweden) and other reagents were from Sigma Co (St. Louis, MO).

2.2. PMN preparation

One volume of human venous blood, heparinized at 10 units/ml, was incubated with one volume of 2% Dextran in saline for 40 min. The supernatant was centrifuged on a cushion of a mixture of 14.1% Nycodenz (*N'-N'-bis*(2,3-dihydroxypropyl)-5-[*N*(2,3-dihydroxypropyl) acetamido]-2,4,6-triodo-isophtalamide), 0.44% NaCl, and 5 mM Tricine/NaOH, pH 7.0 (J.N. Prep from J. Bio Sa, Les Ullis, France). The purified PMN (97%) were subjected to hypotonic lysis, washed and resuspended in Hank's balanced salt solution HBSS) at pH 7.4.

2.3. PMN respiratory burst

The production of superoxide anion was continuously recorded by monitoring the superoxide dismutase-inhibitable reduction of cytochrome *c* [20], using a UVIKON 860 spectrophotometer equipped with a thermostatted (37°C) cuvette holder and a magnetic stirrer. Suspen-

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Abbreviations: PMN, polymorphonuclear leukocytes; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Paf, platelet-activating factor; PMA, phorbol myristate; PKC, protein kinase C; IC_{50}, drug concentration that reduces control values by 50%; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulphonamide.

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sions of 2×10^6 PMN in 2 ml HBSS were incubated in the absence (control) or presence of W-7 before stimulation, under conditions described in the figure legends. The final concentration of drug solvent (DMSO) did not exceed 0.1% and had no discernable effect on RB. The initial rate and total production of superoxide anion are expressed as percentages of control values.

2.4. Statistical analysis

Statistically significant differences between the results of experiments performed in the presence and absence W-7 were determined using Student's paired *t*-tests with a threshold of $P < 0.05$.

3. Results and discussion

Fig. 1 shows compares the effects of W-7 on the initial rate and total production of superoxide induced by 100 nM fMLP. Treatment of PMN for 5 min with W-7 concentrations from 1 to 15 μM, while having no stimulatory effect, strongly enhanced ($P < 0.01$) the total production of superoxide, with a maximal effect reaching approximately 150% of control values. This potentiation also affected the initial rate, but to a lesser extent. These data suggest that biochemical modifications resulting from W-7 action may affect signalling events that control both the initiation and termination processes of the respiratory burst. W-7 concentrations above 20 μM similarly inhibited both parameters of the respiratory burst, in agreement with earlier reports [16–18].

We have previously shown that a potent PKC inhibitor, staurosporine, also primes these parameters of the PMN respiratory burst mediated by fMLP and Paf [10]. However, this priming effect required at least 10 min of PMN pretreatment with the drug [21]. Unlike staurosporine, the enhancing effects of W-7 were observed after both short and long treatment periods (1–40 min) before

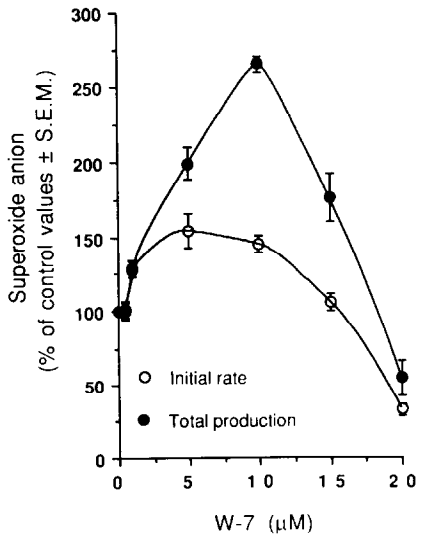

Fig. 1. Effect of W-7 on the fMLP-mediated PMN respiratory burst. PMN were treated at 37°C in the absence (control) or presence of indicated W-7 concentrations for 5 min before stimulation with 0.1 μM fMLP. The initial rate and total amount of superoxide anion production are expressed as % of control values (3.7 ± 0.2 nmol/min and 5.3 ± 0.4 nmol/10^6 cells, respectively).

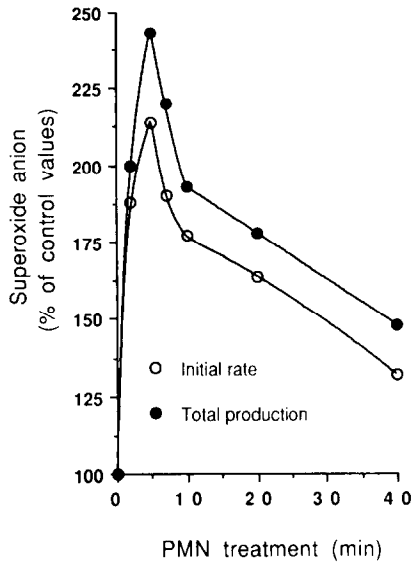

Fig. 2. Effect of the duration of cell treatment on the fMLP-mediated respiratory burst. PMN were treated in the absence (control) or presence of 10 μM W-7 for the indicated periods before stimulation with 0.1 μM fMLP. Initial rate and total production of superoxide anion are the mean of two experiments and are expressed as % of respective controls.

PMN stimulation, indicating that these two drugs may prime the respiratory burst through different mechanisms.

To gain insight into possible sites of action of W-7, its effects were tested on the PMN respiratory burst mediated by three other stimuli known to operate through different pathways: platelet-activating factor (Paf), a chemoattractant which stimulates PMN through membrane receptor stimulation; PMA, a direct PKC activator [22], and the calcium ionophore A23187. The results in Fig. 3 show that W-7 also primed both parameters of the PMN respiratory burst mediated by 1 μM Paf. However, the drug concentrations required were higher than those used with fMLP (Fig. 1). When PMN were stimulated with 160 nM PMA for 10 min, W-7 depressed respiratory burst parameters with an IC_{50} of approximately 8 μM (Fig. 4). W-7 also inhibited the respiratory burst mediated by the calcium ionophore A23187 with an IC_{50} of approximately 30 μM (Fig. 5).

The main observation described above is that low concentrations of W-7 strongly enhanced the production of superoxide by PMN stimulated by two chemoattractants, whereas high drug concentrations were inhibitory. These data partly contrast with previous findings showing only inhibitory effects of W-7 on fMLP-mediated RB [16,18]. Although a small potentiating effect on oxygen consumption of rabbit PMN has been found to occur occasionally [16], the major effects of W-7 are considered to be inhibitory, as the drug also depresses other PMN functions mediated by fMLP such as aggregation, exocytosis [16,17] and directed locomotion [20].

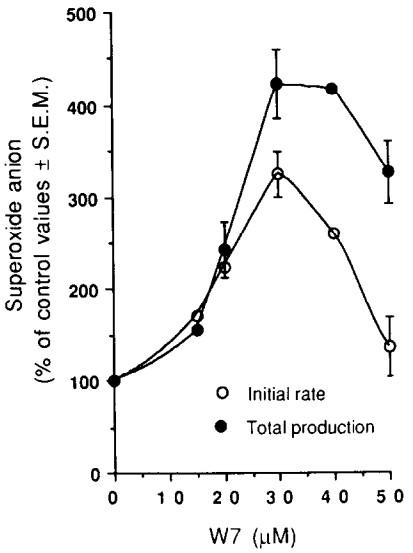

Fig. 3. Effect of W-7 on the Paf-mediated PMN respiratory burst. PMN were treated at 37°C in the absence (control) or presence of indicated W-7 concentrations for 5 min before stimulation with 1 μM Paf. Initial rate and total production of superoxide are expressed as % of control values (2.3 ± 0.1 nmol/min and 2.3 ± 0.2 nmol/10^6 PMN, respectively).

The reason for this descrepancy between W-7 effects in our study and others is difficult to explain. However, one possibility is the use of cells from different sources; for example, elicited rabbit PMN [16] which have been reported to be already primed relative to blood PMN [23].

The ability of W-7 to modulate chemoattractant-mediated RB as a function of the drug concentration suggests that the cellular modifications induced by W-7 affect distinct calmodulin-dependent events that may have a different role in the mechanism of RB stimulation. The comparison of W-7 effects on the respiratory burst mediated by pharmacological stimuli such as A23187 and PMA is informative, given the mechanism by which these agents induced cellular responses. Indeed, W-7 only inhibited the PMN respiratory burst induced by the latter stimulus, whatever the drug concentration (Figs. 4 and 5), in agreement with other reports [20,24]. W-7 also depresses the PMN responses mediated by arachidonic acid [16], a free fatty acid that bypasses the plasma membrane receptor step and acts at a step that precedes calcium mobilization [25]. These data indicate that the biochemical alterations mediated by the drug may occur at a step downstream of calcium mobilization and PKC activation [16]. Supporting this possibility is the recent observation that W-7 inhibits phospholipase D activation in fMLP-stimulated PMN, a finding that was interpreted as being due to inhibition of calmodulin kinase [26]. In fMLP-stimulated PMN, the PLD pathway provides the bulk of phosphatidic acid and diglyceride production [27], and has been linked to respiratory burst stimulation [28,29]. Inhibition of PLD activation by W-7 may thus contribute to the depression of chemoattractant-mediated respiratory burst by W-7 [17,18] (Figs. 1 and 3).

The priming of fMLP-mediated PMN respiratory burst by W-7 occurs at a drug concentration close to its binding constant to purified calmodulin, i.e. approximately 11 μM [30], which indicates that W-7 may exert its effects through association with calmodulin. However, with Paf, higher drug concentrations were required for priming (Fig. 3). This difference reinforces the possibility that the two chemoattractants induce PMN activation through different mechanisms [31]. The fact that W-7 selectively enhanced fMLP and Paf-mediated respiratory burst suggests that the major modifications that lead to RB priming may take place between receptor stimulation and activation of transductional effectors that generate second messengers. This is supported by the observation that W-7 induces activation of phospholipase D in cultured LA-N-2 cells [32]. We have also found that W-7 induces activation of PLD in human PMN (results not shown), as evidenced by production of phosphatidylethanol [33]. Whether this activation occurs through a modification of regulatory components or direct nonspecific interaction of W-7 with PLD remains to be determined. Supporting the former possibility is the observation that calmodulin induces activation of adenylate cyclase [34] through binding to $\beta\gamma$ subunits of Gi [34,35]. Inhibition of calmodulin by W-7 may thus prime the RB through an attenuation of the cyclic AMP-mediated down-regulation of PLD activation, as reported in fMLP-stimulated PMN [36]. Alternatively, W-7 may induce activation of G proteins that regulate PLD

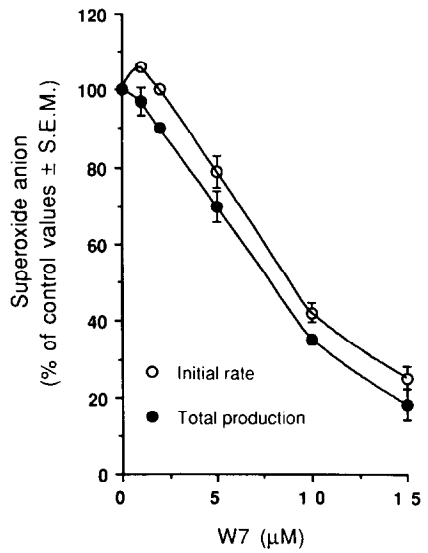

Fig. 4. Effect of W-7 on the PMA-mediated PMN respiratory burst. PMNB were treated at 37°C in the absence (control) or presence of indicated W-7 concentrations for 5 min before stimulation with 160 nM PMA for 10 min. Initial rate and total amount of superoxide are expressed as % of control values (4.5 ± 0.8 nmol/min and 16.9 ± 2 nmol per 10^6 cells, respectively).

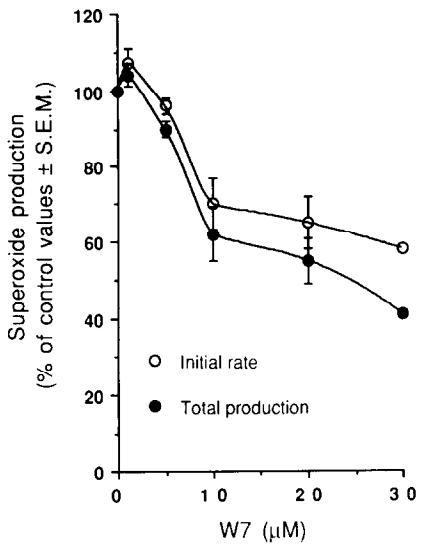

Fig. 5. Effect of W-7 on A23187-mediated PMN respiratory burst. PMN were treated at 37°C in the absence (control) or presence of indicated W-7 concentrations for 5 min before stimulation with 10 μM A23187. Initial rate and total amount of superoxide anion are expressed as % of control values (1.9 ± 0.2 nmol/min and 5.7 ± 0.5 nmol per 10^6 cells, respectively).

activation. Activation of Gi has been proposed as a mechanism by which a potent PKC inhibitor, staurosporine, induces activation of PLD in rabbit PMN [37]. In human PMN, staurosporine also stimulates PLD activation and this may contribute to its priming effect on fMLP- and Paf-mediated respiratory burst [10]. While the biochemical modifications involved in enhancing PLD activation and the respiratory burst remains to be identified, our results suggest that drugs known to antagonize the effects of calmodulin, i.e. some local anesthetics, chlorpromazine and trifluoroperazine, may potentially enhance chemoattractant-mediated physiological PMN functions and therefore their destructive properties.

In summary, low concentrations of the calmodulin antagonist W-7 were found to enhance the respiratory burst of human PMN stimulated by two chemoattractants, fMLP and Paf, wheras higher drug concentrations were inhibitory. This priming effect of W-7 may involve regulation of early signalling events mediated by chemoattractant receptor stimulation.

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