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INHIBITION OF PHAGOCYTOTIC METABOLIC CHANGES OF LEUKOCYTES BY AN INTRACELLULAR CALCIUM-ANTAGONIST 8-(N,N-DIETHYLAMINO)-OCTYL-3,4,5-TRIMETHOXYBENZOATE

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Summary

The role of calcium in regulating the activity of leukocytes to generate and release superoxide was studied by using an intracellular calcium-antagonist, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate. The antagonist inhibited the release of superoxide anions induced by a calcium-ionophore A23187 and the inhibition was relieved by the addition of calcium ions. The release induced by cytochalasin D or by the ingestion of bacteria was similarly inhibited by the calcium-antagonist. The result supports the hypothesis that an intracellular translocation of calcium is regulating the phagocytotic metabolic activity of leukocytes. The release of granule enzymes induced by the ionophore was also inhibited by the calcium antagonist.

Polymorphonuclear leukocytes produce superoxide anions (O_2^-) and hydrogen peroxide during phagocytosis. These active oxygens are known to play an important role in bactericidal function of the cells (1-3). Several reagents, for example cytochalasin E (or D) (4,5) and a calcium ionophore A23187, also induce the metabolic change. The role of calcium in the activation of the oxygen metabolism has been discussed, based on the observations that the enhancement of the oxygen consumption was induced by the calcium-ionophore in the presence of Ca^{2+} (6) and the release of O_2^- induced by digitonin was inhibited by a calcium-chelator EGTA (7). If we assume the role of cytoplasmic Ca^{2+} as a second messenger to regulate the metabolism, it is necessary to consider not only the flux of Ca^{2+} through plasma membrane but also the possibility of an intracellular translocation of the ions between an intracellular storage pool and cytosol.

Abbreviation: TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate.

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A new intracellular calcium antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate(TMB-8) has been synthesized. It blocks contraction of smooth and skeletal muscles induced by electrical stimulation, acetylcholine or caffeine, and the inhibition is relieved by extracellular Ca^{2+} (8,9). An application of this reagent in study of the ATP-secretion from blood platelets was reported: TMB-8 inhibited the secretion induced by either thrombin or the calcium-ionophore and the inhibition was overcome by extracellular Ca^{2+} (10).

In this paper, we report the inhibitory effect of TMB-8 on the activity of leukocytes to generate and release O_2^- and the reversal of its effect by the extracellular Ca^{2+}. The results suggest that an intracellular translocation of Ca^{2+} is regulating the phagocytotic metabolic changes of leukocytes.

METHODS

Polymorphonuclear leukocytes were those of guinea pig peritoneal exudates induced by a caseinate solution (5). The cells were suspended in a calcium-free Tris-Ringer solution (120 mM-NaCl, 5 mM-KCl, 1.2 mM-$MgCl_2$, 20 mM-Tris-HCl buffer, pH 7.4). $CaCl_2$ was added as described in Text. The activity to release O_2^- was assayed by the reduction of cytochrome c essentially as described previously (4). The reduction of cytochrome c was measured by a dual-wavelength spectrophotometer (Hitachi 556) at 550-540 nm with a molar absorption coefficient of 19.1 x 10^3 M^{-1} cm^{-1}. The release of enzymes from granules was studied as described previously (5). The activities of myeloperoxidase, lysozyme, β-glucuronidase and lactate dehydrogenase were assayed by reported procedures (11-14). Superoxide dismutase was prepared from bovine erythrocytes by the method of McCord and Fridovich (15). Cytochalasin D, cytochrome c and phenolphthalein β-glucuronide were purchased from Sigma. Ionophore A23187 and TMB-8 were generous gifts of Eli Lilly Co., and Dr. Shoji Kume, Department of Medicine, Tokyo University, respectively. A23187 and TMB-8 were dissolved in diemthylsulfoxide.

RESULTS AND DISCUSSION

The addition of a calcium-ionophore A23187 to leukocyte suspension induced the release of superoxide anions even without the addition of Ca^{2+} to the medium, which was indicated by a continuous reduction of cytochrome c (Fig. 1, curve a). The reduction could be stopped by the addition of superoxide dismutase. The release was inhibited by an intracellular calcium-antagonist TMB-8, as shown by the curve b and was restored by the subsequent addition of Ca^{2+}, as shown by the curve c.

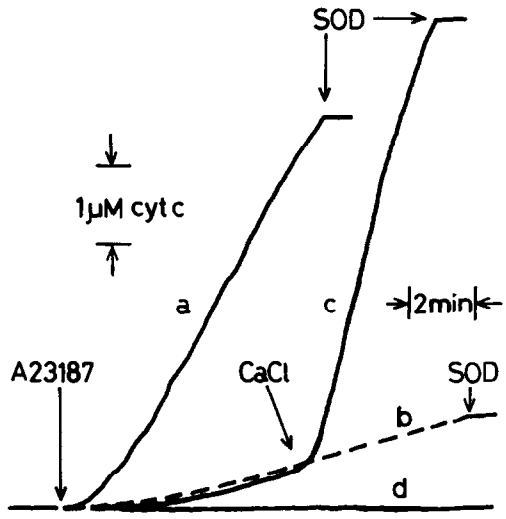

Fig. 1. The release of O_2^- from leukocytes treated with a ionophore A23187. The cells (2 x 10^6) in 1 ml of the Ca^{2+}-free Tris-Ringer solution containing 2 mM glucose and 50 μM cytochrome c were incubated at 37°C with or without TMB-8 (100 μM). The reaction was started by the addition of 5 μM A23187 and the absorbancy change (550-540 nm) was recorded. The reduction of cytochrome c was completely inhibited by 10 ug of superoxide dismutase (SOD). Curves: a) without TMB-8, b) with TMB-8, c) with TMB-8 and subsequently with $CaCl_2$, d) control without A23187.

The effect of extracellular Ca^{2+} concentration on the release of O_2^- induced by A23187 was different in the presence and absence of TMB-8(Fig. 2a). The activity of the cells to release O_2^- was essentially proportional to extracellular Ca^{2+} concentrations when the cells were treated with TMB-8, whereas the activity of the cells not treated with TMB-8 was apparently independent of extracellular Ca^{2+} when the Ca^{2+} concentration was low but it increased linearly with the Ca^{2+} concentration at higher Ca^{2+} concentrations. The release in the absence of TMB-8 and at low concentration of extracellular Ca^{2+} may be ascribed to the effect of A23187 to accelerate the release of Ca^{2+} from an intracellular storage granule to the cytosol, as suggested by Charo et al.(10), whereas the release of O_2^- at higher concentration of extracellular Ca^{2+} may essentially be due to the transport function of the ionophore through plasma membrane. Thus, the percentage of the inhibition caused by TMB-8 at a given concentration of extracellular Ca^{2+} was dependent on the Ca^{2+} concentration

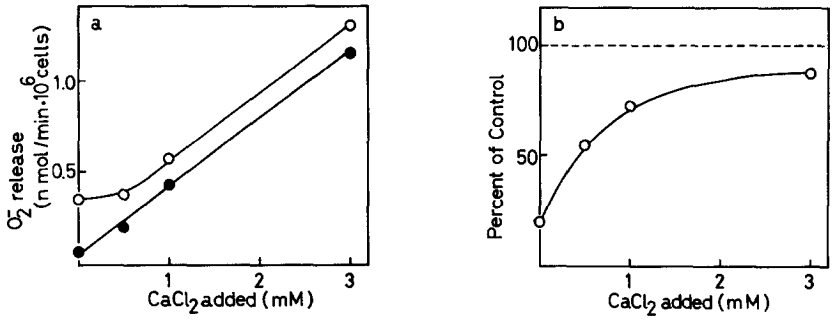

Fig. 2a. The effect of added Ca^{2+} concentration on the O_2^- release induced by A23187 in the presence and absence of TMB-8. The conditions were the same as described for Fig. 1. (●) with TMB-8, (○) without TMB-8.
Fig. 2b. The activity of the cells treated with TMB-8 as percent of the control activity without TMB-8 at a given concentration of Ca^{2+}(from Fig. 2a).

(Fig. 2b): the inhibition was strong in Ca^{2+}-free medium and it decreased gradually with the increase of extracellular Ca^{2+} concentration. This observation may support another hypothesis of Charo et al. (10) that TMB-8 serves as a general antagonist of intracellular Ca^{2+} by inhibiting the release of Ca^{2+} from a storage site.

The inhibition of the O_2^--release by TMB-8 was not limited to the activity induced by the calcium-ionophore. As shown in Table I, the O_2^--release induced by the ingestion of heat-killed E. coli or by the interaction of cytochalasin D was similarly inhibited by TMB-8. This suggests the general role of intracellular Ca^{2+} in the regulation of the O_2^--release reaction. Verapamil which

Table I.The effect of TMB-8 on the O_2^--release activity induced by E. coli, cytochalasin D or A23187.

	Activity (nmol/min per 10^6 cells)		
	E. coli(0.9 mg)	cytochalasin D(10 μg)	A23187(5 nmol)
Control	0.84	1.20	2.49
TMB-8 50 μM	0.49	0.40	2.49
100 μM	0.42	0.13	1.06
200 μM	0.14	0	0.51
300 μM	0.04	0	0.07

The conditions were essentially the same as described for Fig. 1. The inducers were added 5 min after the preincubation with or without TMB-8.

Table II. The effect of TMB-8 on the release of granule enzymes induced by A23187

	Activities of enzymes released (% of total activity)					
	Myeloperoxidase		Lysozyme		β-glucuronidase	
	Control	TMB-8	Control	TMB-8	Control	TMB-8
Control	3.5		4.1		3.4	
A23187						
Ca^{2+}-free	16.2	9.3	14.5	9.3	8.3	7.8
3 mM-Ca^{2+}	59.8	52.1	29.2	21.2	17.3	16.6

Leukocytes (4×10^7) in 2 ml of the Tris-Ringer solution were preincubated for 5 min at 37°C with or without TMB-8 (200 μM) and the reaction was started by A23187 (10 μM). After 10 min, the samples were cooled in ice and the supernatant was separated by centrifugation at 900 x g for 10 min at 4°C. The activity in the supernatant was expressed as the percent of total activity obtained by the disruption of the cells with 0.2 % Triton X-100. No changes in the release of lactate dehydrogenase was observed by the treatments.

has been used to inhibit the influx of extracellular Ca^{2+} (16) showed only 20 % inhibition at 10 μM (induction by cytochalasin D at 1 mM-Ca^{2+}).

TMB-8 also inhibited the release of granule enzymes from the cells (Table II). Myeloperoxidase and lysozyme which are known to be localized in granules, were released from the cells into incubation medium, when the cells were treated with the ionophore A23187, while the release of lactate dehydrogenase, a cytosol enzyme, was essentially unaffected by the treatment. The release of the granule enzymes was accelerated by the addition of Ca^{2+} ions and was inhibited by TMB-8. The inhibition by TMB-8 was strong in calcium-free medium, whereas it was not so apparent in the presence of the added Ca^{2+}. The situation was very much similar to that of the O_2^--release and a similar mechanism is supposed to be operating in the regulation of the both processes. The effect of TMB-8 on the release of another granule enzyme, β-glucuronidase, was not so clear. This may be a reflection of the different localization of myeloperoxidase and β-glucuronidase in guinea pig leukocytes (11). Lysozyme may distribute in the both kinds of granules as in the case of human leukocytes (17) and it may explain the intermediary properties of lysozyme between myeloperoxidase and β-glucuronidase.

There is a general agreement that calcium is a second messenger of several stimulus-secretion or stimulus-contraction coupling processes. Intracellular free calcium can be regulated either by an influx of extracellular Ca^{2+} or by an intracellular translocation of the ions, or by both, but an importance of the intracellular process in short-time metabolic changes has been suggested for various kind of cells (18). The present observation, together with accumulated evidences in the researches of other cells, may allow us to speculate that an intracellular translocation of Ca^{2+} is playing an important role in the regulation of phagocytotic metabolism in polymorphonuclear leukocytes.

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