Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 484-490

INVOLVEMENT OF CALMODULIN IN PHAGOCYTOTIC RESPIRATORY BURST OF LEUKOCYTES

Koichiro Takeshige and Shigeki Minakami
Department of Biochemistry, Kyushu University School of Medicine,
Fukuoka 812, Japan

Received February 10, 1981

Summary: The superoxide release of guinea pig exudate leukocytes induced by phagocytosis or by stimulation with cytochalasin D, digitonin or calcium ionophore A23187 was completely inhibited by the inhibitors of calmodulin-stimulated processes such as trifluoperazine at 10 μM. A particulate NADPH-dependent superoxide-forming enzyme from the cytochalasin D-stimulated cells was also inhibited by the inhibitors and by EGTA. The activation of heart phosphodiesterase by a boiled extract of the cells which was dependent on calcium ions and abolished by trifluoperazine was observed. These results suggest the presence of calmodulin in leukocytes and its possible role in the stimulation of the superoxide formation.

The burst in respiration which is observed in polymorphonuclear leukocytes during phagocytosis or by stimulation of the cells with reagents such as digitonin, cytochalasin D or calcium ionophore A23187 is accompanied by the formation of superoxide radicals. A rise in the cytosol calcium concentrations is considered to be an important factor in the stimulation of the oxidative metabolism(1,2). We have previously reported on the inhibitory effect of an intracellular calcium-antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate on the superoxide release stimulated with A23187 and its reversal by the addition of calcium ions and suggested that an intracellular translocation of the ions is responsible for the stimulation(3). We have also shown by using a fluorescent probe chlortetracycline that the ions are released from an intracellular hydrophobic storage site during phagocytosis(4).

A calcium-dependent modulator protein ubiquitously distributed in tissues of eukaryotes has been considered to be a general intracellular calcium receptor that confers sensitivity on various proteins and mediates calcium-stimu-

0006-291X/81/060484-07$01.00/0

lated reactions(5-8). Several reagents such as trifluoperazine, chlorprothixene, pimozide, prenylamine and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide(W-7) were reported to be selective inhibitors of calmodulin-stimulated reactions(9).

In the present paper, we show that calmodulin is present in polymorphonuclear leukocytes and that the calmodulin-inhibitors strongly inhibit the superoxide release of the cells and the NADPH-dependent superoxide formation by the particulate fraction. The results suggest the involvement of calmodulin in stimulating the oxidative metabolism of the cells during phagocytosis.

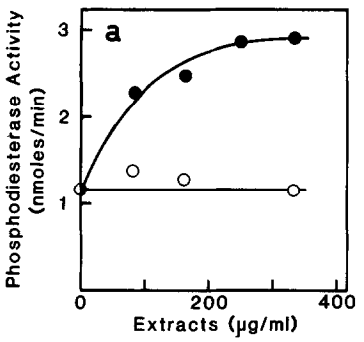
EXPERIMENTAL

Polymorphonuclear leukocytes were those of guinea pig peritoneal exudates induced by 2 % caseinate solution(10). The cells were suspended in a modified Krebs-Ringer phosphate solution(calcium 0.6 mM, pH 7.4). The activity of the cells to release superoxide was measured by the reduction of cytochrome c(10). The particulate fraction was prepared from the homogenate of the resting or the cytochalasin D-stimulated cells as a precipitate between 250 x g for 10 min and 100,000 x g for 30 min by a differential centrifugation in 0.34 M sucrose solution(11). The NADPH-dependent superoxide formation by the particulate fraction was determined by the reduction of acetylated cytochrome c(12). Leukocyte extract for the assay of calmodulin was prepared by sonication of the cells(1.5 ml packed cells in 3 ml of 10 mM Tris-HCl buffer,pH 7.5) for 10 sec, boiling for 5 min and centrifugation at 10,000 x g for 10 min. The supernatant was assayed for calmodulin activity by the activation of bovine heart phosphodiesterase(13). The supernatant itself had no phosphodiesterase activity.

A 23187 was obtained from Calbiochem-Behring Corp. and calmodulin, phosphodiesterase and cytochalasin D from Sigma. W-7 was kindly provided by Prof.H.Hidaka, Department of Pharmacology, Mie University Medical School, trifluoperazine and chlorprothixene from Yoshitomi Pharmaceutical Co.,pimozide by Fujisawa Pharmaceutical Co. and prenylamine from Meiji Confectionary Co. All inhibitors, cytochalasin D and A23187 were dissolved in dimethylsulfoxide.

RESULTS AND DISCUSSION

Presence of calmodulin in leukocytes: We first studied the presence of calmodulin in polymorphonuclear leukocytes because no report was found dealing with this problem. As shown in Fig. 1a, bovine heart phosphodiesterase was activated by the boiled extract of leukocytes in the presence of 50 μM calcium ions to a maximum extent of about 2.5-fold which was equivalent to the maximum activation of the enzyme preparation with an authentic sample of calmodulin.



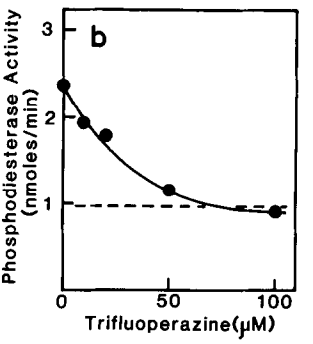


Fig.1a Activation of phosphodiesterase by a boiled leukocyte extract.
Activities of the enzyme(50 μg protein in 1 ml) were assayed in the presence of various amounts of the extract. (●): with 50 μM $CaCl_2$, (○): without calcium and with 1 mM EGTA.

Fig.1b Inhibition of phosphodiesterase by trifluoperazine.
Activities of the enzyme in the presence of the extract(124 μg/ml) and various concentrations of the inhibitor were assayed. The dotted line indicates the activity in the absence of the extract.

The activation was dependent on calcium and no significant activation was observed in the presence of 1 mM ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid(EGTA) without the addition of calcium. The activation by the extract was counteracted by trifluoperazine, an inhibitor of calmodulin-stimulated reactions, as shown in Fig. 1b. The inhibitor at about 70 μM decreased the phosphodiesterase activity to the value without the extract and the half-maximal effect was observed at about 20 μM. The findings indicate that calmodulin is present in the cells.

Inhibition of the superoxide release by calmodulin-inhibitors; When cytochalasin D was added to leukocyte suspension, the release of superoxide radicals was induced as shown by a continuous reduction of cytochrome c sensitive to superoxide dismutase(Fig. 2). A calmodulin-inhibitor trifluoperazine at 10 μM completely inhibited the superoxide release whether it was added before the stimulation or during the active release. The inhibitor at lower concentrations caused the prolongation of the lag time and the decrease of the rate.

Fig. 3a shows effects of various concentrations of calmodulin-inhibitors on the superoxide release induced by cytochalasin D. Trifluoperazine, pimo-

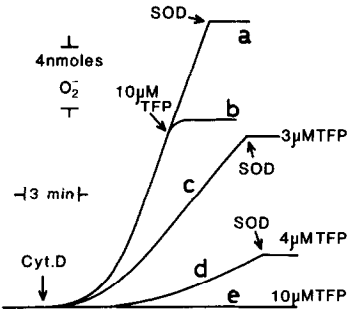
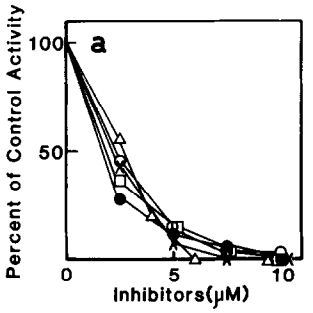


Fig.2 Superoxide release from the cells stimulated with cytochalasin D.
The suspension(2 x 10^6 cells in 1 ml) containing 2 mM glucose and 50 μM ferricytochrome c was preincubated for 5 min at 37°C and the reaction was started by the addition of 5 μM cytochalasin D. The absorbance change at 550-540 nm was recorded with a dual-wavelength spectrophotometer. a): control, b): with 10 μM trifluoperazine added at the point indicated by an arrow. c),d),e): with 3,4,10 μM trifluoperazine added at 2 min before the stimulation of the cells with the cytochalasin. SOD: superoxide dismutase.

zide, chlorprothixene, prenylamine and W-7 were used. The superoxide release activity was progressively decreased by increasing concentrations of these inhibitors and 10 μM of each inhibitor completely inhibited the release. The inhibitors do not act by scavenging the radicals because they did not affect the cytochrome c reduction by the xanthine-xanthine oxidase system. They inhibited not only the release induced by the cytochalasin but also those induced by digitonin, A23187 and heat killed E. coli, as shown with trifluoperazine in Fig. 3b. The release induced by cytochalasin D or A23187 seemed to be slightly more sensitive to the inhibitor than that induced by digitonin or bacteria. The inhibition of the release was not limited to that of guinea pig exudate granulocytes, because the releasing activity of guinea pig peritoneal macrophage or of polymorphonuclear leukocytes from human peripheral blood was similarly inhibited by the inhibitor.

Inhibition of a particulate NADPH-dependent superoxide forming enzyme by calmodulin-inhibitors: We studied the mechanism of the above mentioned inhibition of the cellular superoxide release by examining the effects of calmodu-



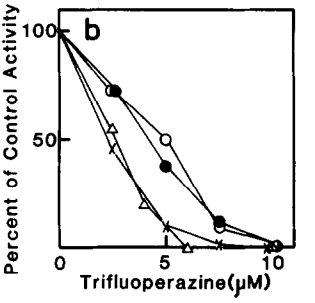


Fig.3a Effects of various calmodulin-inhibitors on the superoxide release induced by cytochalasin D.

The conditions were the same as described for Fig.2, except the inhibitors. The inhibitors were added at 2 min before the induction. The rate of the superoxide release(3.5 nmol/min per 10^6 cells) was set as 100 %. (●): pimozide, (x): chlorprothixene, (△): trifluoperazine, (□): prenylamine, (○): W-7.

Fig.3b Effect of trifluoperazine on the superoxide release induced by various stimulants.

The conditions were as in Fig.2 except the stimulants. The rates stimulated with 5 μg/ml cytochalasin D(△), 10 μg/ml digitonin(○), 0.9 mg/ml heat killed E.coli(●) and 5 μM A23187(x) were 3.1, 5.0, 1.4 and 4.2 nmol/min per 10^6 cells, respectively and they were set as 100 %.

lin-inhibitors on a particulate NADPH-dependent superoxide-forming enzyme which is supposed to be responsible for the superoxide release(14,15). The particulate fractions were prepared from both resting and cytochalasin D-stimulated cells and the activity was assayed by the reduction of acetylated cytochrome c. About 4-fold activation of the enzyme was observed by the stimulation of the cells, in agreement with the proposed mechanism. Thus the activities of the preparations from the resting and the stimulated cells were 0.47 ± 0.09 and 1.87 ± 0.33 nmol/min per mg protein(mean ± SEM, n=3),respectively.

Trifluoperazine inhibited the NADPH-dependent superoxide generating activity of particles from cytochalasin-stimulated cells to the value obtained by particles from resting cells, as shown in Fig. 4. Half-maximal inhibition was observed at about 7 μM which was similar to that required for the 50 % inhibition of the cellular superoxide release(Fig. 3a). Other inhibitors such

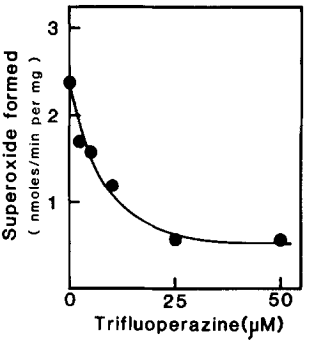


Fig.4 Effect of trifluoperazine on a particulate NADPH-superoxide-forming enzyme.

The particulate fraction was obtained from the cytochalasin-treated cells. The preparation(0.2 mg protein) in 1 ml solution containing 20 mM HEPES buffer,pH 7.5, 70 μM acetylated cytochrome c was preincubated with various concentrations of the inhibitor for 5 min at 37°C and the reaction was started by the addition of 0.5 mM NADPH. The absorbance change at 550-540 nm was recorded and the superoxide-forming rates were calculated by subtracting the reduction rate in the presence of superoxide dismutase.

as prenylamine, pimozide, W-7 and chlorprothixene inhibited the activated enzyme to 50 %, 55 %, 65 % and 75 % respectively, at 10 μM. Calmodulin-stimulated reactions are known to be dependent on calcium and we found the activity of the stimulated enzyme to be decreased by 1 mM EGTA to the value of the resting particles. All these observations support the hypothesis that calmodulin and calcium are essential for the stimulation of the NADPH-dependent superoxide-forming enzyme.

Discussion: Recently, several reports have appeared which indicate a possible involvement of calmodulin in phagocytotic process. Lew and Stossel(16) have shown that calmodulin is involved in the calcium pump of macrophage phagocytic vesicles and Cohen et al.(17) reported the inhibitory effect of phenothiazines and local anesthetics on the cellular superoxide release and on the particulate NADPH-dependent superoxide-forming activity. The latter authors speculated that a change of membrane fluidity caused by the reagents was responsible for the inhibition and did not refer to the role of calmodulin.

Acknowledgement: The study was supported in part by grants from Yamanouchi Foundation of Metabolism and Disease, the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare.

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