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CALCIUM MOBILIZATION IN FLUORIDE ACTIVATED HUMAN NEUTROPHILS

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SUMMARY: Fluoride ion, at concentrations above 10 mM, was found to elicit a rise in intracellular calcium levels in neutrophils, as monitored by changes in Quin 2 fluorescence intensity. The calcium mobilization response was characterized by a lag period of 4 to 10 min. and a prolonged duration of action (greater than 20 min.). In contrast, the chemotactic peptide, formylmethionyl-leucyl-phenylalanine, induced a rise in intracellular calcium concentrations which peaked within 1 min. Preincubation of the cells with 1 µg/ml pertussis toxin resulted in inhibition of the formylmethionyl-leucyl-phenylalanine induced response, but not that mediated by fluoride. Recent evidence suggests that the formylmethionyl-leucyl-phenylalanine receptor is coupled to phospholipase C and phosphoinositide degradation through a guanine nucleotide binding protein susceptible to inhibition by pertussis toxin. Present results suggest that fluoride ion may serve to activate this protein in a manner resistant to inhibition by pertussis toxin.

Although the ability of fluoride ion to activate a respiratory burst in human neutrophils has been reported previously (1, 2, 3), no satisfactory mechanism for the inflammatory action of this halide ion has yet been proposed. In the adenylate cyclase system, fluoride has a well-established role as an activator of both the N_s and N_i guanine nucleotide binding proteins (4) which serve as intermediary units between the cyclase and the receptors for stimulatory and inhibitory hormones, respectively. However, since elevated cAMP levels in the neutrophil are associated with suppression, rather than activation, of the respiratory burst (5, 6), cAMP is unlikely to be the mediator of fluoride action.

Recent reports have implicated the involvement of guanine nucleotide binding proteins in the transduction of those hormonal signals which exploit calcium mobilization as a means of cellular activation (7, 8, 9). Receptor dependent agonists such as the chemotactic peptide, formylmethionyl-leucyl-

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phenylalanine (Fmet-Leu-Phe), induce neutrophil chemotaxis and aggregation, as well as the release of superoxide (O_2^-) and granular enzymes. These responses have been correlated with the turnover of polyphosphoinositides and the mobilization of calcium (10). Notably, the preceding reactions are all inhibited by treatment of cells with pertussis toxin (11, 12, 13, 14), an AB toxin elaborated by Bordetella pertussis, which catalyzes the ADP-ribosylation and concomitant inactivation of the 41,000 MW subunit of N_i (15). N_i has been suggested (13, 14) to act as a coupling factor between agonist receptors and phospholipase C, the enzyme responsible for degradation of phosphoinositides into 1,2-diacylglycerol, the activator of protein kinase C (rev. in 16), and inositol 1,4,5-trisphosphate, a water soluble product believed to be responsible for calcium mobilization from the endoplasmic reticulum (rev. in 9).

In view of fluoride's efficacy as an activator of guanine nucleotide binding proteins, we have examined the possibility that fluoride induced activation of the respiratory burst may be occurring at the level of N_i. If this were the case, fluoride would be expected to mimic the action of agonists of "calcium-mobilizing receptors" by activating phospholipase C with the resultant degradation of polyphosphoinositides and elevation of cytosolic calcium concentrations.

The present data show that fluoride induced an increase in intracellular free calcium levels, as monitored by the fluorescent calcium probe, Quin 2. The calcium mobilization response paralleled that of fluoride activated superoxide production in having a prolonged lag period and sustained duration of action. This finding is consistent with the hypothesis that fluoride elicits a respiratory burst by means of persistent activation of N_i.

METHODS AND MATERIALS

The following materials were purchased from the sources indicated: Quin 2/AM from Calbiochem (San Diego, CA), pertussis toxin (islet activating protein) from List Biological Laboratories (Campbell, CA), Formyl-methionyl-leucyl-phenylalanine (Fmet-Leu-Phe) from Sigma Chemical (St. Louis, MO), and sodium fluoride from Fisher Scientific Co. (Pittsburgh, PA).

Human neutrophils were isolated as previously described (6) and suspended in Hanks' Balanced Salt Solution (HBSS), pH 7.4 and 37° C, at a density of

10×10^6 cells/ml. Intracellular calcium levels were monitored by the Quin 2 fluorescence technique established by Tsien et al. (17). Cell were separated into two groups, one of which was incubated with 1 µg/ml of pertussis toxin, while the other was left untreated. After 30 min., Quin 2/AM, at a final concentration of 10 µM, was added to each cell suspension and the incubation was continued for another 30 min. At the end of the total 1 hour incubation time, cells were centrifuged and washed once in HBSS, then resuspended at a concentration of 2×10^6 cells/ml for fluorescence measurements. The latter were conducted in a Perkin Elmer Model MKF-4 fluorescence spectrophotometer, thermostatically controlled at 37° C. The excitation and emission wavelengths for the fluorescence measurements were 339 nm and 492 nm with 5 nm and 15 nm slits, respectively. Maximal and minimal fluorescence signals were obtained by lysing the Quin 2 loaded cells with Triton X-100 (1%) in the presence of either 1.6 mM calcium (F_{max}) or 2 mM EGTA (F_{min}). The plateau for fluoride mediated calcium mobilization typically lay about half-way between the baseline fluorescence for unstimulated cells and the F_{max} value.

RESULTS AND DISCUSSION

Upon exposure to mM concentrations of NaF, human neutrophils respond with an increase in cytosolic free calcium levels $(Ca^{2+})_i$, as monitored through use of the fluorescent calcium probe, Quin 2 (Fig. 1). Fluoride induced calcium mobilization is characterized by a prolonged lag period of 4-10 min which may reflect the time interval required for the ion to reach its site of action in the membrane. The extended duration of the fluoride evoked calcium response, as compared with that elicited by Fmet-Leu-Phe, is consistent with the fact that fluoride induces the dissociation of N_i into its 35K and 41K subunits in a manner which is not readily reversible (18). Reduction of the lag interval and acceleration of the rate of calcium mobilization were observed to be dose-dependent up to an optimal concentration of 18 mM. The time course of the $(Ca^{2+})_i$ increase paralleled that of the concomitant superoxide release

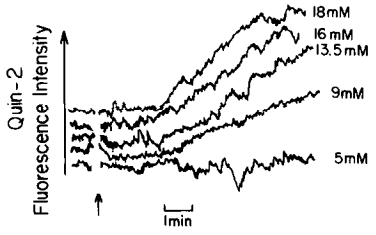

Figure 1: Relationship of Ca^{2+} mobilization to NaF concentration in a 2×10^6 cell/ml suspension of neutrophils. The arrow indicates the time of addition of stimulus. These results depict the tracings obtained from a single experiment which is representative of at least five experiments.

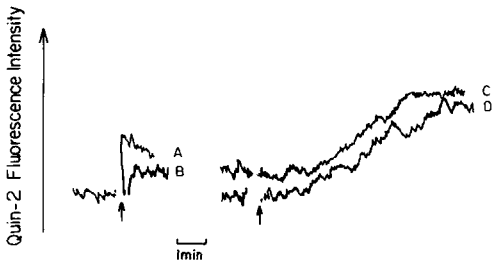

Figure 2: Ca^{2+} mobilization by 1 μM Fmet-Leu-Phe (A and B) and 18 mM NaF (C and D) in the presence (B and D) and absence (A and C) of pretreatment with 1 μg/ml pertussis toxin. The arrows indicate the time of addition of stimulus. These results depict the tracings obtained from a single experiment which is representative of at least three experiments.

reaction (3). At concentrations of 20 mM and above, the Ca^{2+} mobilization response appeared to decline (data not shown), a phenomenon which has also been observed for superoxide production (2).

As reported previously (12), pertussis toxin inhibits the Fmet-Leu-Phe evoked rise in $(Ca^{2+})_i$ (Fig. 2), as well as the associated superoxide production (10), reinforcing the theory of guanine nucleotide binding protein involvement in the transduction pathway. Fmet-Leu-Phe induced calcium mobilization differed from that elicited by fluoride in being of more rapid onset (maximal response within 15 sec) (19), smaller magnitude (by approximately 50%), and rapid termination (1-2 min). The time course of the Fmet-Leu-Phe stimulated rise in $(Ca^{2+})_i$ correlates with that of the associated respiratory burst (3). The comparative brevity of the chemotactic peptide induced responses may be due to down-regulation or desensitization of the Fmet-Leu-Phe receptor and/or inactivation of N_i as a result of GTP hydrolysis (4).

Results illustrated in Fig. 2 show that fluoride activated calcium mobilization was resistant to inhibition by pertussis toxin, suggesting that its mechanism of N_i activation is unaffected by ADP-ribosylation of the protein. Such a finding is consistent with the observation that inhibition of prostaglandin E_1 stimulated adenylate cyclase in the presence of fluoride is unaffected by pertussis toxin (18, 20). It should be noted that Bokoch and Gilman (21) did observe pertussis toxin inhibition of fluoride stimulated

arachidonic acid release. The discrepancy in pertussis toxin effects may be accounted for by the coexistence of distinct subsets of N_i-like guanine nucleotide binding proteins specific to the phospholipase C and phospholipase A_2 dependent pathways. Upon ADP-ribosylation by pertussis toxin these proteins might exhibit a differential sensitivity to fluoride activation. Indeed, the dose-response profile for the calcium-superoxide response differed from that of arachidonic acid release, with optimal concentrations being 18 mM in the case of the former, and 50 mM in the case of the latter. Since inhibition of arachidonate release by pertussis toxin was only marked at fluoride concentrations of 20 mM and above, concentrations at which calcium mobilization and superoxide production were already subject to autoinhibition, it is difficult to correlate these two phenomena.

Although fluoride serves as an activator of the N_s as well as the N_i unit, and is capable of elevating cAMP levels in the neutrophil (3), the alteration in cyclic nucleotide levels cannot be considered responsible for neutrophil activation, as experimental elevation of cAMP levels with dibutyryl cAMP or adenylate cyclase agonists, such as prostaglandins of the E series, does not result in cell activation per se. Indeed, elevated cAMP levels are known to inhibit, rather than potentiate, Fmet-Leu-Phe induced neutrophil responses (5, 6). Our previous finding that fluoride activation of neutrophil responses is unaffected by cAMP or cyclase agonists (3) indicates that the inhibitory effect of cAMP may be occurring at some step in the transduction process prior to N_i, possibly by means of receptor phosphorylation.

Furthermore, it appears that at concentrations above 10 mM, fluoride preferentially stimulates the N_i, rather than the N_s, unit. This is suggested by the results of Katada and co-workers (20) who observed a biphasic effect of fluoride on the adenylate cyclase activity of platelet membranes with increasing concentrations of fluoride first increasing, then decreasing, adenylate cyclase activity. High concentrations of fluoride were also found to inhibit prostaglandin E_1 stimulation of adenylate cyclase. Together these

observations implicate N_i as the molecular site of fluoride interaction during neutrophil stimulation.

In conclusion, we propose that fluoride's ability to elevate cytosolic calcium concentrations and activate a respiratory burst are consequences of its prolonged stimulation of the N_i transducing unit. Activation of N_i would lead to persistent stimulation of phospholipase C, with the resultant breakdown of the principal substrate, phosphatidylinositol 4,5-bisphosphate into the protein kinase C activator, 1,2-diacylglycerol, and the calcium mobilizer, inositol 1,4,5 trisphosphate. In an effort to further substantiate this proposal, we are presently conducting studies of phosphoinositide turnover in the presence of fluoride in the neutrophil.

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