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RELEASE OF CALCIUM FROM MEMBRANES AND ITS RELATION TO PHAGOCYTOTIC METABOLIC CHANGES: A FLUORESCENCE STUDY ON LEUKOCYTES LOADED WITH CHLORTETRACYCLINE

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Summary: A fluorescent probe chlortetracycline was used to monitor the mobilization of intracellular divalent cations of leukocytes. When the chlortetracycline-loaded cells were stimulated with cytochalasin D or E. coli, a fluorescence change ascribable to the release of calcium from the intracellular hydrophobic environment was observed. The dose-response curve of the fluorescence change and that of the superoxide release of the cells were very similar. An intracellular calcium antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate inhibited both metabolic and fluorescence changes in parallel. A supposition that an intracellular mobilization of calcium ions is stimulating the metabolic change was supported.

The increase in respiration which is associated with the production of superoxide anions (O_2^-) was observed in polymorphonuclear leukocytes during phagocytosis or by stimulation of the cells with reagents such as digitonin (1), cytochalasin D (2) or calcium ionophore A23187 (3). The observations that the enhancement of the oxygen consumption was induced by the calcium ionophore in the presence of Ca^{2+} (3) and the O_2^--release induced by digitonin was inhibited by EGTA (1) indicate that calcium ions are involved in the stimulation of the metabolic change. We have previously reported on the inhibitory effect of an intracellular Ca^{2+}-antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) on the O_2^--release and the release of the granule enzymes from leukocytes stimulated with A23187 and its reversal by the influx of Ca^{2+} and suggested that the translocation of Ca^{2+} from an intracellular storage pool to cytosol is stimulating the phagocytotic metabolic changes of the cells (4). A similar inhibitory effect of TMB-8 on the release of the granule enzymes from leukocytes stimulated with phorbol myristate acetate was reported (5).

Abbreviations: CTC, chlortetracycline; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate; EGTA, ethyleneglycol-bis-(-aminoethylether)-N,N,N',N'-tetraacetic acid.

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Fluorescence of chlortetracycline (CTC) is extremely sensitive to the concentrations of divalent cations within the cellular hydrophobic environment (membranes) into which it is preferentially partitioned. The calcium chelate gives stronger fluorescence than the magnesium chelate and the spectra of both chelates are different (6). CTC has been used as a probe to investigate intracellular events associated with the divalent cations and their interactions with membranes such as mitochondria and sarcoplasmic reticulum (7-10). We show in this report that leukocytes loaded with CTC give a fluorescence change which is ascribable to a release of calcium from hydrophobic environment when the cells are stimulated and the fluorescence change is parallel with the change in the O_2^--release. The result supports a supposition that the mobilization of Ca^{2+} from a hydrophobic storage site to cytosol is stimulating the oxidative metabolism of leukocytes.

Experimental

Polymorphonuclear leukocytes were those of guinea pig peritoneal exudates induced by 2 % caseinate solution (11). The cells were suspended in a modified Krebs-Ringer phosphate solution (calcium: 0.6 mM, pH 7.4). Leukocytes (1 x 10^7 cells/ml) were incubated for 30 min at 30°C in the modified Ringer solution containing 2 mM glucose and 100 μM CTC, washed once, resuspended in the Ringer solution containing glucose and CTC, and transferred to a Shimadzu spectrofluorophotometer RF 500 with a constant temperature cuvette holder (37°C). The viability of the cells as measured by the ability to generate superoxide anions was not affected by CTC-treatment. The activity of the superoxide release was measured by the reduction of cytochrome c as described previously (11). Chlortetracycline, oxytetracycline, cytochlasin D and cytochrome c were obtained from Sigma. TMB-8 was a kind gift of Dr. Shoji Kume (Department of Medicine, Tokyo University).

Results and Discussion

Fluorescence change of CTC-loaded leukocytes by stimulation: A rapid decrease of fluorescence was observed when cytochalasin D or heat killed *E. coli* was added to a suspension of CTC-loaded leukocytes, the fluorescence of which was decreasing slowly (Fig. 1a). An essentially similar result was obtained when Ca^{2+} ions were omitted from the suspending solution, indicating that the rapid change in fluorescence is reflecting the intracellular events. The cells loaded with oxytetracycline, a fluorescent reagent structurally similar to CTC but insensitive to divalent cations, did not display such

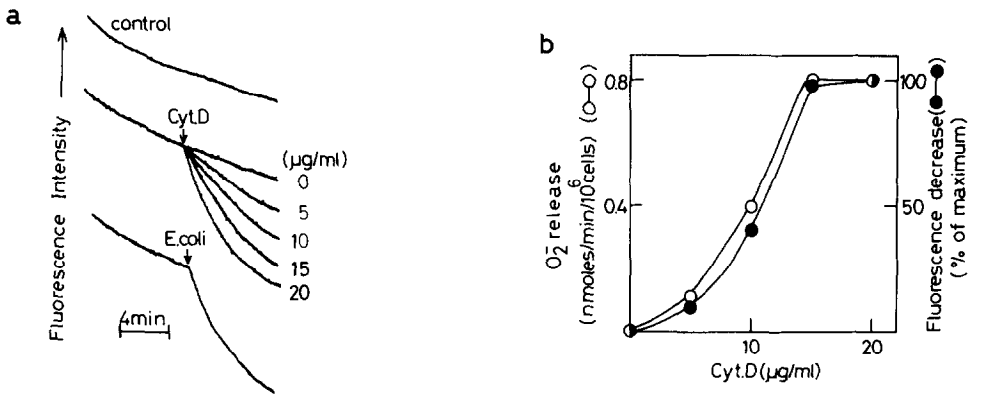

Fig. 1a. Fluorescence change of the CTC-loaded leukocytes during phagocytosis
The cells (1 x 10^7/ml) in the Ringer solution containing 2 mM glucose were incubated at 37°C and the fluorescence was monitored at 514 nm with the excitation wavelength at 401 nm. Cytochalasin D (Cyt D: concentrations given beside the traces) or E. coli (0.9 mg/ml) was added at the points indicated by arrows.
Fig. 1b. Dose dependence of the fluorescence change(●) and the superoxide-release (○) induced by cytochalasin D
The conditions were as in Fig. 1a. The rates of the fluorescence change(arbitrary units) and the superoxide-release (0.8 nmol/min per 10^6 cells) at 20 μg/ml cytochlasin D are set as 100 %.

fluorescence change during phagocytosis. Dose-responses of the fluorescence change and the O_2^--release of the CTC-loaded cells were parallel as shown in Fig. 1b which indicates that both reactions are closely related.

Changes of fluorescence spectra: Because of a difference in fluorescence spectra of CTC chelated to Ca^{2+} and Mg^{2+}, it is possible to distinguish which ions are chelated to CTC in the cells. The excitation and emission maxima of the Mg^{2+} chelate are about 10 nm shorter in wavelength than those of the Ca^{2+} chelate (7). Fig. 2 shows the excitation and emission spectra of the CTC-loaded cells before and after the stimulation with cytochalasin D. Spectra taken 5 min before the stimulation had excitation and emission maxima at 401 nm and 514 nm, respectively, and the fluorescence intensity of the cells decreased greatly after the stimulation, with excitation and emission maxima shifted to 398 nm and 500 nm, respectively. Similar fluorescence changes were observed when the cells were stimulated with E. coli, whereas the resting cells displayed only a moderate change in fluorescence intensity without the shift of the maxima even after incubation at 37°C for 20 min (not

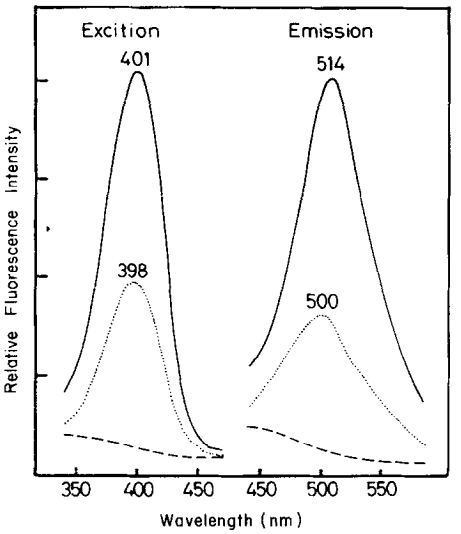

Fig. 2. Excitation and emission spectra of the CTC-loaded leukocytes
The conditions were as in Fig. 1a. The spectra were recorded at 5 min (solid lines) and at 15 min after (dotted lines) the addition of 20 µg/ml cytochalasin D. The spectra of the cells without loading CTC are shown by dashed lines. The excitation and emission wavelengths were set at 401 and 514 nm for the excitation and emission spectra, respectively.

shown). These observations indicate that a large part of the Ca^{2+} in hydrophobic environment (membranes) is displaced by Mg^{2+} when the cells are stimulated. Essentially similar results and interpretations have been reported for pancreatic acinar cells stimulated with caerulein (7) and for peritoneal neutrophiles stimulated with chemotactic factors (8).

Effects of metabolic inhibitors on the fluorescence change: The fluorescence of the CTC-loaded cells were affected by metabolic inhibitors as shown in Fig. 3. Butyryl cAMP which inhibits the O_2^--release (11) strongly inhibited the fluorescence change induced by the stimulation of the cells, whereas cyanide and other respiratory chain inhibitors did not affect the fluorescence in agreement with a previous observation that the O_2^--release is not affected by the inhibitors(11). In contrast, 2-deoxyglucose caused a large decrease of the fluorescence in resting state (Fig. 3b) which was counteracted by glucose at high concentrations. The excitation and emission maxima shifted to shorter wavelength by the addition of deoxyglucose. The fluorescence change in this case was not related to the oxidative metabolic change and a

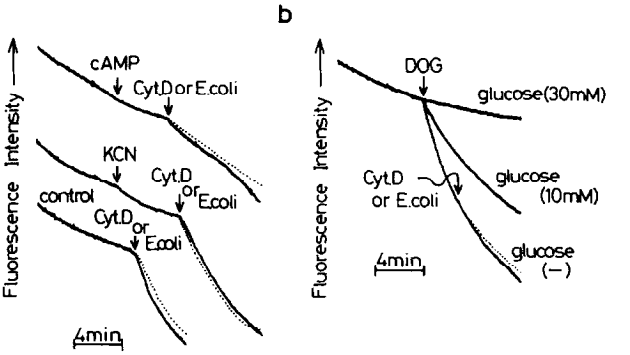

Fig. 3. Effects of cAMP, KCN and 2-deoxyglucose on the fluoresecence change of the CTC-loaded cells
The conditions were as in Fig. 1a. Dibutyryl cyclic AMP(cAMP: 1 mM), KCN (1 mM), 2-deoxyglucose (DOG: 6 mM), cytochalasin D (20 μg/ml: solid lines) and E. coli (0.9 mg/ml: dotted lines) were added at the points indicated by arrows.

subsequent addition of cytochalasin D or bacteria did not cause any decrease of the fluorescence. No O_2^--release was observed when glycolysis was inhibited as reported previously (11). A speculation may be made that the supply of energy by glycolysis is essential for the storage of Ca^{2+} and the ions in the pool are exhausted when glycolysis is inhibited.

Effect of TMB-8 on the fluorescence change: The relation between the release of the membrane-Ca^{2+} and the phagocytotic metabolic change of releasing O_2^- was further studied by using an intracellular Ca^{2+} antagonist TMB-8. As shown in Fig. 4a, when the CTC-loaded cells were treated with TMB-8 (100 μM) for 5 min, the fluorescence decrease and the shift of the excitation and emission maxima due to the stimulation disappeared. TMB-8 also inhibited the O_2^--release induced by cytochalasin D as described previously (4). The titration of the cells with TMB-8 gave essentially the same curve both for the fluorescence change and the O_2^--release (Fig. 4b) which suggests that the release of Ca^{2+} from an intracellular hydrophobic pool to cytosol is an essential process in the oxidative metabolic change of the stimulated cells.

A concept that calcium functions as a link between stimuli and secretion in various cells has generally been accepted. The mobilization of intracellular Ca^{2+} ions or an influx of extracellular Ca^{2+} seems to be regulating

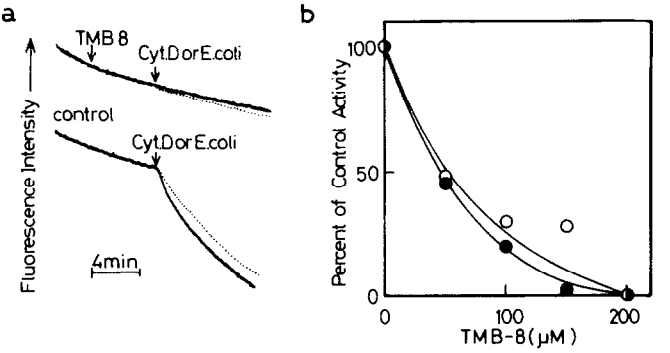

Fig. 4a. Effect of TMB-8 on the fluorescence of the CTC-loaded cells
The conditions were as in Fig. 3, except that TMB-8 (100 μM) was added at the points indicated by an arrow.
Fig. 4b. Effects of TMB-8 on the fluorescence change (●) and the superoxide release (o) of the CTC-loaded cells induced by cytochalasin D
The cells were stimulated with cytochalasin D at 5 min after treatment with various concentrations of TMB-8.

various functions of leukocytes such as chemotaxis (8) and the induction of the oxidative metabolism (3,4). The present study using CTC as an intracellular probe of divalent cations may provide a further evidence for the translocation of the membrane-Ca^{2+} and its close association with the induction of the metabolism to form superoxide anions in leukocytes.

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