

Relationship of Phosphatidylinositol Bisphosphate Hydrolysis to Calcium Mobilization and Functional Activation in Fluoride-treated Neutrophils

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Abstract

Sodium fluoride (20 mM) effected rapid hydrolysis of phosphatidylinositol bisphosphate (PIP₂) in human neutrophils. Intracellular free Ca²⁺ levels increased after PIP₂ hydrolysis but before respiratory burst activation. Both the increase in intracellular free Ca²⁺ levels and the extent of functional activation were dependent on the availability of extracellular Ca²⁺. The rate of F⁻-stimulated PIP₂ hydrolysis, however, was not affected when the rise in cytosolic Ca²⁺ was severely limited by depletion of extracellular Ca²⁺. Fluoride caused the specific hydrolysis of PIP₂ in isolated neutrophil plasma membranes. This effect occurred in the presence of low levels of available Ca²⁺ and was accompanied by the release of inositol phosphates. We conclude that PIP₂ hydrolysis is an early event in the response of neutrophils to F⁻. This response is not Ca²⁺-regulated but may lead to an influx of Ca²⁺ from the extracellular medium. Activation of a PIP₂-specific phospholipase independent of a change in cytosolic free Ca²⁺ levels may be the initial event in the stimulus-response pathway triggered by fluoride.

Introduction

The involvement of the phosphatidylinositol (PI)¹ cycle in neutrophil signal transduction and its role in Ca²⁺ mobilization in these cells has been a matter of considerable controversy. While several studies have demonstrated that certain agonists, including *n*-formyl-methionyl-leucyl-phenylalanine (FMLP), opsonized zymosan, ionophore A 23187, and hexachlorocyclohexane, do effect hydrolysis of phosphoinositides in neutrophils and other myeloid cells (1–15), it is not clear whether these changes result from, are independent of, or lead to mobilization of intracellular Ca²⁺. Conditions under which hydrolysis of neutrophil phosphoinositides is not accompanied by Ca²⁺ mobili-

zation (16) and increases in intracellular free Ca²⁺ levels occur in the absence of PI cycle activation have been described (17). Several investigators have concluded that phosphoinositide hydrolysis and other phospholipid changes in stimulated neutrophils are dependent on prior Ca²⁺ mobilization (1, 5, 12–14). Others have provided evidence in support of the view that phosphoinositide hydrolysis is an early event in the activation sequence that does not require prior Ca²⁺ mobilization and may in fact lead to release of Ca²⁺ from intracellular stores (2–4, 7–9, 11). In support of this possibility, Smith et al. recently demonstrated that FMLP, in the presence of guanine nucleotides and low levels of ionized calcium (10 nM) activates a phospholipase C that catalyzes the hydrolysis of phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂) in neutrophil plasma membrane preparations (18). While such evidence suggests that phosphoinositide hydrolysis is not secondary to Ca²⁺ mobilization, it has also been noted that membrane phospholipase C activity is also efficiently induced by Ca²⁺ (19). Furthermore, it is clear that phosphoinositide hydrolysis can be directly stimulated by elevating the intracellular free Ca²⁺ concentration in phagocytic cells (14). Thus, the relationship of phospholipid changes to neutrophil Ca²⁺ mobilization remains unsettled.

Most studies of the involvement of the PI cycle in neutrophil signal transduction performed to date have employed FMLP as the cell stimulus. However, there are certain problems and limitations inherent in experiments in which this stimulus is exclusively used. First, neutrophil responses to FMLP occur almost immediately after stimulation (20–23). Thus, the temporal relationship of individual events, specifically Ca²⁺ mobilization, phospholipid changes, and functional activation, are extremely difficult to compare with accuracy. Second, the results of Ca²⁺-depletion experiments are difficult to interpret because neutrophil responses to FMLP are only partially dependent on the presence of extracellular Ca²⁺. Extracellular Ca²⁺ depletion only partially inhibits functional responses and intracellular free Ca²⁺ elevation provoked by FMLP (17, 21, 24–25). To prevent totally the rise of intracellular free Ca²⁺ effected by FMLP, potent pharmacological agents that potentially have many nonspecific effects on cell function or exhaustive methods of cellular Ca²⁺ depletion have been employed. The significance of results obtained using such methods is not entirely clear. Finally, neutrophil responses to FMLP are inhibited by islet-activating protein (pertussis toxin), an agent which specifically interferes with the function of certain guanine nucleotide regulatory proteins (24, 26–29). Neutrophil responses to other metabolic stimuli are not similarly inhibited by pertussis toxin (24, 29–30). Thus, results with FMLP may reflect activation of biochemical pathways and regulatory mechanisms specific to that stimulus. While these pathways are certainly important, they may not be involved in transduction of messages from other metabolic stimuli.

In the present study, we examined the relationship of PI cycle activation to Ca²⁺ mobilization and functional activation

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1. *Abbreviations used in this paper:* FMLP, *n*-formyl-methionyl-leucyl-phenylalanine; mBSS, modified Hanks' balanced salt solution; PA, phosphatidic acid; PC, phosphatidylcholine; Pi, inorganic phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; TLC, thin layer chromatography.

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in neutrophils stimulated with 20 mM sodium fluoride. Sodium fluoride was chosen for several reasons. First, the response of superoxide generation induced by fluoride develops very slowly, commencing after an activation period of 8–10 min at 37°C (31, 32). Cellular processes induced during this lag period have not been defined. Second, neutrophil functional responses to 20 mM NaF are almost totally dependent on the presence of extracellular Ca^{2+} (31, 32). Finally, NaF induces a slow rise in neutrophil intracellular free Ca^{2+} (30). Although this rise in cellular Ca^{2+} begins after a distinct lag period, it seems to occur sooner than the commencement of O_2^- release and may play a pivotal role in the activation process.

In this investigation, we first determined if the PI cycle was activated when neutrophils were exposed to fluoride by comparing individual phospholipid and inositol phosphate levels in stimulated and resting cells. Then, we defined the kinetics of NaF-induced changes of individual PI cycle metabolites to determine if the changes occurred before, during, or after Ca^{2+} mobilization and functional activation. Finally, the relationship of PI cycle activation to Ca^{2+} mobilization was investigated by altering Ca^{2+} availability. The results clearly demonstrate that while hydrolysis of phosphatidylinositol bisphosphate can occur in the absence of a detectable increase in intracellular free Ca^{2+} , secondary phospholipid changes that may play an important role in functional activation depend on Ca^{2+} mobilization in F^- -treated neutrophils.

Methods

Carrier free $^{32}\text{P}_i$ (orthophosphoric acid in water) and [^3H]myoinositol were obtained from New England Nuclear, Boston, MA. Phospholipid standards, including phosphatidic acid (PA), PIP_2 , PIP, PI, phosphatidylcholine (PC), and phosphatidylserine (PS) were obtained from Sigma Chemical Co., St. Louis, MO. ^{32}P -labeled inositol trisphosphate, containing low but detectable amounts of $^{32}\text{P}_i$ and ^{32}P -inositol bisphosphate was generated by Ca^{2+} treatment of ^{32}P -labeled erythrocyte membrane ghosts according to the method of Downes and Michell (33). Preparations of labeled inositol phosphates were characterized using anion exchange chromatography with Dowex 1-X8 and by high performance liquid chromatography on a Partisil SAX-1 column as described below.

Measurement of ^{32}P -labeled phospholipids. Neutrophils were prepared from human blood after Ficoll-Hypaque density gradient centrifugation (21). Cells were washed and resuspended in modified Hanks' balanced salt solution (mBSS) containing 10 mM Hepes instead of phosphate buffer at a concentration of 1×10^8 cells/ml. Cells were then incubated with $^{32}\text{P}_i$ (500 $\mu\text{Ci}/\text{ml}$) for 90 min at 37°C, washed three times with mBSS, and resuspended in the same buffer at a concentration of 2×10^7 cells/ml. The phosphate-free buffer used during cell preparation and incubation of cells with $^{32}\text{P}_i$ contained 0.5 mM CaCl_2 . CaCl_2 (0.5 mM) was included or excluded from the media used to wash and resuspend labeled cells as indicated in the results. In some experiments, cells were washed three times in mBSS containing 2.5 mM EDTA after labeling with $^{32}\text{P}_i$. Cells washed with mBSS containing EDTA (Ca^{2+} -depleted cells) were resuspended in Ca^{2+} -free mBSS which also contained 2.5 mM EDTA.

Phospholipids were extracted from ^{32}P -labeled cells after stimulation with fluoride. In these experiments, 0.5 ml of the cell suspension (10^7 cells) was treated with 20 mM NaF (or NaCl) in a final volume of 1.0 ml at 37°C. At indicated times thereafter, 5.0 ml of chloroform/methanol/concentrated HCl (20:40:1) were added to stop the reactions and extract lipids. After 30 min, phases were resolved by addition of 1 ml of H_2O and 1 ml of chloroform, the organic phase recovered, washed twice with water and dried under nitrogen. Dried extracts were solubilized in 25 μl of chloroform/methanol (90:10) and spotted on precoated 20×20 -cm thin layer chromatography (TLC) plates of silica gel 60 on a polyester base (Sigma Chemical Co.). Phospholipid standards were also dissolved

in chloroform/methanol (90:10) and spotted on TLC plates. Plates were developed in one dimension with chloroform/methanol/20% methylamine (60:36:10), dried, and exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) overnight to localize radioactivity, as previously described (1, 10). Phospholipid standards were localized by spraying developed plates with molybdenum blue (0.65% molybdenum oxide in 4.2 M sulfuric acid). Phospholipids, including PIP_2 and PIP, were well separated by this technique. Preliminary experiments revealed that pretreatment of TLC plates with 1% potassium oxalate did not enhance separation of PIP_2 and PIP in this system. R_f values were ~ 0.63 for PC, 0.45 for PA, 0.36 for PI, 0.23 for PIP, and 0.13 for PIP_2 . Spots corresponding to the position of each radioactive lipid localized by autoradiography were cut from the polyester plates, dropped into vials containing 20 ml of Scintilene (Fisher Scientific Co., Pittsburgh, PA), and assayed for ^{32}P activity by scintillation counting.

Neutrophil inositol phosphate levels. Inositol phosphates, including inositol monophosphate, inositol bisphosphate, and inositol trisphosphate were measured in cells prelabeled with [^3H]inositol using methods similar to those described by diVirgilio et al. (9). Cells were resuspended at a concentration of $3.0 \times 10^7/\text{ml}$ in mBSS containing 10 mM Hepes and 3% fetal bovine serum. [^3H]inositol was added at a concentration of 50 $\mu\text{Ci}/\text{ml}$ and the cells were incubated in an atmosphere of 5% CO_2 in air for 4 h. In some experiments, preincubation with [^3H]inositol was extended to 16 h. Cells were then washed three times, resuspended in mBSS (3.0×10^7 cells/ml), and stimulated with 20 mM fluoride. Resting inositol phosphate values were determined using cells exposed to NaCl under similar conditions. The volume of cells used for each determination was 300 μl . NaF or NaCl was added from 150 mM stock solutions. LiCl (10 mM) had no influence on inositol phosphate recovery and was not used in the experiments reported. After stimulation, incubations were terminated by addition of an equal volume (347 μl) of cold 15% TCA. The acid-treated suspensions were extracted four times with 4 ml of diethyl ether, centrifuged, and neutralized with sodium tetraborate. Samples were diluted to 4 ml and applied to a 1-ml bed volume column of Dowex-1 (AG 1-X8, 200-400 mesh, formate form) as previously described (33). After washing the column with 0.1 M formic acid, inositol monophosphate, inositol bisphosphate, and inositol trisphosphate were eluted with 15 ml of 0.2, 0.5, and 1.0 M ammonium formate (each in 0.1 M formic acid), respectively. The resolution of the columns was confirmed with [^{32}P]inositol trisphosphate prepared from erythrocyte membranes. Fractions (2 ml) were collected and assayed for radioactivity after dilution in 4 ml of Ready Solv MP scintillation fluid (Beckman Instruments Inc., Fullerton, CA).

Intracellular Ca^{2+} determinations with Fura-2. Neutrophils were loaded with the intracellular Ca^{2+} probe Fura-2 by incubation with Fura-2 acetoxymethyl ester (Fura-2 AM) as described by Kruskal et al. (34). Fura-2 AM, obtained from Molecular Probes Inc., Junction City, OR, was dissolved in dimethylsulfoxide at a concentration of 1.0 mM. Cells were incubated in mBSS (containing 1.0 mM CaCl_2) with 5 μM Fura-2 AM for 30 min at 37°C, washed twice, and resuspended in mBSS at a concentration of 10^7 cells/ml. Fluorescence measurements were made in a MPF-66 spectrofluorimeter (Perkin-Elmer Corp., Norwalk, CT) set at 37°C. Ca^{2+} was added or cells were depleted of Ca^{2+} before fluorescence measurements as indicated. In most experiments, fluorescence changes were monitored continuously after stimulation of 2×10^6 cells in 1 ml using an excitation wavelength of 340 nm, an emission wavelength of 510 nm and a slit width of 10 nm. In addition, the fluorescence ratios at 340 and 380 nm excitation in resting and stimulated cells are expressed, because these ratios reflect relative intracellular free Ca^{2+} levels (35). In control experiments, we determined that fluoride ions had no direct effect on Fura-2 fluorescence at either 340 or 380 nm.

Neutrophil superoxide generation. Superoxide release was measured by quantitating superoxide dismutase inhibitable reduction of cytochrome *c* as previously described (21). Cells were treated in a manner identical to that used for phospholipid determinations except for the addition of cytochrome *c*. In control experiments, cytochrome *c* was found to have no influence on phospholipid changes induced by exposure of neutrophils to NaF.

Subcellular fractionation. Release of radioactivity and hydrolysis of phosphoinositides in cell-free membrane preparations from ^{32}P -labeled neutrophils was determined using methods similar to those described by Cockcroft et al. (19). Cells were preincubated with ^{32}P as described above, washed, and resuspended in buffer containing 0.13 M NaCl, 0.34 M sucrose, and 1.0 mM EGTA. Cells were then sonicated with three 15-s bursts at 30% power in a Fisher 300 sonic dismembrator. Sonicates were centrifuged at 800 g for 10 min to remove intact cells and debris. The supernatants were layered over a cushion of 40% sucrose (wt/vol) and centrifuged in a swinging bucket rotor at 150,000 g for 30 min. The top layer (sample application zone) was removed and the entire 40% sucrose layer containing plasma membranes was saved. Whereas activity of the plasma membrane marker alkaline phosphatase was increased over five-fold (determined as units per milligram protein) in these preparations, activity of the granule enzyme markers lysozyme and myeloperoxidase, while detectable, was diminished to < 5% of that in whole-cell lysates. The tightly packed pellet that contained most of the azurophilic and specific granule markers was discarded. Membranes were diluted with 5 vol of 20 mM Hepes in 1 mM EGTA (pH 7.0), recentrifuged for 30 min at 150,000 g, and resuspended in 20 mM Hepes–0.1 mM EGTA (pH 7.3) at a concentration of ~ 5 mg protein/ml. Labeled membranes (100 μl) were incubated with 20 mM NaF or NaCl in a final volume of 1.0 ml of 20 mM Hepes–0.1 mM EGTA. In some experiments, membranes were exposed to 0.5 mM CaCl_2 instead of 20 mM NaF. After 10 min, incubations were terminated by the addition of 250 μl cold 15% TCA, and the precipitate was pelleted by centrifugation (80,000 g, 10 min). Pellets were extracted with chloroform/methanol/HCl and chromatographed as described above to separate cellular phospholipids. Supernatants of the TCA extracts were assayed for radioactivity by determination of Cherenkov radiation. After counting, supernatants were extracted with ether to remove TCA, neutralized, diluted with 3 vol of water, and analyzed by high performance liquid chromatography. Samples were applied to a Partisil 1-SAX anion exchange column and eluted with a gradient of ammonium formate as described by Hawkins et al. (36). Fractions of 1.0 ml were collected and assayed for radioactivity in a liquid scintillation counter. The elution positions of inositol phosphates and inorganic phosphate were confirmed using standards from ^{32}P -labeled erythrocytes. Inositol-1-monophosphate is not detected by this method since the 1-phosphate is not labeled (see references 33 and 37). In each separation, the elution position of the first peak of radioactive material corresponded to that of $^{32}\text{P}_i$ from New England Nuclear. The identity of fractions thought to contain inorganic phosphate was further confirmed using the ammonium molybdate organic extraction method described by Downes et al. (37). Inositol bisphosphate and inositol trisphosphate were well resolved from each other and from $^{32}\text{P}_i$ by the method described.

In some experiments, F^- -stimulated release of inositol phosphates from neutrophil membranes was quantitated using [^3H]inositol instead of $^{32}\text{P}_i$ to prelabel neutrophils. Intact cells (1 ml, 3×10^7 cells/ml) were incubated with 50 μCi of [^3H]inositol for 4 h at 37°C , washed, resuspended in sucrose/saline/EGTA buffer and then sonicated, as described above. Membranes prepared from these cells were incubated in the presence and absence of 20.0 mM NaF for 10 min at 37°C and pelleted by centrifugation. Supernatants (1.0 ml) were diluted to 5.0 ml, applied to the gravity flow columns of Dowex-1, and eluted as described in experiments with extracts of intact cells. Radioactivity was determined after dilution of each fraction in 2 vol of Beckman Ready Solv MP scintillation fluid.

Results

Oxidative activation and Ca^{2+} mobilization. NaF was found to stimulate a substantial burst of O_2^- release by neutrophils in the presence of extracellular Ca^{2+} . As shown on Fig. 1, the oxidative response started ~ 8 min after addition of NaF and continued for over 30 min. The response was markedly suppressed when the availability of extracellular Ca^{2+} was limited. Total release of O_2^- during a 30-min incubation was inhibited by 76% when

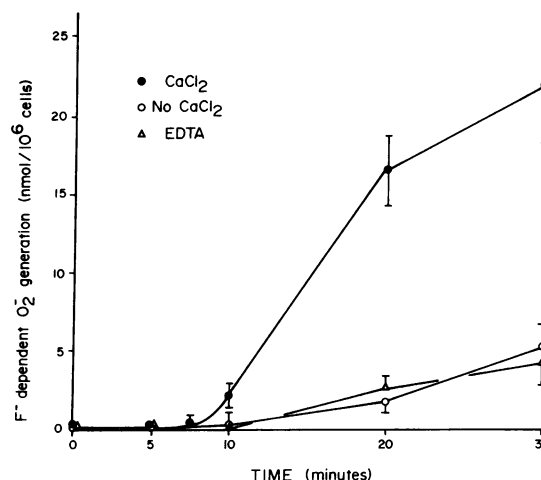


Figure 1. Kinetics of F^- -stimulated neutrophil superoxide generation. Cells were prepared in media containing 0.5 mM CaCl_2 , no added CaCl_2 , or 2.5 mM EDTA (and no added CaCl_2) and stimulated with 20 mM NaF. Data indicate mean $\pm$ SEM of five separate experiments. The coefficient of variation of replicate samples within each experiment was < 10%. The values plotted reflect the amount of superoxide released above that released by resting cells incubated at 37°C . The maximal release of O_2^- by unstimulated cells observed in these experiments was 2.5 nmol/ 10^6 cells/30 min.

extracellular Ca^{2+} was removed. Ca^{2+} depletion with EDTA inhibited oxidative responses by > 80%.

Changes in intracellular free Ca^{2+} levels were monitored spectrofluorometrically after exposure of Fura-2-loaded neutrophils to 20 mM NaF. When cells were exposed to NaF in the presence of extracellular Ca^{2+} (0.5 mM), intracellular Ca^{2+} levels began to rise ~ 2 min after stimulation, as indicated by an increase in relative fluorescence intensity at 340 nm excitation (Fig. 2). Ca^{2+} levels in F^- -treated neutrophils continued to increase slowly over the next 10 min. When Ca^{2+} was not included in the suspending medium, the Fura-2 response was markedly attenuated (Fig. 2). Further attenuation of the Fura-2 response was obtained when cell Ca^{2+} levels were depleted with EDTA.

The determination of cytoplasmic free Ca^{2+} concentrations from Fura-2 fluorescence responses is problematic (35, 38). Much of this difficulty is due to the effects of the cytoplasm on fluorescence properties of the dye. Thus, Ca^{2+} levels calculated from ratios of fluorescence at 340 and 380 nm may be much lower than levels actually present (39). However, if the extent of cytoplasmic interference of the fluorescence signals does not markedly change when the cells are stimulated, then the actual fluorescence ratios at 340 and 380 nm excitation should relate linearly to intracellular free Ca^{2+} levels (38). Thus, these ratios are a reliable indicator of the intracellular free Ca^{2+} concentration. In three experiments, the ratios of fluorescence at 340 and 380 nm excitation in resting and F^- -stimulated neutrophils were, respectively, as follows: 1.75 ± 0.13 (resting), 3.02 ± 0.13 (F^- -stimulated) for cells in media with Ca^{2+} ; 1.41 ± 0.12 , 1.93 ± 0.07 for cells in Ca^{2+} -free media; 1.43 ± 0.09 , 1.71 ± 0.05 for cells in media with EDTA. Whereas determination of actual cytoplasmic free Ca^{2+} levels from these ratios awaits definition of the effect of the intracellular environment on Fura-2 fluorescence, the results strongly indicate that levels of intracellular free Ca^{2+} of cells prepared in EDTA and exposed to F^- were lower than

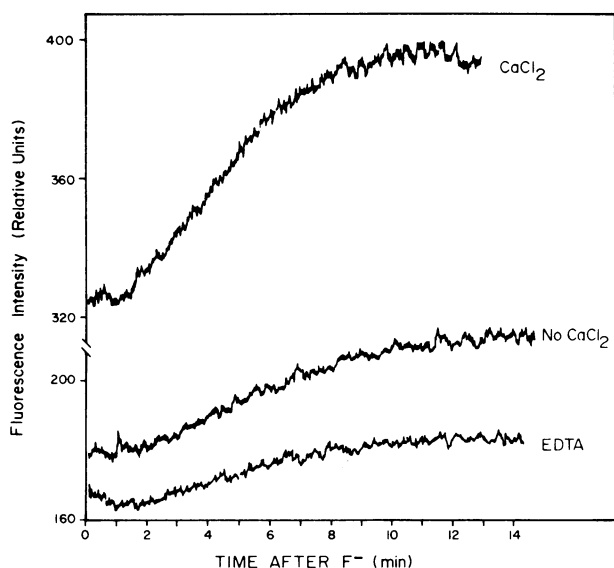


Figure 2. Cytosolic free Ca^{2+} in F^- -treated neutrophils. Tracings represent actual fluorometric recordings (excitation 340 nm), of Fura-2-loaded neutrophils exposed to 20 mM NaF in the presence and absence of extracellular Ca^{2+} at 37°C . In the lower tracing, cells were depleted of Ca^{2+} by washing in the presence of 2.5 mM EDTA. Substitution of EGTA for EDTA led to similar results (not shown). The illustrated tracings are one of three taken under each condition in this experiment. Each of the three were essentially identical. Results similar to those illustrated have been obtained in two additional experiments. In the absence of stimulus, fluorescence levels did not increase during the 10-min incubation period.

levels of intracellular free Ca^{2+} in unstimulated neutrophils in media with Ca^{2+} .

Phospholipid changes in F^- -treated neutrophils. The above experiments demonstrated that rises in cytosolic Ca^{2+} precede oxidative activation in F^- -stimulated neutrophils and depend heavily on the availability of extracellular Ca^{2+} . If F^- effects phospholipid changes in neutrophils typical of those resulting from phospholipase C activation, the relationship of these changes to Ca^{2+} mobilization may therefore be assessed by comparing the kinetics of phospholipid changes in the presence and absence of extracellular Ca^{2+} . Table I demonstrates that F^- does effect substantial alterations in the phospholipid composition of ^{32}P -labeled neutrophils. These changes include significantly decreased levels of ^{32}P -labeled PIP_2 and increased levels of labeled PA and PI. PIP levels decreased moderately in neutrophils incubated with F^- for 5 min at 37°C , but a similar decrease in PIP levels occurred in unstimulated cells. Very little change in PA and PI occurred in neutrophils incubated at 37°C in the absence of F^- . No significant changes in other phospholipids, including phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, were observed in fluoride-treated neutrophils (not shown).

Fluoride-induced changes in neutrophil phospholipids were accompanied by inositol phosphate generation. When neutrophils preincubated with ^3H]inositol for 4 h were exposed to 20.0 mM sodium fluoride, markedly increased levels of inositol monophosphate and inositol bisphosphate were recovered in cell extracts in comparison with values recovered in extracts of unstimulated cells (Table II). Levels of inositol trisphosphate were only slightly increased in F^- -treated neutrophils, possibly

Table I. Changes in Phospholipid Levels in Neutrophils Exposed to F^- for 5 min

Phospholipid	% Change during incubation	
	NaF	NaCl
PIP_2^*	-49.3 ± 6.9	-6.7 ± 1.4
PIP^*	-4.6 ± 11.6	-3.8 ± 8.5
PI^*	$+43.3 \pm 21.3$	$+2.5 \pm 1.9$
PA^*	$+62.9 \pm 11.5$	$+1.1 \pm 1.1$

^{32}P -labeled cells were incubated at 37°C for 5 min with 20 mM NaF or, for control values, NaCl. Incubations were carried out in the presence of 0.5 mM extracellular calcium. Lipids were then extracted separated by thin layer chromatography and localized by autoradiography. Values show the percent change ($\pm\text{SD}$) in each lipid from preincubation values ($n = 3$). * Differences between F^- and Cl^- values significant at $P < 0.03$ (Student's t test). † Difference between F^- and Cl^- values not statistically significant by ($P > 0.05$).

as a result of degradation by cellular phosphatases (37). In an attempt to increase levels of inositol phosphates (specifically inositol trisphosphate) in F^- -treated neutrophils, preincubation of cells with ^3H]inositol was extended 16 h as suggested by DiVirgilio et al. (9). Cells incubated overnight at 37°C retained $\sim 60\%$ of their functional responsiveness to fluoride as assessed by measurement of O_2^- release (not shown). However, overnight preincubation did not markedly alter the pattern of inositol phosphate release effected by sodium fluoride (Table II).

Phospholipid changes and Ca^{2+} mobilization. Fig. 3 A shows the kinetics of PIP_2 hydrolysis in neutrophils exposed to 20 mM

Table II. Inositol Phosphate Generation by Fluoride-treated Neutrophils

Inositol phosphate	Percent increase*	
	4-h preincubation†	16-h preincubation‡
Inositol monophosphate	50.1 ± 7.6	43.6 ± 3.2
Inositol bisphosphate	66.1 ± 8.6	58.6 ± 4.04
Inositol trisphosphate	10.0 ± 3.2	4.2 ± 5.9

Neutrophils preincubated with ^3H]inositol were exposed to 20 mM NaF in the presence of 0.5 mM CaCl_2 for 5 min at 37°C . Control values were obtained by using NaCl in place of NaF. After incubation, cellular inositol phosphates were liberated with TCA and lipid-soluble material extracted with diethyl ether. Inositol phosphates were separated by anion exchange chromatography of the aqueous extracts.

* Values reflect the percent increase in radioactivity in fractions isolated from F^- -treated neutrophils in comparison to unstimulated cells (mean $\pm\text{SD}$, $n = 3$). † The actual counts recovered in each inositol phosphate fraction from unstimulated cells in these three experiments were as follows: inositol monophosphate, 597 ± 56 ; inositol bisphosphate, 256 ± 19 ; inositol trisphosphate 137 ± 16 . Differences in inositol monophosphate and inositol bisphosphate levels in resting and F^- -stimulated cells were statistically significant ($P > 0.01$, Student's t test). Differences in inositol trisphosphate levels were not significant ($P > 0.1$). ‡ The actual counts recovered in fractions from unstimulated cells in these experiments were: inositol monophosphate, 971 ± 35 ; inositol bisphosphate, 349 ± 22 ; inositol trisphosphate, 125 ± 26 . Differences in inositol monophosphate and inositol bisphosphate levels in resting and F^- -stimulated cells were statistically significant ($P < 0.01$) but differences in inositol trisphosphate levels were not ($P > 0.1$).

NaF. PIP₂ levels began decreasing immediately after stimulation. Consumption of PIP₂ was virtually complete within 5 min of stimulation. PIP₂ hydrolysis was not hindered under conditions which limited Ca²⁺ mobilization; as shown in Fig. 3 A, neither the rate nor the extent of PIP₂ hydrolysis was affected when cellular Ca²⁺ was depleted by prior washing of cells in EDTA. Similar results were observed from cells extensively washed with Ca²⁺-free media in the absence of chelator and for cells washed in Ca²⁺-free media containing EGTA instead of EDTA (not shown).

Whereas Ca²⁺ depletion had no influence on F⁻-stimulated PIP₂ hydrolysis, other phospholipid changes effected by F⁻ were at least partially dependent on elevated cytosolic Ca²⁺ (Fig. 3, B-D). Fig. 3 B illustrates the kinetics of appearance of ³²P-labeled phosphatidic acid in F⁻-treated neutrophils. PA levels began to increase soon after stimulation and subsided after 5 min. Removal of Ca²⁺ delayed the initial rise in PA by ~ 2.5 min. A second phase of PA synthesis began ~ 10 min after stimulation in the presence of Ca²⁺. Late appearance of PA was strongly dependent on the availability of extracellular Ca²⁺.

Increases in PI levels of neutrophils exposed to NaF were also partially dependent on extracellular Ca²⁺. After a rapid initial increase, PI levels slowly increased for up to 30 min when extracellular Ca²⁺ was present (Fig. 3 C). In the absence of extracellular Ca²⁺, no further increase in PI levels occurred after 10

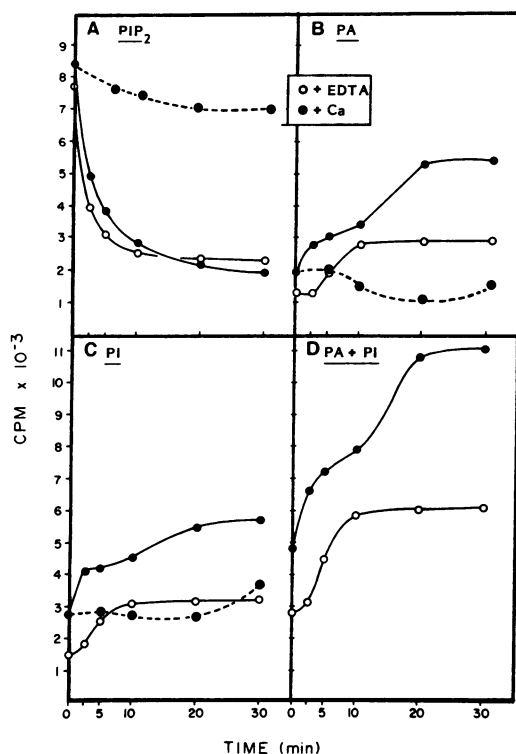


Figure 3. Kinetics of phospholipid changes in F⁻-treated neutrophils. Neutrophils were exposed to 20 mM NaF either in the presence of 0.5 mM extracellular Ca²⁺ or after depletion of cellular Ca²⁺ levels with EDTA before extraction of lipids. Radioactivity (cpm) associated with PIP₂ (A), PA (B), and PI (C) are shown. Dotted lines show radioactivity levels in lipids recovered from unstimulated neutrophils incubated at 37°C. (D) cpm recovered with PA and PI in F⁻-treated neutrophils were summed. The data shown are from a single representative experiment. Two additional experiments have yielded similar results.

min. When plotted together, late increases in PA and PI levels in F⁻-stimulated cells displayed a strong dependence on the presence of extracellular Ca²⁺ (Fig. 3 D).

Ca²⁺ and F⁻-mediated hydrolysis of phosphoinositides in neutrophil membranes. To examine the influence of F⁻ on membrane phospholipids in a cell-free system, we isolated a light-membrane fraction from ³²P-labeled neutrophils, washed it by ultracentrifugation in a Ca²⁺-free medium and resuspended it in buffer containing 0.1 mM EGTA and no added calcium. Ca²⁺ (0.5 mM) effected the breakdown of over 40% of both PIP₂ and PIP in these membranes (Fig. 4) but had minimal effects on other labeled phospholipids. Associated with this effect was a considerable release of radioactivity from the membranes. Radioactivity released from membranes exposed to 0.5 mM Ca²⁺ for 10 min was 56.5±8.4% higher (mean±SD, n = 3) than radioactivity released from untreated membranes. Radioactivity released into the supernatants was analyzed by anion exchange chromatography on a Partisil SAX column. The released activity was recovered in distinct fractions corresponding to inositol trisphosphate, inositol bisphosphate, and inorganic phosphate (Fig. 4). Similar results have been reported by Cockcroft et al., who used Dowex-1 anion exchange columns to analyze water soluble radioactivity released when neutrophil membranes were exposed to Ca²⁺ (19).

Sodium fluoride (20 mM) similarly provoked a substantial release of radioactivity from neutrophil membranes. Radioactivity in supernatants of fluoride-treated membranes was 43±11% higher (mean±SD, n = 5) than radioactivity in supernatants of untreated membranes. Increasing the concentration of EGTA from 0.1 to 1.0 mM did not alter F⁻-stimulated release of membrane radioactivity (not shown). The distribution of radioactivity released when neutrophil membranes were exposed to F⁻ is illustrated in Fig. 4. Like supernatants of Ca²⁺-treated membranes, most of the ³²P activity released was recovered as inorganic phosphate. The remainder was recovered in fractions containing inositol bisphosphate and, to a lesser extent, inositol trisphosphate. Associated with liberation of membrane radioactivity, 20 mM F⁻ effected the specific hydrolysis of membrane PIP₂. Unlike Ca²⁺, F⁻ had little influence on levels of ³²P-labeled PIP in membrane preparations. Levels of other phospholipids, including PI and PA were only slightly altered after incubation of neutrophil membranes with F⁻ (Fig. 4).

The ability of F⁻ to effect release of radioactivity from [³H]inositol-labeled neutrophil plasma membranes was also determined. In two experiments, 20.0 mM NaF increased the release of radioactivity from [³H]inositol-labeled membranes by > 50%. The distribution of radioactivity in these supernatants was analyzed by Dowex-1 anion exchange chromatography. Supernatants of resting membranes contained detectable amounts of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate. Levels of each of these three inositol phosphates were consistently and significantly increased by exposure to F⁻. F⁻ approximately doubled release of [³H]inositol monophosphate and [³H]inositol bisphosphate in this system. Inositol trisphosphate levels in supernatants of F⁻-treated membranes were 52% higher than levels recovered in supernatants of resting membranes (Table III).

Discussion

This report demonstrates that fluoride induces phospholipid changes in neutrophils consistent with those known to result

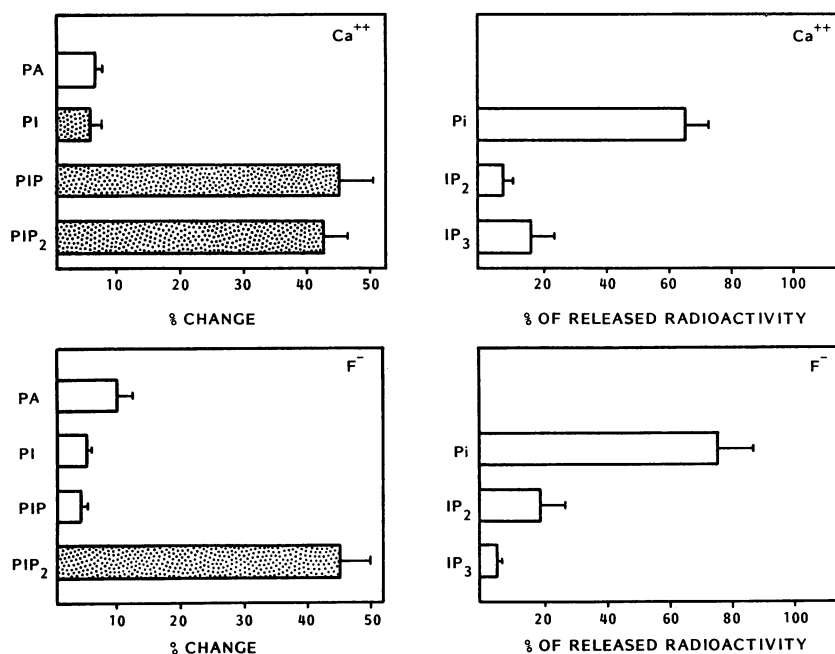


Figure 4. Comparison of fluoride- and Ca^{2+} -stimulated inositol phosphate release and phosphoinositide hydrolysis in neutrophil membranes. Plasma membranes isolated from ^{32}P -labeled neutrophils were washed, resuspended in 20 mM HEPES-0.1 mM EGTA (pH 7.4), and exposed to 0.5 mM Ca^{2+} (upper graphs) or 20 mM NaF (lower graphs) for 10 min at 37°C . Inositol phosphates liberated during incubation were separated by high performance liquid chromatography; lipids were analysed by thin layer chromatography. In graphs of membrane lipid levels (left), stippled bars represent decreases and open bars increases in radioactivity in comparison with lipids in membranes incubated for 10 min with 20.0 mM NaCl. Inositol phosphate graphs (right) indicate the portion of membrane radioactivity released during incubation with Ca^{2+} or F^- that was recovered in each inositol phosphate or inorganic phosphate fraction after chromatography. In supernatants of F^- -treated membranes, the differences in inositol monophosphate and inositol bisphosphate levels but not in inositol trisphosphate levels were statistically significant ($P < .05$). Values reflect results with one membrane preparation. Similar results have been obtained in one additional experiment. In the illustrated experiment, total ^{32}P released from membranes was 44% above control for Ca^{2+} -treated membranes and 32% above control for F^- -treated membranes.

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from phosphatidylinositol cycle activation (40–42). In comparison to unstimulated cells incubated at 37°C , fluoride-treated cells rapidly lost ^{32}P -labeled PIP_2 and accumulated phosphatidic acid, phosphatidylinositol, and soluble inositol phosphates. Phospholipid changes and inositol phosphate generation effected by fluoride may result from the action of PIP_2 -specific phospholipase C. Diacylglycerol, generated as a result of PIP_2 hydrolysis, may rapidly be converted to phosphatidic acid by neutrophil diacylglycerol kinase. Phosphatidylinositol generated in fluoride-treated neutrophils is probably derived from subsequent metabolism of newly synthesized phosphatidic acid. The other product of PIP_2 hydrolysis by phospholipase C, namely inositol trisphosphate, was not recovered in appreciable quantities in fluoride-treated neutrophils. We did, however, detect newly synthesized inositol bisphosphate and inositol mono-

phosphate. Our failure to detect substantial levels of labeled inositol trisphosphate may be a result of the action of cellular phosphatases. While the finding by Storey et al. that F^- inhibits degradation of intracellular inositol trisphosphate (43) argues against this possibility, limited or transient inositol trisphosphate availability is consistent with the apparent minimal release of stored intracellular Ca^{2+} in F^- -treated neutrophils, as discussed below. The failure of $[^3\text{H}]$ inositol to incorporate as rapidly as ^{32}P into the pool of phosphoinositides available as a substrate for hydrolysis by PIP_2 -specific phospholipase C may also have contributed to the low levels of labeled inositol trisphosphate detected. Others have noted that whereas phosphoinositides labeled with ^{32}P are rapidly hydrolyzed in FMLP-treated neutrophils, $[^3\text{H}]$ inositol phosphates are exceedingly difficult to detect (4, 5, 7, 9).

There is considerable disagreement concerning the relation-

Table III. F^- -stimulated Release of $[^3\text{H}]$ Inositol Phosphates From Neutrophil Membranes

Inositol phosphate	Experiment 1			Experiment 2		
	Resting	F^-	% Increase	Resting	F^-	% Increase
	cpm	cpm		cpm	cpm	
Inositol monophosphate	168	378	125	141	280	99
Inositol bisphosphate	210	430	105	180	336	86
Inositol trisphosphate	250	394	58	220	322	46
Total inositol phosphates	628	1202	91	541	938	73

Membranes were isolated from $[^3\text{H}]$ inositol-labeled neutrophils and incubated with 20.0 mM NaCl (resting) or NaF for 10 min at 37°C . Total radioactivity in F^- -treated supernatants was 74.8% higher than that of resting supernatants in experiment 1 and 53% higher in experiment 2. Activity in each inositol phosphate fraction is expressed as counts per minute, as determined by liquid scintillation counting. The counting intervals were adjusted so that each count was obtained with a deviation of $< 2\%$. The total counts recovered in inositol phosphate fractions were $\sim 80\%$ of those applied to the column. The remainder of activity was recovered in fractions containing free inositol and glycerophosphoinositol, eluted with water and 60 mM ammonium formate/5 mM sodium tetraborate, respectively. Differences in levels of each inositol phosphate recovered after Dowex-1 anion exchange chromatography were statistically significant ($P < 0.05$).

ship of calcium influx or mobilization, phosphatidylinositol cycle turnover, and functional activation in stimulated neutrophils. In an early study, Volpi et al. observed that changes in ^{32}P -PIP₂ and PIP levels induced by FMLP were markedly reduced when extracellular calcium was limited (1). The authors did not measure cytosolic Ca²⁺ but discounted the possibility that breakdown of PIP₂ in FMLP-stimulated neutrophils led to intracellular Ca²⁺ release. Serhan et al., on the other hand, found that some phospholipid changes in FMLP-treated neutrophils were not inhibited by chelation of extracellular Ca²⁺ and concluded that these changes were therefore independent of intracellular Ca²⁺ mobilization (2). Using methods to extensively deplete both intra- and extracellular Ca²⁺, Cockcroft et al. concluded that initial changes in phosphatidylinositol metabolism in FMLP-stimulated neutrophils did, in fact, result from Ca²⁺ mobilization (5). With similar methodology, Dougherty et al. concluded, however, that PIP₂ breakdown in FMLP-stimulated neutrophils possibly leads to but does not depend on Ca²⁺ mobilization (3). Using ionomycin in the presence of EGTA to deplete cells of Ca²⁺, diVirgilio et al. demonstrated that FMLP effected inositol phosphate release when Ca²⁺ mobilization was severely curtailed (9). However, Rossi et al. observed that Ca²⁺-depleted neutrophils failed to respond to FMLP with activation of phosphoinositide hydrolysis (12). Lew et al. recently concluded that FMLP-triggered phosphatidylinositol bisphosphate phosphodiesterase (phospholipase C) activation does require Ca²⁺ but does not depend on a previous or concomitant rise in intracellular free Ca²⁺ levels (11).

Very few studies have addressed this controversy by examining neutrophil PI cycle activation and changes in cytosolic Ca²⁺ mobilization with an alternative stimulus. In a study with monocytes, Moscat et al. found that phospholipid changes and inositol phosphate release promoted by ionophore A23187 were entirely dependent upon Ca²⁺ influx (14). Thus, the authors concluded, elevated cytosolic Ca²⁺ may be sufficient to induce the PI response. However, our results clearly show that stimulated PIP₂ hydrolysis in neutrophils can occur before and in the absence of marked elevations in cytosolic Ca²⁺. The kinetics of phospholipid alterations, cytosolic Ca²⁺ changes, and O₂⁻ generation in F⁻-treated neutrophils are consistent with the possibility that Ca²⁺ influx results from PIP₂ hydrolysis and leads to functional activation. As shown in Fig. 5, cytosolic free Ca²⁺ levels did not begin to increase until ~ 1.7 min after addition of F⁻ to neutrophils, a time when PIP₂ hydrolysis was well underway. Whereas this appears to be an unusually long delay for two related functions, it is possible that a certain threshold level of PIP₂ hydrolysis is required before Ca²⁺ influx can begin. Removal of extracellular Ca²⁺ severely limited F⁻-stimulated in-

creases in cytosolic Ca²⁺ but had no effect on PIP₂ hydrolysis. This observation, especially when considered in light of the kinetics of each response, demonstrates that PIP₂ hydrolysis was not a result of cytosolic Ca²⁺ increases in F⁻-treated neutrophils. PIP₂ hydrolysis is an early event in the response of neutrophils to NaF and may lead to functional activation by promoting an influx of extracellular Ca²⁺. It is possible that structural changes or phosphatidic acid generation resulting from PI cycle activation are responsible for increased Ca²⁺ permeability in F⁻-treated neutrophils.

While it is clear that breakdown of PIP₂ occurred independently of cytosolic Ca²⁺ elevation, it is also apparent that secondary phospholipid alterations occur as a consequence of Ca²⁺ influx. As shown in Fig. 3 a second phase of PA and PI generation was observed in F⁻-stimulated cells. This delayed increase was dependent on the presence of extracellular Ca²⁺. Dougherty et al. similarly observed a partial Ca²⁺ dependence of PA and PI synthesis in FMLP-treated HL-60 cells (3). In a recent study, Ohta et al. concluded that delayed release of arachidonic acid and generation of inositol monophosphate in FMLP and Ca²⁺ ionophore-stimulated neutrophils was a consequence of Ca²⁺-dependent activation of nonspecific phospholipases (8). Lew et al. recently demonstrated that Ca²⁺, mobilized in HL-60 cells as a result of FMLP-triggered phospholipase C activation, markedly influences the metabolism of inositol phosphates (11). Ca²⁺ may have profound effects on other PI cycle intermediates. The pathways involved in the Ca²⁺-dependent phospholipid alterations documented in this report have not been identified and the processes involved may be quite complex. Ca²⁺ may activate cellular phospholipases or alter their specificity. In addition, the cation may have profound effects on kinases or other enzymes involved in the synthesis of phosphatidic acid and phosphatidylinositol.

We propose that the reaction sequence triggered by F⁻ involves specific hydrolysis of PIP₂, influx of extracellular Ca²⁺, and activation of the superoxide-generating enzyme. The biochemical processes that initiate this sequence and connect each event to the next remain to be defined. Strnad and Wong have concluded that the effects of F⁻ on neutrophil Ca²⁺ mobilization are a result of the ion's effects on certain guanine nucleotide regulatory proteins (30). While there is little direct evidence to support this interesting hypothesis, Blackmore et al. have demonstrated that F⁻ mimicks the effects of Ca²⁺-mobilizing hormones on isolated hepatocytes, possibly by activating a pertussis toxin-resistant guanine nucleotide regulatory protein involved in activation of PIP₂-specific phospholipase C (44). The induction of neutrophil O₂⁻ generation and Ca²⁺ mobilization

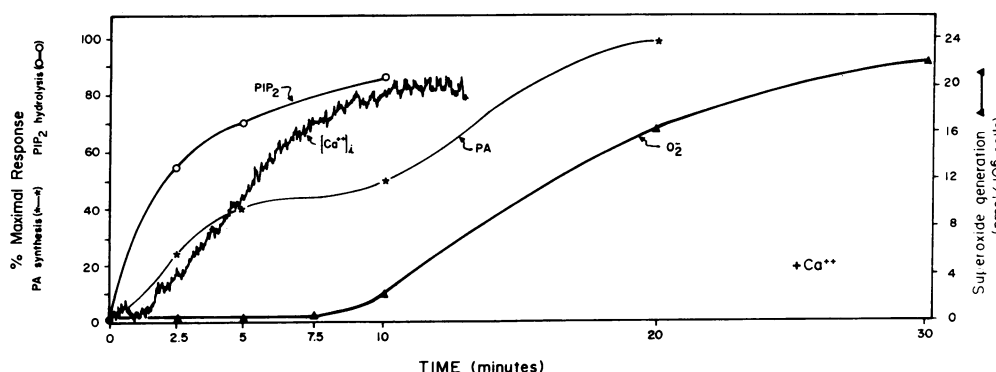


Figure 5. Temporal relationship of phosphatidylinositol bisphosphate hydrolysis, phosphatidic acid synthesis, Ca²⁺ mobilization, and superoxide generation in F⁻-treated neutrophils. Data are recast from the responses of cells in Ca²⁺-replete media of Figs. 1, 2, and 3. The kinetics of phosphatidylinositol bisphosphate hydrolysis and phosphatidic acid synthesis are shown as the percent of maximal (30 min) response.

by F^- is also resistant to pertussis toxin (30). In addition, Bokoch and Gilman demonstrated that F^- causes a substantial release of arachidonate acid in neutrophils (45). This effect was blocked by prior treatment of cells with pertussis toxin. It has recently been demonstrated that F^- induces PIP_2 hydrolysis in WRK 1 cell membranes in a manner consistent with its effects on the guanine nucleotide binding proteins N_s , N_i , and transducin (46). This effect was attributed to the influence of F^- , together with trace levels of Al^{+3} , on PIP_2 -linked guanine nucleotide regulatory proteins. In these studies, the concentration of fluoride which induced optimal PIP_2 hydrolysis was 20 mM. Our data demonstrate that fluoride similarly effects specific hydrolysis of PIP_2 in isolated neutrophil plasma membranes. Fluoride-induced PIP_2 hydrolysis in neutrophil plasma membranes occurred when the availability of Ca^{2+} was severely limited by excess chelators. The mechanism of fluoride-mediated PIP_2 hydrolysis, the role of guanine nucleotide-binding proteins in this process, and the relevance of fluoride-stimulated PIP_2 hydrolysis in isolated membranes to the response in intact cells are not known. With respect to the latter problem, it is noteworthy that F^- effected the hydrolysis of PIP_2 nearly exclusively in both isolated membranes and whole cells. Ca^{2+} , on the other hand, effected the hydrolysis of both PIP and PIP_2 . Levels of both PIP and PIP_2 decrease quickly when intact neutrophils are stimulated with FMLP (1, 8, 15). FMLP receptor-ligand interactions, via certain guanine nucleotide regulatory proteins, effect hydrolysis of both PIP and PIP_2 in isolated neutrophil membranes, possibly by lowering the Ca^{2+} requirement of phospholipase C (18). Whether or not the effects of fluoride on intact neutrophils or neutrophil plasma membranes are due to its effects on a novel guanine nucleotide regulatory protein remains to be proven. Rapid reversibility of the F^- effect on intact neutrophils (31) mitigates against the argument that G proteins are the exclusive mediators of neutrophil activation by F^- , because current models of AlF_4^- -induced G protein activation (47) do not account for rapid reversibility. Our results do, however, support the conclusion that F^- induces the hydrolysis of PIP_2 in neutrophil membranes, an event which may be an early if not initial response in one of the metabolic pathways leading to functional activation.

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