Phosphorylase a Activity As an Indicator of Neutrophil Activation by Chemotactic Peptide

JOAN L. SLONCZEWSKI, MARY W. WILDE, and SALLY H. ZIGMOND
Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT The activity of glycogen phosphorylase, an enzyme that is activated by both cAMP and calcium, was used as an indicator of the state of the cytoplasm after chemotactic stimulation of polymorphonuclear leukocytes (neutrophils). The activity of the enzyme showed a clear dependence on cytoplasmic calcium. Addition of the calcium ionophore A23187 caused a 4–5-fold increase in activity of phosphorylase a. In the absence of external Ca^{2+}, A23187 caused only brief transient activation of phosphorylase; probably reflecting release of sequestered intracellular Ca^{2+}. Addition of the chemotactic peptide N-formylnorleucylleucyl-phenylalanine (FNLLP) caused a transient 2–3-fold activation of the enzyme. The dose-dependence of activation by FNLLP showed a peak at 10^{-8} M, near the K_d of the receptor for FNLLP. The phosphorylase activity peaks by 90 s and then declines, returning to basal levels by 20 min after stimulation with 10^{-8} M peptide and by 60 min with 10^{-7} M peptide. This finding suggests that the cells do not need to maintain elevated cytoplasmic calcium levels to exhibit stimulated locomotion. Thus, if calcium continues to modulate the motility, there either must be highly localized changes that are not detected in measures of the total cytoplasm, or the sensitivity to calcium must be variable such that basal levels are sufficient to maintain locomotion. Cells loaded with the fluorescence calcium probe quin2 (0.6 mM) in the presence or absence of external Ca^{2+} had elevated phosphorylase levels before addition of FNLLP. Thus, the presence of quin2 may alter the cytoplasmic Ca^{2+} level, and it clearly alters some aspects of the neutrophil physiology. Phosphorylase a appears to be a sensitive, nonperturbing indicator of the cytoplasmic calcium levels.

Chemotactic peptides are known to have both acute and chronic effects on polymorphonuclear leukocytes, neutrophils. The acute effects include cell ruffling, adhesion, and secretion of granule enzymes (34, 44). These responses occur within seconds of peptide addition, reach peak levels in ~30 s, and then decline toward baseline levels even in the continued presence of peptide. The chronic effects of the peptide include a stimulated rate of locomotion (chemokinesis), and a directional response to a gradient of peptide (chemotaxis) (27, 39, 40, 42, 43). These responses continue for hours in the presence of peptide. The sequence of molecular events that mediate the various responses is a topic of much current interest. Early molecular events include a rapid change in phosphatidylinositol metabolism and increases in cytoplasmic Ca^{2+} and cyclic AMP levels. These changes occur rapidly and reach their peak levels before receptor binding has reached its plateau. These responses then decline toward baseline levels. Whether there is a persistent small elevation of these or other mediators that regulate the motile responses over long time periods is unknown.

If the Ca^{2+} level continuously mediates the motile response stimulated by peptide, one might expect to find a persistent increase in the steady-state Ca^{2+} levels in cells in the presence of chemotactic factor. It is difficult to measure the activity of free cytoplasmic Ca^{2+}. Neutrophils are too small for practical use of intracellular ion electrodes. $^{45}Ca^{2+}$ labels whole-cell calcium, including bound sources, albeit with differing kinetics (25). The membrane-bound fluorescence probe chlortetracycline has been used to observe the chemotactic factor–induced depletion of a bound Ca^{2+} pool whose location is not well defined (24, 31). The soluble fluorescence probe quin2 has provided much useful information on cytoplasmic free Ca^{2+}, but its use has several drawbacks, most importantly the chelating and buffering properties of the probe itself (4, 26, 35). There is evidence that the presence of quin2 alters the actual or apparent free Ca^{2+} level, and that it stimulates

phosphoinositide metabolism (17).

To study the long-term effects of the chemotactic peptides, we sought a sensitive, quantitative, nonperturbing indicator of the state of the cytoplasm. We believe that the enzymatic activity of glycogen phosphorylase satisfies a number of these criteria: (*a*) The enzyme is endogenous, thus modifications of it occur in a cell undisturbed by any buffering or foreign agents. Since the modification is covalent, the enzyme (and thus the state of the cytoplasm) can be assayed at any time by lysing the cells into medium in which the enzymatic activity is stable. (*b*) The enzyme is known to have a cytoplasmic location, thus it is susceptible to the same mediators as is the motile machinery of the cell. (*c*) The enzymatic activity is positively regulated by both Ca^{2+} and cAMP, two second messengers known to be altered by chemotactic peptides (1–3, 12–15, 18–20, 29, 32, 38). The phosphorylase activity depends upon the enzyme itself being phosphorylated. Phosphorylase kinase can be activated by a cAMP-dependent kinase or by calcium binding directly to a calmodulin-like subunit of the enzyme. As is described below, it appears that the enzymatic regulation in leukocytes stimulated by chemotactic peptide is sensitive to changes in Ca^{2+}, and that the peptide-induced changes in cAMP do not, on their own, activate the enzyme. (*d*) The enzymatic activity can be quantitated as a monitor of relative levels of cell activation (22). The total enzyme present can be assayed by including 5′AMP which activates the unactivated phosphorylase, phosphorylase b, to 80% of the activity of the phosphorylated enzyme, phosphorylase a.

We now report that: (*a*) in neutrophils as in hepatocytes the activity of the enzyme increased in response to the A23187, in a Ca^{2+}-dependent manner; and (*b*) the enzyme activity was rapidly increased by addition of the chemotactic peptide N-formylnorleucylleucylphenylalanine (FNLLP)[1] in a manner parallel but slightly slower then the changes in calcium measured by quin2 (25). (*c*) Removal of the peptide causes the enzymatic activity to rapidly return to basal levels. (*d*) These rapid changes were blocked by pertussis toxin which is known to block the quin2 response but not the cAMP rise (5, 15). The best (although not exclusive) explanation for these findings is that changes in phosphorylase a activity reflect changes in cytoplasmic Ca^{2+}.

We have used this enzyme to monitor the steady-state activation of cells maintained in chemotactic peptides. The enzymatic activity is no higher in cells maintained in FNLLP than in control medium. Our findings suggest that if Ca^{2+} is involved in mediating the persistent motile functions of the neutrophil, this mediation must occur at basal calcium levels.

MATERIALS AND METHODS

Media and Chemicals: Hank's balanced salt solution with 10 mM HEPES buffer replacing bicarbonate, at pH 7.2 (Hank's-HEPES), was prepared from reagent grade chemicals. A23187 and FNLLP were obtained from Sigma Chemical Co. (St. Louis, MO). Quin2-AM and quin2 acid were obtained from Calbiochem-Behring Corp. (La Jolla, CA). [^{14}C]Glucose-1-phosphate (290 mCi/mmol) was obtained from Amersham-Searle. Pertussis toxin (islet-activating protein) was obtained from List Biochemical Laboratories Inc. (Campbell, CA). Dibutryl cAMP and 8-bromo-cAMP were obtained from Sigma Chemical Co.

Cells and Treatments: Rabbit peritoneal neutrophils obtained as described previously (41) were washed in saline and in Hank's-HEPES medium, and resuspended at 2×10^7 cells/ml in Hank's-HEPES medium with 0.5% fatty acid–free bovine serum albumin (BSA; Sigma Chemical Co.). The cells were incubated 30 min with mild stirring at 37°C before the experiment was initiated. For experiments with cells in Ca^{2+}-free medium, the Ca^{2+} in Hank's-HEPES was replaced with 1 mM EGTA. At each time point, 200 μl of cell suspension was pipetted into 50 μl sample buffer (250 mM NaF/25 mM EGTA/25 mM EDTA/250 mM PIPES, pH 7.0) on ice. Samples were then assayed for phosphorylase a activity immediately or frozen at −20°C and later thawed for assay.

For experiments using adherent cells, 1.5-ml aliquots of suspension at 5×10^6 cells/ml were pipetted into 5-cm culture dishes. The cells settled 20–30 min at room temperature, then the surrounding medium was aspirated and replaced with 0.5 ml fresh Hank's-HEPES. The dishes were moved to a 37°C room for the experiment. To maintain the cells in medium with a minimum of secreted products, the medium was replaced with fresh medium every 2 min throughout the experiment. For each time point, medium was aspirated from the dish, the dish was placed on ice, and 0.5 ml of ice-cold sample buffer diluted with saline was added (immediately). The dishes were scraped in the cold with a rubber policeman, and the samples were stored frozen for phosphorylase a assay.

For quin2 studies, cells (10^7/ml) were incubated in either buffer A (150 mM NaCl, 5 mM KCl, 1.29 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) (20), or buffer B (147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 3 mM $MgSO_4$, 1 mM $MgCl_2$, pH 7.3; Sklar buffer), for 60 min at 37°C. Cells were then centrifuged, resuspended in either buffer A or buffer B (with 1.5 mM $CaCl_2$ added), and equilibrated for 15 min at 37°C before peptide addition. The quantity of quin2 loaded in cells was determined by reading emission at 492 nm (excitation 339 nm) in the presence of 0.2% Triton with or without 5 mM EGTA. Quin2 free acid was used as a standard.

Cells (10^7/ml) were loaded with pertussis toxin (200 ng/ml) in Hank's HEPES without Ca^{2+} or Mg^{+2} and containing 0.1% BSA for 2 h at 37°C. They were spun, resuspended at 2×10^7 cells/ml in Hank's-HEPES with 0.1% BSA, and equilibrated for 15 min at 37°C. Peptide was added and samples were taken at various times for phosphorylase assay.

Phosphorylase a Assay: The activity of glycogen phosphorylase a was determined by following [^{14}C]glucose-1-phosphate incorporation into glycogen, based on the method of Borregard and Herlin (8, 37). Samples were sonicated to break >90% of the cells, then centrifuged in the cold to remove large particles. 30 μl of sonicate was added to 60 μl of assay buffer (15 mM [^{14}C]glucose-1-phosphate [1 μCi/ml]/1.5% rabbit liver glycogen/150 mM NaF/100 mM PIPES, pH 6.4) and incubated at 30°C for 30–90 min depending on the range of activity expected. To determine the [^{14}C]glucose-1-phosphate incorporation into glycogen, 75 μl of each reaction mixture was spotted onto a Whatman No. 1 filter paper circle (Whatman, Inc., Clifton, NJ) and dried under a heat lamp. Unreacted substrate was washed out by soaking the filters in 66% ethanol, using at least 4 ml per sample, with four changes. The ^{14}C was counted in a New England Nuclear (Boston, MA) Econofluor in a Beckman scintillation counter (Beckman Instruments, Inc., Palo Alto, CA), with an efficiency of 65%.

RESULTS

A23187 Raises Phosphorylase Activity

The effect of A23187 on phosphorylase was examined by adding ionophore to a suspension of neutrophils in the presence of 1.3 mM Ca^{2+} (Fig. 1). Addition of 2×10^{-6} M A23187 induced a rise in activity of phosphorylase that reached a plateau after 10 min. The maximal activity reached was ~4–5 times the basal level. A lower concentration of ionophore (2×10^{-7} M) caused a much slower rise in enzyme activity, presumably due to a slower influx of Ca^{2+}. With 1 mM EGTA present and no Ca^{2+} in the external medium (Fig. 2), 5×10^{-6} M A23187 caused a slight transient increase in phosphorylase a followed by return to the basal level. Similar results were obtained in Ca^{2+}-free medium without EGTA.

Glycogen phosphorylase activity was also modulated by cAMP. The presence of millimolar concentrations of the cAMP analogues 8-Br-cAMP or dibutyryl cAMP activated glycogen phosphorylase. Both peptide and A23187 were able to stimulate the activity further. The peptide-induced response in the presence of 5 mM dibutyryl cAMP was similar in time course and magnitude to that seen in control cells. In the

[1] *Abbreviation used in this paper:* FNLLP, N-formylnorleucylleucylphenylalanine.

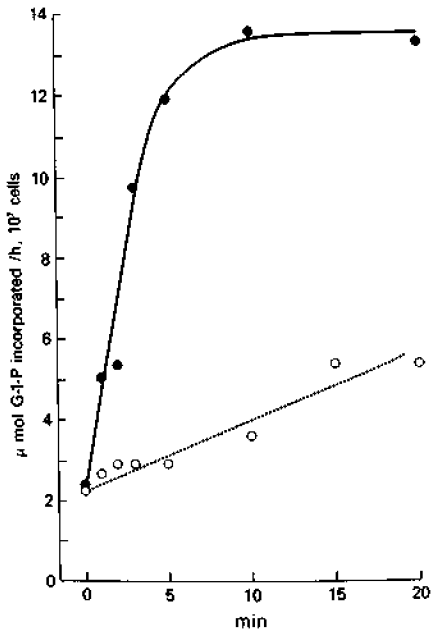

FIGURE 1 Time course of calcium ionophore A23187 activation of phosphorylase a. Neutrophils were resuspended at 1.5×10^7 cells/ml in medium containing 1.3 mM calcium. A23187 was added at time zero, at 2×10^{-6} M (●), or at 2×10^{-7} M (○). For all figures: G-1-P, glucose-1-phosphate.

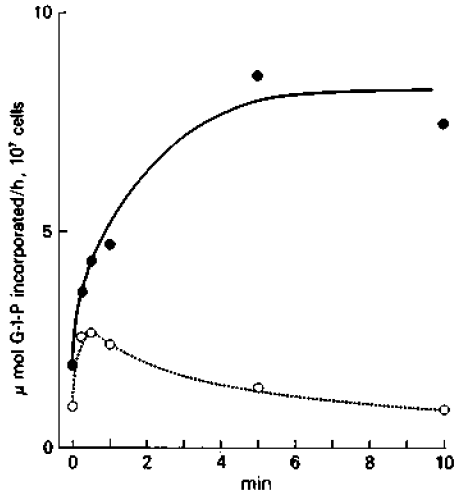

FIGURE 2 Activation of phosphorylase a by A23187 in the presence and absence of extracellular Ca^{2+}. The neutrophil suspension at 2×10^7 cells/ml was preincubated 15 min at 37°C in medium containing 1.3 mM Ca^{2+} (●) or no calcium and 1 mM EGTA (○). 5×10^{-6} M A23187 was added at time zero.

presence of millimolar concentrations of 8-Br-cAMP, the stimulation by low levels (5×10^{-8} M) of A23187 was greater than that seen in control cells (data not shown). This result suggests that the calcium sensitivity of the kinase may be increased by the cAMP analogues.

Transient Elevation of Phosphorylase a by FNLLP

The addition of the chemotactic peptide FNLLP caused a transient rise in phosphorylase a (Fig. 3). In the presence of external Ca^{2+}, the peak of activation was reached at 1–1.5 min, with a return to nearly basal levels by 6 min. The maximum amount of increase was typically 3–4-fold. In the absence of external Ca^{2+}, a rise in phosphorylase a activity was still observed. The peak activity occurred earlier (0.5–1 min), and the overall levels of activity were lower. Thus, while the enzyme did show a dependence upon extracellular Ca^{2+}, the chemotactic peptide did not require an external pool of Ca^{2+} in order to raise the level of phosphorylase a. This finding is consistent with the observation by others that chemotactic factors release Ca^{2+} from an internal pool and increase the permeability of the plasma membrane to Ca^{2+}.

The FNLLP dose-dependence of phosphorylase a activation in the presence or absence of external Ca^{2+} is shown in Fig. 4. The range of peptide concentration between minimal and maximal effect was 5×10^{-10}–10^{-8} M in the presence of external Ca^{2+} (Fig. 4*A*). The degree of activation was less at 10^{-7} M than at 10^{-8} M. 10^{-5} M peptide gave a response similar to that at 10^{-7} M. A23187 (10^{-6} M) given as stimulus

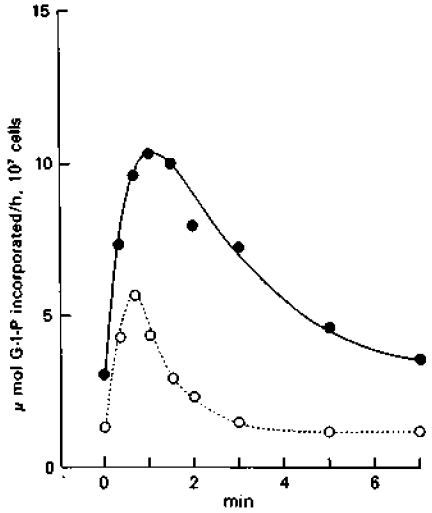

FIGURE 3 Time course of FNLLP activation of phosphorylase a in medium containing 1.3 mM Ca^{2+} (●) or no calcium and 1 mM EGTA (○). Cell concentration was 3×10^7 cells/ml; the suspensions were incubated 30 min at 37°C before 10^{-7} M peptide was added.

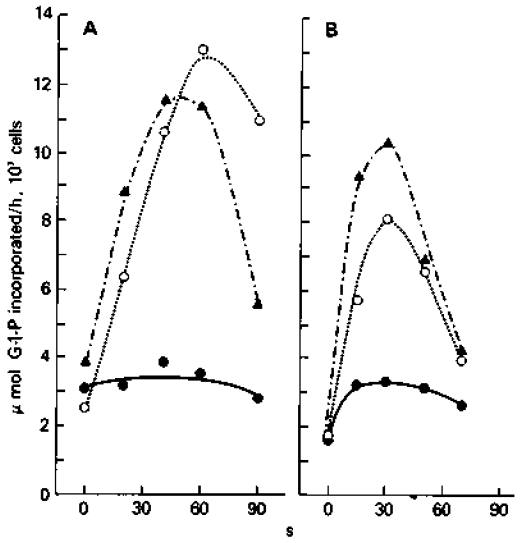

FIGURE 4 Dose-dependence of FNLLP activation of phosphorylase a in the presence (*A*) or absence (*B*) of external Ca^{2+}. Conditions were the same as for Fig. 3. Concentration of FNLLP was 5×10^{-10} M (●); 10^{-8} M (○); 10^{-7} M (▲).

in the presence of external Ca^{2+} activated phosphorylase to levels higher than any concentration of peptide, thus this finding indicates that the peak activity reached in 10^{-8} M peptide is not a result of maximally stimulated enzyme. In the absence of external calcium, the response continued to increase up to 10^{-7} M peptide.

Persistent Activation of Phosphorylase by FNLLP

It is known that cell locomotion is stimulated for several hours in the presence of FNLLP (43). Therefore, if calcium levels are involved in the stimulation of locomotion, we would expect to find a difference between the steady-state levels of Ca^{2+} in cells incubated with or without FNLLP. We sought to test this hypothesis by comparing the steady-state levels of phosphorylase a activity in the presence and absence of FNLLP.

To improve the sensitivity of these experiments, the phosphorylase a assay was adapted to cells plated on dishes. The use of plated cells had several advantages over cells in suspension. Cell clumping, which occurs when peptide is added to cells in suspension, was avoided. Furthermore, the medium surrounding the cells could be removed and replaced rapidly (within 5 s). By repeatedly changing the medium on the plated cells, we could keep the control cells in fresh medium free of activators that might be released by the cells themselves and the peptide-treated cells in fresh peptide free of digestion products and other possible activators. The initial transient increase in phosphorylase activity induced by FNLLP in plated cells was similar to that observed in cells in suspension (Fig. 6).

Cells incubated in 2×10^{-7} or 2×10^{-8} M FNLLP for 60 min have phosphorylase activities similar to that of control cells (Fig. 6). The return of the activity to baseline depends on the peptide concentration. In 2×10^{-8} M peptide, the level returns to baseline within 20 min, while in 2×10^{-7} M peptide the activity remains above baseline at 20 min but returns to baseline by 60 min. In this experiment, the medium was exchanged every 10 min to ensure that neither peptide degradation nor depletion was contributing to the decline. Analysis of protein on the dishes indicated that there was no cell loss during the prolonged incubations. Thus, there does not appear to be a permanent change in the levels of cytoplasmic calcium in cells maintained in peptide.

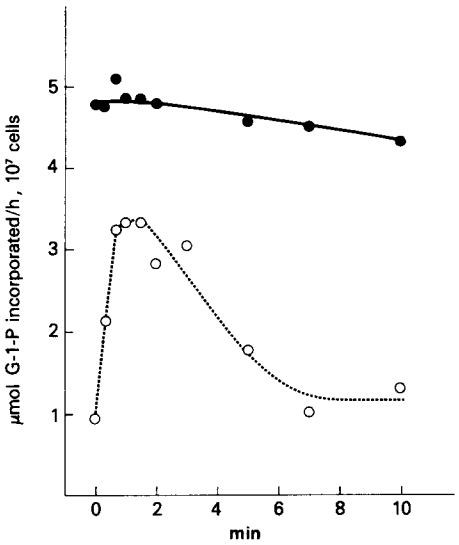

FIGURE 5 Effect of intracellular quin2 on activation of phosphorylase a by FNLLP. Neutrophils suspended at 10^7 per ml in Hank's-HEPES medium were incubated with (●) or without (○) 20 μM quin2-AM for 60 min at 37°C, then resuspended in medium without quin2. The intracellular content of quin2 acid was determined to be 0.6 mM, based on comparison of quin2 content in Triton-lysed cells with a standard sample of quin2 acid (29). The remaining cells were incubated with stirring at 37°C for 30 min before addition of 10^{-7} M FNLLP.

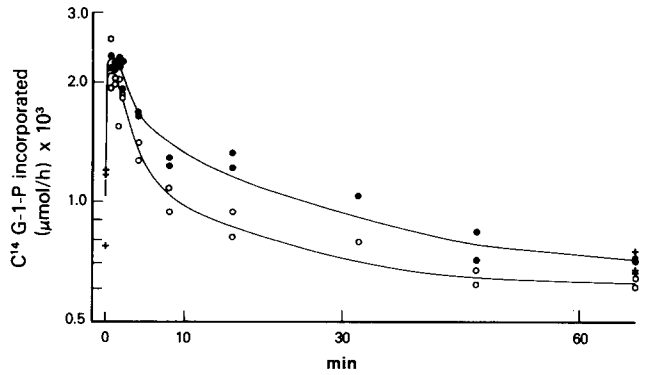

FIGURE 6 Time course of the log of phosphorylase activity of cells maintained in peptide. Cells were plated on petri dishes and incubated at 37°C for times up to 67 min in Hank's (+), 2×10^{-8} M FNLLP (○), or 2×10^{-7} M FNLLP (●). The medium on the dishes was replaced every 10 min. At various times the phosphorylase activity of the cells was measured in sonicates of 10^7 cells/ml.

It is known that neutrophils respond to a decrease in concentration of chemotactic factor by ceasing to locomote and withdrawing their pseudopods (40). Removal of peptide must cause some change in the state of the cytoplasm. We investigated whether this change would be reflected by a change in phosphorylase a levels. Cells incubated for 20 or 60 min in medium containing peptide were switched to fresh medium devoid of peptide. The phosphorylase a level of these cells decreased within 1.5 min. The levels of phosphorylase observed in these cells sometimes went below that of cells in control medium.

Pertussis Toxin Treatment Blocks Phosphorylase Activity Induced by FNLLP

Treatment of neutrophils with pertussis toxin (islet-activating protein) blocks the peptide-induced rise in Ca^{+2} but not the cAMP rise (5, 15). We used treatment with pertussis toxin to evaluate the effect of the rise in cAMP on phosphorylase activity. The activation of phosphorylase by 10^{-8} M FNLLP was completely blocked in cells treated with 200 ng/ml pertussis toxin for 2 h (Fig. 7). Basal levels of phosphorylase activity were not affected. Both A23187 (10^{-6} M) and dibutryl cAMP (5 mM for 30 min at 37°C) could activate the phosphorylase in pertussis toxin–treated cells to the same level as untreated cells.

FNLLP Activation of Phosphorylase in Presence of Quin2

It was of interest to observe the peptide-induced activation of phosphorylase a in the cells loaded with quin2, a fluorescence probe commonly used as an indicator of peptide-induced modulation of Ca^{2+} level (20, 26, 38). Cells were loaded with quin2 by incubation for 60 min in the presence

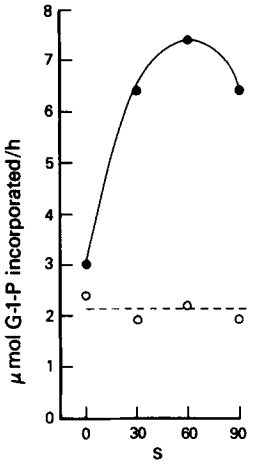

FIGURE 7 Peptide-induced activation of phosphorylase is blocked by treatment with pertussis toxin. Cells were incubated in Hank's-Ca^{+2}-Mg^{+2}, with 0.1% BSA in the presence (O) or absence (●) of 200 ng/ml pertussis toxin for 2 h at 37°C. They were spun, resuspended in Hank's with Ca^{+2} and Mg^{+2}, and equilibrated 15 min at 37°C. Peptide (10^{-8} M FNLLP) was added and samples were taken at the time points indicated.

of 20 μM quin2-AM, resulting in intracellular accumulation of 0.6 mM quin2 acid. This concentration is typical of those used for measurement of intracellular free Ca^{2+}. We found a substantial elevation of phosphorylase a in the quin2-loaded cells as compared with the control (Fig. 5). Upon addition of 10^{-7} M FNLLP, little or no further activation of enzyme was observed. The quin2-loaded cells could release lysosomal enzymes upon peptide treatment in the presence of 5 μg/ml cytochalasin B, confirming that the cells can function when loaded with quin2.

DISCUSSION

We have used phosphorylase as a nonperturbing indicator of cytoplasmic activation by chemotactic peptide. We find that this enzyme which is activated by addition of peptide returns to basal levels in the continued presence of peptide. This suggests that the peptide-stimulated locomotion of cells occurs when the mean cytoplasmic calcium levels are at basal levels.

Usefulness of Phosphorylase Assay

Since we were interested in investigating the small, long-term effects of peptide on cells, we needed a marker that would not itself perturb the cells and would be sensitive enough to detect small changes. We focused on changes in calcium levels since a considerable amount of biochemical evidence suggests that calcium is a regulator of cell motility. However, there is no evidence that calcium levels are altered over the time course that peptide stimulates cell locomotion. Quin2 has been useful for many studies on calcium levels in neutrophils since it is a highly specific probe of cytoplasmic calcium. However, two properties of quin2 limited its usefulness to us in this study. First, it does perturb the cell. It is a calcium buffer and can be used to lower cytoplasmic calcium levels. It has also been shown to stimulate phosphatidylinositol metabolism in lymphocytes (16), and we report here that the presence of quin2 stimulates the phosphorylase activity. The second difficulty with quin2 is its inability to detect long-term small changes in levels of calcium. Although sensitive at physiological calcium concentrations, the baseline quin2 signal often declines over a long time course making it difficult to detect small changes between treated and untreated cells.

Glycogen phosphorylase is an endogenous enzyme whose activity is highly regulated. Both calcium and cAMP are known to positively modulate the enzyme activity; thus the enzyme has the potential to be sensitive to both of these second messengers. Furthermore, since the regulation involves phosphorylation of the enzyme, the level of activation can be measured at any time by lysing cells and assaying the enzyme activity in medium that prevents further modification of the enzyme. As will be discussed below, in neutrophils stimulated by chemotactic peptide the enzymatic activity appears to be sensitive to changes in Ca^{+2} but not cAMP. However, because of the complexity of the regulation of the steady-state activity of glycogen phosphorylase (which involves the activation of phosphorylase kinase and the activity of several phosphatases each of which may also be regulated), we cannot ascribe the changes in activity to a single factor. We propose that phosphorylase a is a useful indicator of the activation of the cytoplasm of neutrophil and that it is particularly sensitive to calcium.

Basis of Phosphorylase Activation

The activity of the phosphorylase in neutrophils is clearly sensitive to changes in cytoplasmic calcium. The early phase of enzyme activation by A23187 in medium containing Ca^{2+} (Fig. 1) has a nearly linear time course for either 2×10^{-7} M or 2×10^{-6} M ionophore. The initial slope shows roughly a 10-fold increase for the 10-fold increase in concentration of ionophore. This is consistent with a dependence of phosphorylase a activation on the Ca^{2+} influx through pores created by the A23187. At the higher concentration (2×10^{-6} M), the phosphorylase activity eventually leveled off at a plateau about 4–5 times the original basal level. This saturation point may represent the limit of available phosphorylase b substrate in the cell or a maximal steady-state between kinase and phosphatase activities. In the absence of external Ca^{2+}, addition of A23187 resulted in a brief transient activation of enzyme (Fig. 2). The result is probably due to the release of cellular bound Ca^{2+} into the cytoplasm and subsequent loss from the cell. Our interpretation is consistent with the reported ability of A23187 to deplete an internal Ca^{2+} pool required for lysosomal enzyme release (10).

Activation of phosphorylase could also reflect changes in cAMP. It is known that cAMP induces phosphorylation of the Ca^{2+}-dependent phosphorylase kinase; as a result of this phosphorylation the kinase is converted to a more active form (12). We find that analogues of cAMP do stimulate the phosphorylase activity in neutrophils. In one study, the kinase isolated from neutrophils no longer required calcium for activity if it was fully phosphorylated (33). However, we found that cells treated with concentrations of cAMP analogues that maximally stimulated the phosphorylase activity still responded to peptide and to A23187. In fact, the responses to low levels of A23187 were increased, suggesting an increased sensitivity of the kinase to calcium. An increased sensitivity of the phosphorylated kinase to calcium has been observed in muscle (11).

The small transient elevation of cAMP known to occur during FNLLP stimulation (29) does not appear to activate the phosphorylase. We have shown that cells treated with pertussis toxin do not increase their phosphorylase activity upon the addition of peptide. Pertussis toxin does not block the peptide-induced rise in cAMP but does block the calcium rise as observed with quin2 (6, 16). The inability of peptide to activate the phosphorylase in the pertussis toxin confirms the conclusions of Borregard et al. that small cAMP increases are insufficient to cause covalent alterations of the phospho-

rylase kinase (8, 9). They isolated the phosphorylase kinase from cells undergoing phagocytosis. These cells show a small increase in cAMP similar to that caused by peptide. The isolated kinase does not show the properties of the phosphorylated kinase (9). Therefore, it appears unlikely that the activation of phosphorylase a induced by phagocytosis or chemotactic peptides is mediated by cAMP.

The level of phosphorylase activity could also be modulated by the activity of the phosphatases. We have not investigated regulation of the phosphatases. The rapid decrease in phosphorylase activity, both after the transient rise and upon removal of peptide, suggest that the phosphatases are active and that the steady-state level of the phosphorylase activity does reflect the current or at least recent kinase activity.

There is a report that the phosphorylase can be activated by 0.5 μg/ml phorbol myristate acetate, suggesting a protein kinase C–dependent pathway of activation (9). Whether this pathway represents a direct activation of the enzyme or whether it represents an indirect effect due to increased leukotriene production or raised calcium levels (28) is not clear. Although phorbol myristate acetate at nanomolar levels does not raise cellular Ca^{2+} as measured by quin2, Sha'afi et al. have shown that (1 μg/ml) phorbol myristate acetate does cause a quin2 response (17, 21, 23, 28).

Thus, it appears that the phosphorylase a levels do reflect cytoplasmic calcium levels. However, since the activity of this enzyme is regulated at many levels, its activity cannot be ascribed uniquely to the calcium levels. The phosphorylase a activity can be safely used as an indicator of an activation of the neutrophil cytoplasm for which calcium appears to be a primary modulator.

Peptide Dose-dependence for Activation

We found that addition of a chemotactic peptide caused transient activation of phosphorylase in the presence or absence of external Ca^{2+} (Fig. 4). The dose-dependence of this transient phosphorylase a activation has several interesting features. In the presence of external calcium, the enzyme activity peaked at $\sim 1 \times 10^{-8}$ M FNLLP and decreased at the higher concentration (10^{-7} M). The quin2 signal has also been reported to be maximal after stimulation by peptide at concentrations near the K_d of binding (20, 30). Thus, this level of stimulation by peptide may be sufficient to cause the maximal change in cytoplasmic calcium levels. This concept proposed by Sklar and co-workers is supported by the fact that the quin2 signal reaches its peak level within 10–30 s of peptide addition. This time point is well before receptor occupancy has plateaued (30). The phosphorylase activity peaks somewhat later (60–90 s), possibly reflecting the time course of the kinase activity after the calcium is raised. However, some cellular responses including actin polymerization (36) and phospholipase A_2 activation (7) continue to increase as peptide concentrations are raised above the K_d. Thus, these responses must either be dependent on features of receptor transduction separate from the cytoplasmic calcium levels or may depend on the duration as well as the magnitude of the raised calcium levels. In the absence of calcium, phosphorylase activity was not maximally stimulated by peptide until the concentration was in excess of the K_d. The reason for the difference in the dose-response curve is not clear.

Significance of Results for Cell Locomotion

We found that phosphorylase activity of cells maintained in peptide for 20–60 min returned to baseline. These findings suggest that cytoplasmic Ca^{2+} returns to a prestimulus level in the continued presence of FNLLP. Thus, if calcium continues to modulate motility, there must either be highly localized changes that are not detected in the total cytoplasm or the cell's sensitivity to calcium must be variable such that basal Ca^{2+} levels are sufficient to maintain locomotion. It is possible that a decrease in cell Ca^{2+} upon removal of peptide plays a role in the negative motile response (cessation of locomotion and collapse of the lamellipodia) which follows withdrawal of peptide from the medium of locomoting cells (40).

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