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EXTRACELLULAR ATP TRIGGERS SUPEROXIDE PRODUCTION IN HUMAN NEUTROPHILS

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SUMMARY: The effects of ATP on the concentration of cytosolic calcium ($[Ca^{2+}]_i$) were examined with respect to early events associated with activation of the superoxide (O_2^-)-generating system in human neutrophils. Addition of ATP to cytochalasin B-treated neutrophils resulted in two sequential increase in $[Ca^{2+}]_i$: an initial phase presumably related to the mobilization of Ca^{2+} from intracellular stores and a second phase dependent upon the presence of extracellular Ca^{2+}. The second phase was associated with an increase in the rate of O_2^- production, which also required the presence of extracellular Ca^{2+}. The results suggest that increased Ca^{2+} influx may act to trigger a cascade of Ca^{2+}- sensitive events, leading to stimulated O_2^- production.

An increasing number of studies have described a variety of actions of extracellular ATP on the function of intact cells (for review see Ref. 1). Recently ATP at physiological concentrations has been found to augment superoxide production (O_2^-) by human neutrophils stimulated with chemotactic peptide f-Met-Leu-Phe (2-5). The mechanism by which ATP exerts its effect is unclear, but recent studies (2,5) implicate early increases in cytosolic calcium concentration ($[Ca^{2+}]_i$). These observations raise the possibility that ATP may act by binding to specific receptors, designated P_2-purinoceptors.

In separate studies, we have found that ATP, as well as other nucleotide triphosphates such as UTP and ITP, induce a rapid and transient increase in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores (6). We report here that, in the presence of cytochalasin B, ATP can trigger O_2^- production by human neutrophils. The present studies were further designed to examine the causal relationship between changes in $[Ca^{2+}]_i$ and the activation of the O_2^- generating system.

MATERIALS AND METHODS

Chemicals—Fura 2 acetoxymethyl ester (fura 2) was purchased from Dojindo Laboratories, Kumamoto, Japan. ATP was from Oriental Yeast Co., Ltd., Tokyo, Japan. Cytochrome C (type III) was from Sigma Chemical. Co., St.Louis, MO. Cytochalasin B was from Aldrich Co., Milwaukee, WI.

Preparation of the neutrophils—The cells were isolated from fresh citrated blood by dextran sedimentation followed by Conray-Ficoll gradient centrifugation (7) and hypotonic lysis of erythrocytes. The cells were suspended in a HEPES buffer containing 135 mM NaCl, 5 mM KCl, 5 mM glucose, and 20 mM HEPES (pH 7.4), and stored on ice until use. For the experiments listed below, aliquots were obtained from the cell suspensions up to 8 hr after preparation.

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Measurements of superoxide generation—O_2^- production by human neutrophils was monitored continuously by the reduction of ferricytochrome c at 550-540 nm using a dual-wavelength spectrophotometer (Hitachi 557) as previously described (8).

Measurements of $[Ca^{2+}]_i$ with fura 2—Neutrophils were loaded with fura 2 by incubating a cell suspension with 2 μM fura 2/AM for 30 min at 33°C. The cells pelleted by a low speed centrifugation were washed twice and resuspended in the HEPESA buffer. The cell suspension was stored on ice until the experiments were performed. Measurements were performed on a Hitachi F-3000 spectrofluorometer, in a stirred plastic cuvette maintained at 37°C. The excitation and emission wavelengths were 340 nm and 500 nm, respectively. Free $[Ca^{2+}]_i$ was calculated by using the equation $[Ca^{2+}]_i = Kd(F - Fmin)/(Fmax - F)$, where F is the fluorescence intensity of the dye in the cells, Fmax and Fmin are the intensities at saturating and zero calcium concentrations, respectively. The dissociation constant (Kd) of fura 2 for Ca^{2+} was assumed to be 224 nM at 37°C (9). Fmax and Fmin were determined empirically by the addition of Triton X-100 (0.05%) and EGTA (10 mM), respectively.

RESULTS AND DISCUSSION

We examined the effect of ATP on O_2^- production by neutrophils. At the concentrations used (0.1-500 μM), ATP did not by itself result in O_2^- production, as reported by others (5). However, if neutrophils were pretreated with cytochalasin B (5 μg/ml) for 1 min, the cells showed a significant increase in O_2^- production. Fig. 1 shows the representative time courses of ATP-induced O_2^- production. The response to ATP was characterized by a definite lag period of 30 - 45 s prior to the onset of measurable O_2^- production and by the rapid termination of O_2^- production. The rapid termination of O_2^- production was not due to metabolic conversion of ATP since a similar pattern was obtained with ATPγS (adenosine 5'-*O*-(3-thiotriphosphate)), a non-hydrolyzable analogue (not shown). Fig. 1 also shows that the presence of extracellular Ca^{2+} is a critical element of ATP-induced O_2^-production since reduction of the extracellular Ca^{2+} concentration by short-term chelation with 2.5 mM EGTA essentially abolished the response.

The effects of ATP on $[Ca^{2+}]_i$ in neutrophils were monitored using a fluorescent Ca^{2+} probe fura 2. Fura 2-loaded neutrophils, in the presence of 1 mM Ca^{2+}, demonstrated a rapid but

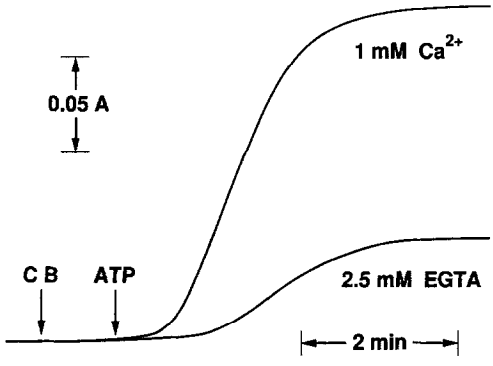

Fig. 1. Time course of ATP-induced O_2^- production by human neutrophils. Neutrophils (1x10^6 cells/ml) were preincubated with 1 mM $CaCl_2$ for 5min at 37°C in the absence (lower) or presence (upper) of 2.5 mM EGTA, and then treated with cytochalasin B (CB, 5 μg/ml) followed after 1 min by ATP (10 μM). The results illustrated are from a typical experiment that was repeated at least ten times using the cells of different donors. Qualitatively similar results were observed for each preparation, although variations among different donors were noted. This variation could be in part attributed to differences in cell age (up to 8 hr after preparation). During this storage time, the response to ATP progressively increased in both rate and magnitude.

transient rise in fluorescence after the addition of 10 μM ATP, with no detectable lag period (Fig. 2A). When the extracellular Ca^{2+} concentration was reduced by short-term chelation with 2.5 mM EGTA, an increase in fura 2 fluorescence was also obtained, indicating that the mobilization of intracellular Ca^{2+} stores contributes to the rise in $[Ca^{2+}]_i$ (not shown). Addition of cytochalasin B (5 μg/ml) to fura 2-loaded cells caused a small increase in $[Ca^{2+}]_i$, in agreement with previous observations (10-12). When ATP was added to these cytochalasin B-treated cells, there was a biphasic increase in $[Ca^{2+}]_i$ that consisted of an initial peak 10 - 15 s after the ATP addition and a broad shoulder of elevated Ca^{2+} 60 - 120 s after stimulation (Fig. 2B). The secondary peak seen in the presence of Ca^{2+} may be attributed to a stimulated entry of Ca^{2+} from the extracellular medium, since it was completely abolished by short-term chelation with 2.5 mM EGTA (Fig. 2C). It therefore appears that the second phase correlates with O_2^- production in its sensitivity to cytochalasin B and to extracellular Ca^{2+}. In contrast, the initial peak was observed also in the presence of EGTA, though partially inhibited. Thus the increase was thought to be primarily from intracellular stores as was seen in the absence of cytochalasin B.

To define the temporal relationship of changes in $[Ca^{2+}]_i$ to the stimulation of O_2^- production, we compared the time course of the two events. The initial increase in $[Ca^{2+}]_i$ occurred almost instantly, peaking within 15 s of activation, and was followed by a second, prolonged increase, which reached a maximum 90 s after stimulation. In contrast, O_2^- production was

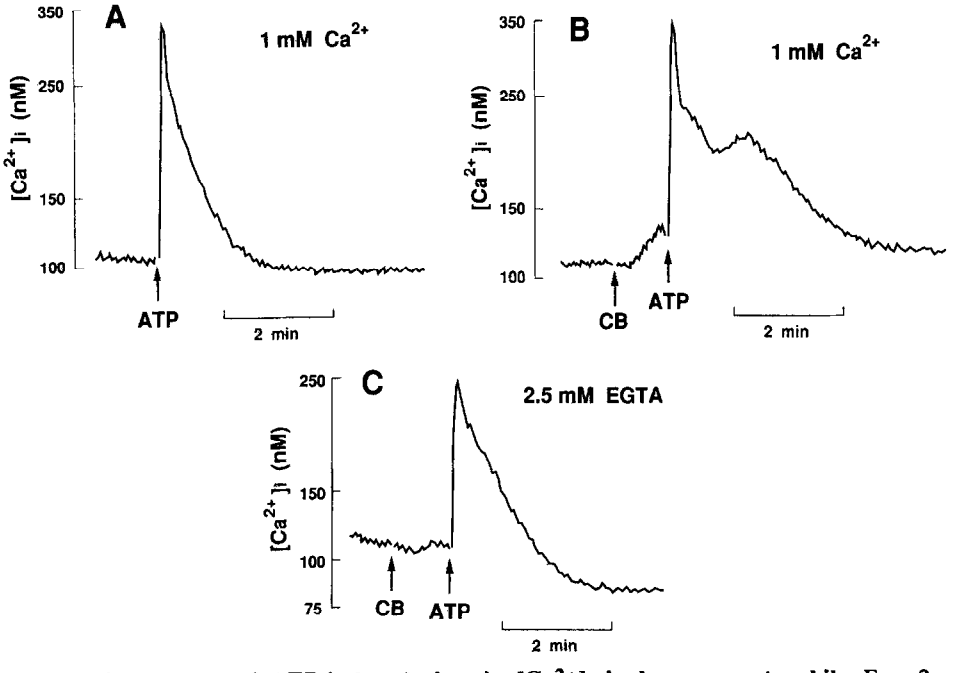

Fig. 2. Time course of ATP-induced rises in $[Ca^{2+}]_i$ in human neutrophils. Fura 2-loaded neutrophils ($2x10^6$ cells/ml) were preincubated with 1 mM $CaCl_2$ for 10 min at 37°C. Trace A represents cells stimulated with ATP (10 μM) alone, while traces B and C represent cells pretreated with cytochalasin B (CB, 5 μg/ml) for 1 min prior to the addition of ATP. In trace C, EGTA was added (to 2.5 mM) to chelate extracellular Ca^{2+} 2 min prior to the addition of CB. As was seen in the O_2^- production, we noticed that the secondary peak in trace B was apparently irreproducible and was extremely variable in extent in various batches of cells. The three experiments were carried out with aliquots of the same batch of fura 2-loaded neutrophils.

detectable only after 30 s and then increased linearly from 45 s to 120 s. It can therefore be concluded that the initial phase precedes the onset of detectable O_2^- production, and that the second phase coincides with the increase in the rate of O_2^- production.

The results reported here indicate that human neutrophils can be induced by exposure to micromolar concentrations of ATP to produce O_2^- when preincubated with cytochalasin B. ATP, *per se*, provoked a transient increase in $[Ca^{2+}]_i$, but did not induce O_2^- production unless the cells were treated with cytochalasin B. Therefore, our results support the view (8,10,13) that an increase in $[Ca^{2+}]_i$ may be necessary but not sufficient for O_2^- production in neutrophils. Pozzan et al.(13) suggested that the activation of O_2^- production by neutrophils in response to f-Met-Leu-Phe requires an additional intracellular signal besides Ca^{2+}. One candidate is likely to be diacylglycerol (DG), a metabolite of phosphoinositide breakdown. DG is considered to be the physiological activator of the ubiquitous Ca^{2+}-phospholipid-dependent protein kinase C, perhaps acting synergistically with Ca^{2+} (14). In this context, it is of interest that cytochalasin B causes an elevation in f-Met-Leu-Phe-stimulated DG generation which correlates augmentation of O_2^- production (15-16). Moreover, cytochalasin B have been demonstrated to prolong the increase in $[Ca^{2+}]_i$ when stimulated with high concentrations of f-Met-Leu-Phe (11,12), which is very similar to that shown here in ATP-stimulated neutrophils. Taken together, it can be hypothesized that cytochalasin B exerts its effect on DG levels and O_2^- production by augmenting Ca^{2+} mobilization (16). The findings of the present work reinforce the hypothesis with respect to the action of cytochalasin B on ATP-stimulated neutrophils. Additional studies will be required to demonstrate that cytochalasin B does indeed enhance DG levels in ATP-stimulated neutrophils.

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