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CALMODULIN INHIBITORS, W-7 AND TFP, BLOCK THE CALMODULIN-INDEPENDENT ACTIVATION OF NADPH-OXIDASE BY ARACHIDONATE IN A CELL-FREE SYSTEM

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Summary: The calmodulin inhibitor, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), or trifluoperazine inhibited not only Fc_γ-receptor mediated cytosolic free Ca^{2+} increase and O_2^- generation in macrophages, but also an arachidonate-induced activation of NADPH-oxidase in a cell-free system. Although these results suggested the involvement of Ca^{2+}-calmodulin system, the cell-free activation of NADPH-oxidase occurred in the presence of EGTA and addition of calmodulin had no effect. Furthermore W-7 shifted the optimal concentration of arachidonate required for the activation to a higher level, suggesting that W-7 may block the interaction between arachidonate and NADPH-oxidase system rather than inhibiting a Ca^{2+}-calmodulin system.

When neutrophils and macrophages are stimulated by a chemotactic formyl peptide, immune complexes or complements, they release active oxigens which have important roles in bactericidal mechanisms of phagocytes (1,2).

We previously showed that a cross-linking of Fc_γ receptor ($Fc_\gamma R$) induces a rapid and transient increase of cytosolic free Ca^{2+} ($[Ca^{2+}]i$) and superoxide anion (O_2^-) generation in macrophages (3). The $[Ca^{2+}]i$ increase is thought to be required for subsequent Ca^{2+} dependent pathway such as the activation of calmodulin system or protein kinase C (C-kinase), and various antagonists have been used to study the roles of Ca^{2+}-calmodulin and C-kinase in cell activation. Thus, the calmodulin inhibitors, trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), were reported to inhibit respiratory burst and degranulation in neutrophils (4-7), and Wright and Hofman suggested that calmodulin-dependent pathway rather than C-kinase is essential for the neutrophil activation (7). But it is still unknown how Ca^{2+} -calmodulin acts in a cell activation actually.

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We reported recently that arachidonate seems to act as a main intracellular activator of NADPH-oxidase (3). In this paper, we investigated the effects of calmodulin and calmodulin inhibitors on the $Fc_\gamma R$-mediated induction of $[Ca^{2+}]i$ increase and O_2^- generation, and on the arachidonate-nduced NADPH-oxidase activation in a cell-free system. The results showed that inhibition of the NADPH-oxidase activation by W-7 in a cell-free system is not due to an inhibition of Ca^{2+}-calmodulin but may be due to a blocking of the interaction of arachidonate with the NADPH-oxidase system.

MATERIALS AND METHODS

Assay of O_2^- generation. Macrophages were obtained by glass adherence method from guinea pig peritoneal exudate cells induced by paraffin oil as described (3). O_2^- release was determined by spectrophotometric measurement of the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c as described (3). In brief, macrophages (5×10^5 /ml) were prewarmed in a HEPES-saline buffer (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 5.5 mM glucose, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, pH 7.2) containing 80 μM cytochrome c at 37°C for 5 min. After stimulation, the increase of A_{550} was measured continuously. $Fc_\gamma R$-mediated O_2^- generation was triggered by the addition of 50 μg $F(ab')_2$ fragment of rabbit anti-guinea pig Fab_γ to guinea pig IgG2-bound macrophages.

Assay of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$). Cytosolic free Ca^{2+} concentration was determined by measuring the fluoresence of Quin-2 loaded cells by a modification (3) of the procedure of Tsien et al. (8).

Disruption and fractionation of macrophages. Cells were disrupted by sonication and postnuclear (800 x G supernatant (sup.)) and particulate (100,000 x G pellet) fractions were prepared as described previously (3). Activated NADPH-oxidase was obtained from the 100,000 x G pellet of the arachidonate (1000 μM)-stimulated 800 x G sup. (5×10^7 cell eq./ml).

Assay of NADPH-oxidase activity in a cell-free system. The activity of the cellular fractions was measured by the SOD-inhibitable cytochrome c reduction in the presence of NADPH (200 μM) and FAD (10 μM) at 25°C as described (3). Calcium free buffer (65 mM phosphate buffer, 2 mM EGTA, 0.17 M sucrose, 2mM NaN_3, pH 7.2) or calcium containing buffer (20 mM Tris, 1 mM $CaCl_2$, 0.17 M sucrose, 2 mM NaN_3, pH 7.2) was used as the assay buffer.

Reagents. Cytochrome c (Type III), sodium arachidonate, 12-O-tetradecanoyl phorbol 13-acetate (TPA) and N-formyl-methyl-leucyl-phenylalanine (FMLP) were obtained from Sigma, St. Louis, MO., A23187 from Calbiochem, La. Jolla, CA., N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) from Biochemicals Co., Tokyo, and trifluoperazine (TFP) from Wako Pure Chemical Industries, Osaka. Purified calmodulin of bovine brain was kindly provided by Dr. E. Miyamoto of Kumamoto University Medical School.

RESULTS

Inhibition of the $Fc_\gamma R$-mediated increase of $[Ca^{2+}]i$ and O_2^- generation by calmodulin inhibitors. The cross-linking of the $Fc_\gamma R$ caused a rapid and transient increase of $[Ca^{2+}]i$ and O_2^- generation. Both reactions were dose-

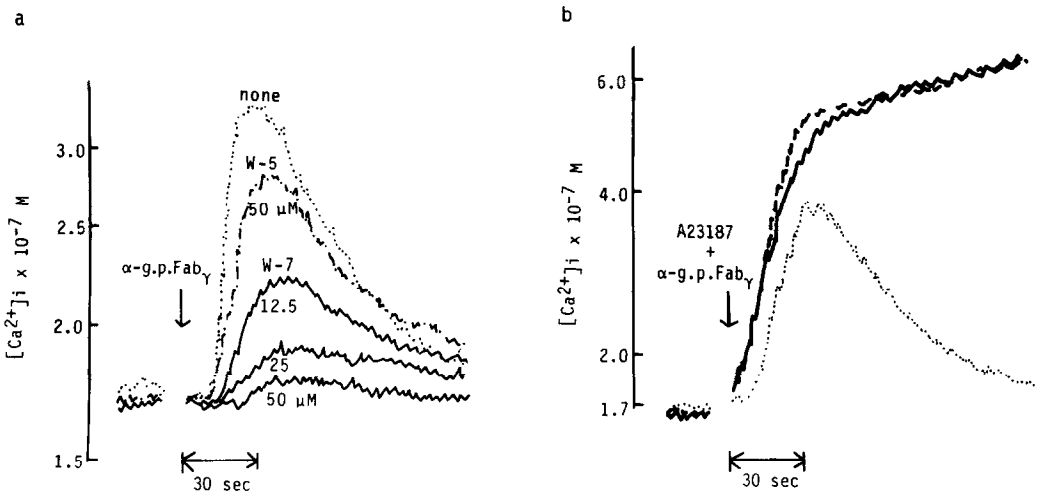

Fig. 1. Effect of W-7 on Fc$_\gamma$R-mediated [Ca^{2+}]i increase. a; IgG2-bound cells loaded with Quin-2 (2.5 x 10^6 cells/ml) were preincubated with W-7 or W-5 at 37°C for 2 min in an assay cuvette, and stimulated with 100 μg/ml of F(ab')$_2$ fragment of rabbit anti-guinea pig Fab$_\gamma$ antibody (α-g.p.Fab$_\gamma$) in the presence of W-7 (——), W-5 (—·—) or none (·····). b; 0.24 μM A23187 was added at the time of stimulation. A23187 + α-g.p.Fab$_\gamma$ (– – –), A23187 + α-g.p.Fab$_\gamma$ + 100 μM W-7 (——), and α-g.p.Fab$_\gamma$ alone (.....).

dependently inhibited by a calmodulin inhibitor, W-7 (Fig. 1a, Fig. 2). But the inhibition of the O_2^- generation did not seem to be due to the inhibition of the increase of [Ca^{2+}]i, because the O_2^- generation in the presence of 100

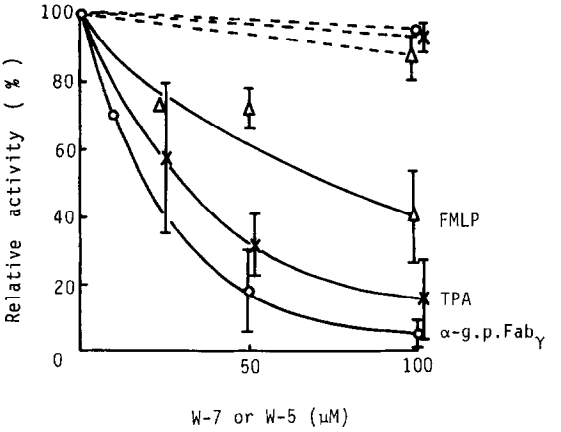

Fig. 2. Effect of W-7 on the O_2^- generation induced by various stimulants. Cells (5x10^5 cells/ml) were preincubated with W-7 (——) or W-5 (– – –) at 37°C for 2 min and stimulated with 50 μg/ml α-g.p.Fab$_\gamma$ (○), 2 ng/ml TPA (✕), 10^{-6}M FMLP (△): O_2^- generating activity by these stimulations in the absence of inhibitors were ; 0.63 + 0.32 (n=3), 3.67 + 0.74 (n=3) and 4.08 + 2.20 nmole cyt c reduced/min/5 x 10^5 cells (n=2), respectively.

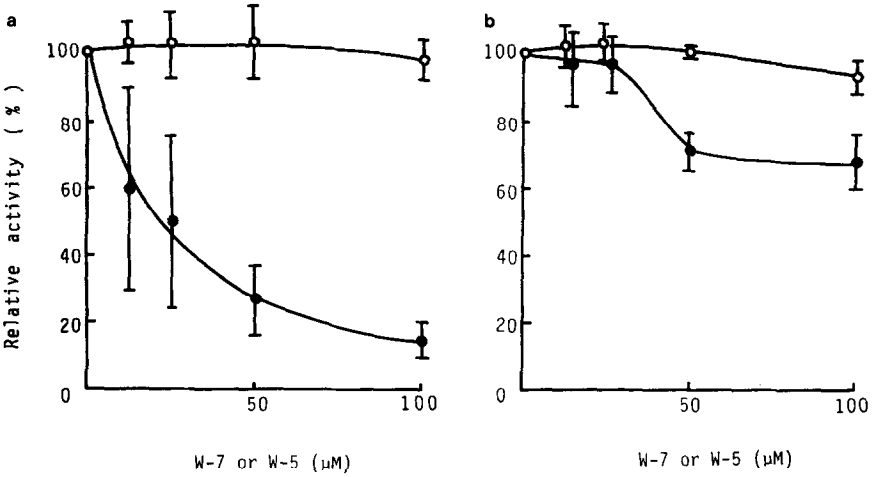

Fig. 3. Effect of W-7 on arachidonate-induced NADPH-oxidase activation in cell lysates and on previously activated NADPH-oxidase. a; A postnuclear fraction (800 x g sup.) of unstimulated cells ($1x10^6$ cell eq./ml) was stimulated by 25 μM arachidonate in the presence of W-7 (●) or W-5 (○). Assays were performed in the presence of 2 mM EGTA. NADPH-oxidase activity of control was 1.16 + 0.37 nmole cyt c reduced/min/$1x10^6$ cell eq. (n=4). b; A postnuclear fraction (5×10^7 cell eq./ml) was incubated with 1000 μM arachidonate at 25°C for 15 min to activate NADPH-oxidase, and the 100,000 x G pellet was resonicated and used as previously activated NADPH-oxidase. Assays were performed in the presence of 2 mM EGTA. Control activity was 0.84 + 0.25 nmole cyt c reduced/min/1 x 10^6 cell eq. (n=4).

μM W-7 was not restored (data not shown) when the $[Ca^{2+}]i$ was increased sufficiently by the addition of a calcium ionophore, A23187 (Fig. 1b). The O_2^- generation induced by TPA and FMLP was also inhibited by W-7 (Fig. 2) and by another inhibitor, TFP (5-10 μM) (data not shown).

The effect of calmodulin and calmodulin inhibitors on NADPH-oxidase activation in a cell-free system. As shown in Fig. 3a, W-7 inhibited dose-dependently the NADPH-oxidase activity stimulated by arachidonate in a postnuclear fraction of unstimulated cells in the presence of 2mM EGTA, while W-7 had only a weak effect on the previously activated NADPH-oxidase (Fig. 3b). Similarly, TFP (10 μM) inhibited the arachidonate-induced NADPH-oxidase activity but only weakly the activated NADPH-oxidase (data not shown).

Calmodulin (10-100 μg/ml) did not induce the NADPH-oxidase activation by itself nor did it enhance the arachidonate-induced activation in the presence or absence of $CaCl_2$ (Table 1). An enough amount of calmodulin (10 or 100 μg/ml) to recover the phosphodiesterase activity in the presence of 25 μM W-7

Table 1. Effects of calmodulin and W-7 on arachidonate-induced NADPH-oxidase activation in cell lysates[a]

Stimulants and inhibitors	NADPH-oxidase activity (nmole cyt c reduced/min/1x10[6] cell eq.)	
	1 mM $CaCl_2$[b]	2 mM EGTA[b]
		(%)
none[c]	0.09 ± 0.02	0.16 ± 0.03
CaM(10 µg/ml)	0.17 ± 0.01	0.09 ± 0.09
CaM(100 µg/ml)	0[d]	0.13[d]
$C_{20:4}$(25 µM)	0.40 ± 0.12	1.04 ± 0.14 (100)
$C_{20:4}$(25 µM) + CaM(100 µg/ml)	0.18 ± 0.03	0.93[d] (89)
$C_{20:4}$(25 µM) + W-7(25 µM)	N.D.[e]	0.42 ± 0.17 (40)
$C_{20:4}$(25 µM) + CaM(10 µg/ml) + W-7(25 µM)	N.D.	0.43 ± 0.17 (41)
$C_{20:4}$(25 µM) + CaM(100 µg/ml) + W-7(25 µM)	N.D.	0.28[d] (27)
$C_{20:4}$(25 µM) + W-5(25 µM)	N.D.	1.04 ± 0.14(100)

a, A postnuclear fraction (800 x G sup.) of unstimulated cell lysats was stimulated by arachidonate ($C_{20:4}$) or calmodulin (CaM).
b, Calcium-containing buffer and -free buffer are described in MATERIALS AND METHODS.
c, NADPH (200 µM) alone was added.
d, Result of a single experiment. All other data (means ± S.D.) were obtained from three or four independent experiments.
e, N.D., not done.

could not cancel the inhibitory effect of 25 µM W-7 (Table 1) or 10 µM TFP (data not shown) on the activation of NADPH-oxidase.

The above results show that the inhibition of the cell-free activation of NADPH-oxidase by calmodulin inhibitors was not due to an inhibition of Ca^{2+}-calmodulin system or a direct inhibition of the NADPH-oxidase activity.

Effect of calmodulin inhibitors on the dose-related activation of NADPH-oxidase by arachidonate in cell lysates. Arachidonate activated the NADPH-oxidase in 800 x G sup. with an optimal concentration of 25-50 µM (Fig. 4). The optimal concentration of arachidonate shifted to 50-75 µM in the presence of 50 µM W-7 (Fig. 4). Similar effect was observed with 10 µM TFP (data not shown), suggesting that W-7 and TFP competitively inhibit the interaction of arachidonate with the NADPH-oxidase system.

DISCUSSION

Our study showed that W-7 inhibited both the $Fc_\gamma R$-mediated increase of $[Ca^{2+}]i$ and the O_2^- generation in macrophages (Fig. 1a, Fig. 2). Since it was

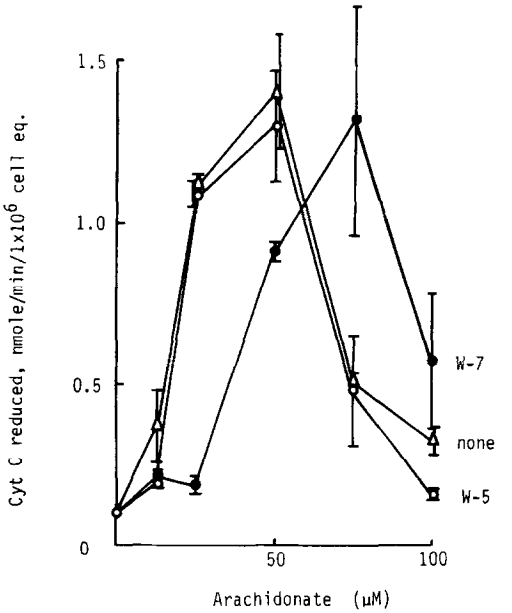

Fig. 4. Effect of W-7 on the dose-related activation of NADPH-oxidase in cell lysates by arachidonate. A postnuclear fraction (800 x G sup.) of unstimulated cells (1 x 10^6 cell eq./ml) was stimulated by various concentrations of arachidonate in the presence of 50 μM W-7 (●), 50 μM W-5 (O) and none (Δ). Assays were performed in the presence of 2 mM EGTA.

shown recently that generation of $I(1,3,4,5)P_4$ which is reported to induce Ca^{2+} influx (9) is mediated by the phosphorylation of $I(1,4,5)P_3$ by a calmodulin dependent protein kinase (10), the inhibition of $[Ca^{2+}]i$ increase by W-7 could be due to its inhibitory effect on calmodulin. However, the inhibition of the O_2^- generation by W-7 was not due to a blocking of the $[Ca^{2+}]i$ increase because the O_2^- generation was still inhibited by W-7 when $[Ca^{2+}]i$ was increased by a Ca^{2+} ionophore, A23187 (Fig. 2b).

It is known, recently, that fatty acids including arachidonate induce the NADPH-oxidase activation in cell lysates (11,12). In our study, W-7 and TFP inhibited the arachidonate-induced NADPH-oxidase activation in a cell-free system (Fig. 3a) but this activation seems to be independent of a Ca^{2+}-calmodulin pathway because, 1) Ca^{2+}-calmodulin system requires at least $Ca^{2+}(>10^{-6}M)$ (13), but arachidonate could activate the NADPH-oxidase in the presence of 2 mM EGTA, 2) purified calmodulin did not affect the arachidonate-induced activation nor could it restore the NADPH-oxidase

activity inhibited by W-7 or TFP (Table 1). Furthermore, calmodulin had no effect on the previously activated NADPH-oxidase in our study (data not shown) although Jones et al. reported that calmodulin enhanced the NADPH-oxidase activity of TPA-stimulated neutrophil membrane (14).

Additionally, W-7 and TFP were reported to have some inhibitory effects on C-kinase (15,16) but our previous study denied the role of C-kinase in an arachidonate-induced NADPH-oxidase activation in a cell-free system (3).

Bromberg and Pick reported that an anionic polar head and a long hydrophobic alkyl chain of fatty acids might be important for the NADPH-oxidase activation (17). In this respect, our result that W-7 shifted the optimal concentration of arachidonate for the NADPH-oxidase activation to a higher concentration (Fig. 4) suggests that W-7 and TFP may be blocking the interaction of arachidonate with the NADPH-oxidase system.

In whole cells, the inhibitory effects of the calmodulin inhibitors on the $Fc_{\gamma}R$-mediated O_2^- generation might be due to not only the inhibition of Ca^{2+}-calmodulin pathway but also the interference with the interaction of arachidonic acid released with the membrane NADPH-oxidase system.

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REFERENCES

1. Badwey, J.A., and Karnovsky, M.L. (1980) Ann. Rev. Biochem. 49, 695-726.
2. Goldstein, I.M., Roos, D., Kaplan, H.B., and Weissmann, G. (1975) J. Clin. Invest. 56, 1155-1163.
3. Sakata, A., Ida, E., Tominaga, M., and K. Onoue, K. (1987) J. Immunol. 138, 4353-4359.
4. Takeshige, K., and Minakami, S. (1981) Biochem. Biophys. Res. Commun. 99, 484-490.
5. Alobaidi, T., Naccache, P.H., and Sha'afi, R.I. (1981) Biochim. Biophys. Acta 675, 316-321.
6. Curnutte, J.T., Badwey, J.A., Robinson, J.M., Karnovsky, M.J., and Karnofsky, M.L. (1984) J. Biol. Chem. 259, 11851-11857.
7. Wright, C.D., and Hoffman, M.D. (1986) Biochem. Biophys. Res. Commun. 135, 749-755.

8. Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982) J. Cell Biol. 94, 325-334.
9. Irvine, R.F., Letcher, A.J., Heslop, J.P., and Berridge, M.J. (1986) Nature 320, 631-634.
10. Yamaguchi, K., Hirata, M., and Kuriyama, H. (1987) Biochem. J. 244, 787-791.
11. Bromberg, Y., and Pick, E. (1984) Cell. Immunol. 88, 213-221.
12. Tanaka, T., Kanegasaki, S., Makino, R., Iizuka, T., and Ishimura, Y. (1987) Biochem. Biophys. Res. Commun. 144, 606-612.
13. Hidaka, H., Yamaki, T., Totsuka, T., and Asano, M. (1979) Mol. Pharmacol. 15, 49-50.
14. Jones, H.P., Ghai, G., Petrone, W.F., and McCord, J.M. (1982) Biochim. Biophys. Acta 714, 152-156.
15. Tanaka, T., Ohmura, T., Yamakado, T., and Hidaka, H. (1982) Mol. Pharmacol. 22, 408-412.
16. Wise, B., Glass, D. B., Chou, C.-H.J., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F., and Kuo, J.F. (1982) J. Biol. Chem. 257, 8489-8495.
17. Bromberg, Y., and Pick, E. (1985) J. Biol. Chem. 260, 13539-13545.