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CYTOCHALASIN E DIMINISHES THE LAG PHASE IN THE RELEASE OF SUPEROXIDE BY HUMAN NEUTROPHILS*

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Incubation of human neutrophils with cytochalasin E at $\geq$ 0.30 μM for 10 minutes diminishes the latency period for superoxide release by these cells upon subsequent stimulation with phorbol-12-myristate-13-acetate. Treatment of neutrophils with cytochalasin E at $\geq$ 2.5μM virtually eliminates this latency period under the circumstances of our assay. The utility of this compound for studies concerning the sequence of the biochemical events that occur during stimulation of neutrophils is intimated.

Human neutrophils produce substantial quantities of superoxide (O_2^-)[1] and H_2O_2 during phagocytosis or upon perturbation of their plasmalemma by a variety of surface active agents (e.g. phorbol-12-myristate-13-acetate (PMA), polyunsaturated fatty acids) (for review, see 1-3). Upon addition of PMA to human neutrophils, there is a latency period (lag phase) of 45-60 seconds before a linear rate of O_2^- release is achieved (4,5). Lag periods in cellular O_2^- release have also been reported for a variety of other stimuli (e.g. fluoride, opsonized zymosan, N-formylmethionyl-leucylphenylalanine) (e.g. 4, 6-8). This latency is thought to represent the time required for the assembly and/or activation of the O_2^--producing oxidase system(s) (e.g. 4-6).

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[1]The abbreviations used are: O_2^-, superoxide; PMA, phorbol-12-myristate-13-acetate.

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Several compounds have recently been reported to augment this lag period (e.g. chlorpromazine, trifluoperazine, tetracaine) (5). Such reagents are useful in determining the sequence of events (e.g. depolarization vs. O_2^- production) consequent upon the interaction of neutrophils with stimuli (e.g. 9). To date, however, the only situations reported to diminish the lag period other than increases in the amounts of PMA used, are temperature increases within the range 26 to 46°C (4). In this communication, we report that cytochalasin E can diminish the lag phase in O_2^- release by human neutrophils maximally stimulated with PMA, in a concentration dependent fashion.

Experimental Procedures

Materials

Ficoll-Paque was obtained from Pharmacia Inc., Piscataway, N.J. Cytochalasin E (from Rosellinia necatrix) was purchased from Sigma Chemical Co., St. Louis, MO. Stock solutions of this compound at 2.0 mg/ml were prepared in dimethyl sulfoxide. Sources of all other materials are described elsewhere (10).

Methods

Preparation of Neutrophils - Human neutrophils were routinely purified from 50 ml of whole blood by the dextran sedimentation method of Skoog and Beck (11), with a modification previously described (12). Hypotonic lysis was performed twice to ensure removal of erythrocytes. The resulting cells were suspended in 2.5 ml of Dulbecco's medium (13) lacking divalent cations (138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) and purified further by layering the sample (2.5 ml) over 3.0 ml of Ficoll-Paque in a 16 x 125 mm plastic Falcon tube and centrifuging them through this gradient at 13,000 RPM for 20 min at 4°C (International centrifuge, model PR-2) (14). The neutrophils, which pellet at the bottom of the tube, were resuspended, washed twice, and maintained on ice in modified Dulbecco's medium (described above). Cells were pelleted between washes by centrifugation at 1,000 RPM for 10 min at 4°C.

This procedure resulted in cell preparations that contained >95% neutrophils. Cell viability was always >90%, as measured by exclusion of trypan blue.

Superoxide Release - Superoxide was measured by following the superoxide dismutase inhibitable reduction of ferricytochrome c at 550 nm in a double beam spectrophotometer (15). Cells were incubated in the assay medium (1.0 ml) for 10 min at 37°C before the reactions were initiated by the addition of PMA (1.0 µg/ml). Dimethyl sulfoxide (solvent for cytochalasin) was present in all assays at a constant concentration of 0.25% (v/v). This amount of dimethyl sulfoxide did not significantly affect either the lag time or the final rate of O_2^- release. The assay conditions have been described in detail (16).

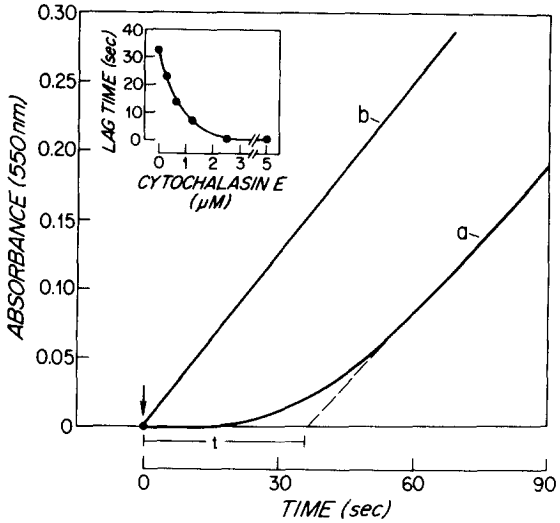

Figure 1 Effect of cytochalasin E on the latency period of superoxide release by human neutrophils.

Reaction progress curves demonstrate the effect of cytochalasin E on the superoxide dependent reduction of cytochrome c by human neutrophils stimulated with PMA (1μg/ml) which was added last to initiate the reactions (bold arrow). Curve "a" shows the initial portion of the progress curve for 1.5 x 10^6 cells/ml in the absence of cytochalasin E. The time (t) required to reach the maximal activity (lag time) was approximated by back-extrapolation to zero absorbance change, as described elsewhere (e.g. 4, 5). Incubation of cells with cytochalasin E (2.5μM) prior to stimulation abolished the lag (curve "b"). The inset shows the effect of various concentrations of cytochalasin E on the lag time. Cells were incubated with cytochalasin E for 10 minutes prior to stimulation.

Results and Discussion

Upon exposure to a "saturating" concentration of PMA (1μg/ml) (10), the initial release of O_2^- by human neutrophils is not linear, but increases over a period of time until the maximal rate is achieved. This time-dependent process results in the appearance of a distinct lag phase in the reaction progress curves (Figure 1). The time required (lag time) for attainment of the maximal rate of O_2^- release under our conditions can be approximated (see Figure 1, & ref. 4, 5) to be 34.8 ± 4.3 S.D. seconds (n=6). This value is comparable to those noted previously with PMA (4,5).

Incubation of neutrophils with cytochalasin E ($\geq$ 0.30 μM) for 10 minutes prior to stimulation with PMA markedly reduces the lag-time (Figure 1 inset). Concentrations of cytochalasin E $\geq$ 2.5 μM virtually eliminate the lag under our assay conditions. The procedure utilized to calculate the lag

time does not take into account the time required for mixing the cells with PMA (ca. 5-7 sec). Since this mixing time is short compared to the lag time, and since the mixing procedure was identical in the presence and absence of cytochalasin E, the data of Figure 1 demonstrate that the effects of cytochalasin E represent a real diminution of the lag-phase.

Kitagawa et al (17) have reported that cytochalasin E alone at 10 μM stimulates O_2^- release by human neutrophils. We calculate the rate of O_2^- release in that study (17) to be ca. 3.0 nmol O_2^-/min/10^7cells [calculated utilizing an extinction coefficient of 20,000 M^{-1} cm^{-1} for cytochrome c (reduced minus oxidized forms (18)]. Under our assay conditions, exposure of cells to cytochalasin E alone at $\leq$ 5 μM does not stimulate O_2^- release when monitored for periods of 10-15 minutes. However, cytochalasin E at 10 μM does stimulate cellular O_2^- release (3.4 ± 1.0 S.D. nmol O_2^-/min/10^7 cells)- a value virtually identical to that of the previous study (17). This stimulation is small by comparison with the effect of the PMA used here. The rate of O_2^- release from human neutrophils stimulated with PMA is 68.6 ± 14.7 S.D. nmol O_2^-/min/10^7 cells (10). Preincubation of human neutrophils for 10 min with cytochalasin E at concentrations <2.5 μM does not affect this rate, while diminishing the lag-time. Preincubation of cells with cytochalasin E at concentrations $\geq$2.5 μM produces a variable increase in the rate of O_2^- release upon stimulation with PMA (20-40%), without a lag.

Nakagawara et al (19) have reported that incubation of guinea pig peritoneal neutrophils with cytochalasin E (10 μM) results in the migration of both the azurophilic and specific granules from the perinuclear region to the cell periphery and subsequent degranulation. Studies of a combined biochemical and cytochemical nature to determine if analogous changes occur in human neutrophils may illuminate the question of whether such events (i.e. contact between organelles and/or membrane fusion) (e.g. see 20) are a part of the "activation" process of the enzyme system(s) responsible for O_2^- release by these cells.

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