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Evidence for *N*-formyl chemotactic peptide-stimulated GTPase activity in human neutrophil homogenates

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Neutropil homogenates contained a high affinity guanosine triphosphatase (GTPase) that was stimulatable (+27%) by the addition of 100 nM *N*-formyl chemotactic peptide (CHO-pep), but not by 1 $\mu g \cdot ml^{-1}$ phorbolmyristate acetate (PMA). Kinetic analysis of the stimulation demonstrated an apparent lagtime of 14.3 ± 6.9 s between the addition of CHO-pep and the optimal GTPase stimulation. The GTPase activity (but not CHO-pep-stimulated GTPase activity) was preserved in a highly purified plasma membrane fraction of the homogenate. From these observations we suggest that both a high affinity guanine nucleotide binding protein and GTPase are closely associated with the plasma membrane CHO-pep receptor. The possibility that GTPase activity may influence guanine nucleotide regulation of adenylate cyclase during CHO-pep stimulation of neutrophils is discussed.

Neutrophil *Plasma membrane* *Chemotactic peptide* *Phorbol esters* *GTPase* *Cyclic AMP*

1. INTRODUCTION

Human polymorphonuclear leukocytes contain a sensory transduction system which initiates chemotaxis and microbicidal activity to certain inflammatory stimuli [1]. Although biochemical characterization of the transduction process has yet to be elucidated, there is evidence that cyclic nucleotides are involved in the stimulation, since there is a 2–3-fold transient increase in intracellular cAMP which is maximal within 20 s of exposure of neutrophils to CHO-peptides [2]. Intracellular cAMP elevation does not appear to be a sufficient condition for neutrophil activation as dibutyryl cAMP is not a stimulus [3], and phorbolesters, while fully activating cell function, do not elevate intracellular cAMP. However, manipulation of intracellular cAMP concentration utilizing phosphodiesterase inhibitors or addition of dibutyryl cAMP alters the kinetics and magnitude of cellular response to ligand [3].

Hormonal regulation of adenylyl cyclase activity is mediated by both inhibitory (N_i) and stimulatory (N_s) guanine triphosphate-binding proteins [4,5]. Termination of the hormone-mediated regulatory capacity of N_i and N_s may occur by hydrolysis of bound GTP, by a specific high affinity GTPase [5–9]. The locus of the GTPase activity (with respect to N_i and N_s) and its regulation during hormone-dependent guanine nucleotide modulation of cyclase activity remains unknown.

In the human neutrophil, the cAMP system may involve N-binding proteins, since it was reported [10] that the non-hydrolysable analogue of GTP (GppNHp) reduced the affinity of the formyl peptide receptor for CHO-pep by increasing the dissociation rate constant. Furthermore, authors in [11] reported that purified neutrophil plasma membrane adenylate cyclase activity was activated by incubation with guanine nucleotides.

Here we demonstrate that homogenates from human neutrophils contain a CHO-peptide-activatable GTPase. From the kinetics of this activation, we speculate that GTPase activation is involved in the termination sequence of cyclase activation by CHO-peptide–N_s complex.

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2. MATERIALS AND METHODS

2.1. *Preparation of cells*

Human neutrophils were prepared from freshly drawn venous blood as in [12]; 120–250 ml blood yielded between 0.2–1 $\times$ 10^9 cells of greater than 95% purity. Cells were suspended at a concentration of 10^8 ml^{-1} in a buffer containing 5 mM KCl, 147 mM NaCl, 1.9 mM KH_2PO_4, 1.1 mM K_2HPO_4; 5.5 mM glucose, 1.5 mM $CaCl_2$, 0.3 mM Mg SO_4 and 1 mM $MgCl_2$ (pH 7.4) at room temperature.

2.2. *Neutrophil protease inhibition*

The cell suspension was treated with 0.5 $ml \cdot ml^{-1}$ diisopropylfluorophosphate (DFP, Sigma, St. Louis, MO) for 5 min with occasional agitation. The cells were then sedimented at 1000 $\times$ *g* for 5 min. This procedure abolishes all serine protease activity [13].

2.3. *Homogenization by nitrogen cavitation*

The cell pellet was then rapidly dispersed at 2.5 $\times$ 10^7 cells $\cdot$ ml^{-1} in a homogenizing buffer containing 50 mM imidazole–HCl, 3 mM $MgCl_2$, 0.5 mM EGTA, 5% Ficoll 400 (Parmacia, Uppsala), 2 mM mercaptoethanol (pH 6.7) at 37°C. The cell suspension was placed in a 50-ml polyethylene tube with a cylindrical stir bar in a pre-cooled Parr cell disruption bomb (Parr Instruments, Moline, IL). The stirred cell mixture was equilibrated for 10 min at 400 lb/in^2 N_2 at 0°C and then lysed. The foamy layer was gently dispersed in a glass homogenizer. Ninety percent of the starting volume was recovered, and microscopic examination of the lysate revealed no intact cells. The homogenate was utilized in GTPase assays without further purification.

2.4. *Preparation of plasma membranes*

Purified plasma membranes were prepared from DFP-treated cells as in [14], and suspended in 50 mM imidazole-HCl, 2 mM β-mercaptoethanol, 3 mM $MgCl_2$, 0.1 mM EGTA, 5% Ficoll 400 (pH 7.4) at 4°C at a protein concentration of 1 $mg \cdot ml^{-1}$.

2.5. *Preparation of CHO-peptide and PMA solutions*

The peptide utilized here was the derivatized fluoresceinated hexapeptide (FLPEP) [15]. PMA was from Sigma. The peptide and PMA were stored at stock solutions at −20°C in DMSO. Aliquots were added to Gey's physiologic buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA) such that the peptide concentration was 3.4 $\times$ 10^{-6} M and PMA was 68 $\times$ 10^{-3} g/l. The final concentration of DMSO in the GTPase assay was 0.9 $\mu l \cdot ml^{-1}$, and an aliquot of Gey's-BSA buffer containing DMSO was added to control assay systems for GTPase activity in the absence of peptide or PMA.

2.6. *Measurement of levels of cAMP in human neutrophils*

cAMP levels in stimulated cells were measured with the RIA kit from NEN (Boston, MA). Cells (5 $\times$ 10^6 ml^{-1}) were equilibrated for 7 min at 37°C prior to stimulation with 10 nM FLPEP. After stimulation, aliquots of the cell suspension were withdrawn at timed intervals and transferred to a glass test tube heated to 150°C in a bath of ballotini beads (Atlas Chemical, San Diego, CA). The sample boiled within 2 s, inhibiting further alteration of the cAMP levels. The analysis from this point on essentially followed that in [16].

2.7. *GTPase assay*

The assay conditions were similar to those in [6]. The assay media contained 0.5 μM [γ-^{32}P]GTP (15–20 mCi/μmol, New England Nuclear, MA), 5 mM $MgCl_2$, 0.8 mM App(NH)p (Sigma), 4 mM creatine phosphate (Sigma), 51 units of creatine phosphokinase (Sigma), 2 mM β-mercaptoethanol, 0.1 mM EGTA, 50 mM imidazole–HCl (pH 6.7) at 37°C; 850 μl of this assay medium were transferred into a water jacketed 3 ml stirred plastic chamber at 37°C. To this was added 50 μl of a solution containing CHO-peptide (final concentration 100 nM) or an identical solution but in the absence of peptide. The assay was initiated by the addition of either an 800 μl aliquot of cell homogenate or plasma. In studies with PMA, a 25 μl aliquot of the drug was added to both the pre-equilibrating homogenate and to the assay media 60 s before initiation of the assay.

At 10- or 15-s intervals following the initiation of the assay, 100 μl aliquots were removed from the stirring chamber and immediately mixed with 0.1 ml 2.5% SDS (with stirring to terminate the

GTP hydrolysis). The aliquots were frozen on dry ice for 16 h. The extent of GTP hydrolysis in the thawed samples was determined as in [6], utilizing charcoal columns to adsorb nucleotides from each reaction mixture. Hydrolysed phosphate eluates were collected and analyzed for ^{32}P in a Searle liquid scintillation spectrometer with wide open counting windows. Background ^{32}P contaminating the [γ-^{32}P]GTP was subtracted from all values and usually fell in the range of 3–8% of total ^{32}P.

3. RESULTS

A representative plot of the time course of GTP hydrolysis in the presence and absence of the derivatized hexapeptide is shown in fig. 1. A clear activation of GTP hydrolysis is observed in the presence of peptide. In table 1 are summarized the data from 4 separate experiments utilizing cells from 4 different donors. Since the mean regression coefficients from activity plots plus and minus peptide were both very close to +1, and were not significantly different from one another, it can be concluded that the presence of the peptide produced a sustained simulation of GTPase activity of about 27% over the time course of the experiment. Furthermore, an apparent lag time for peptide stimulation of GTPase activity can be calculated from the extrapolated intersection of the activities in each set of data (table 1), yielding mean lag time of activation of 14.3 ± 6.9 s. The addition of PMA (1 $\mu g \cdot ml^{-1}$) to the homogenates 1 min prior to the initiation of the assay did not alter GTPase activity (table 1). In separate studies, we found that a PMA concentration of $\mu g \cdot ml^{-1}$ fully activated superoxide production after a lag time of about 60 s.

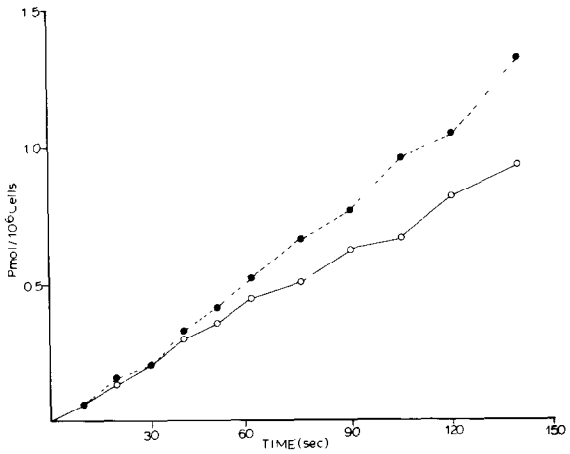

Fig. 1. Kinetics of 0.25 μM GTP hydrolysis in the presence (●) and absence (○) of 100 nM chemotactic hexapeptide by human neutrophil homogenates at 37°C.

The GTPase activity in purified plasma membranes is shown in fig. 2. Since GTP hydrolysis is observed in the presence of 0.25 μM GTP it can be

Table 1

Activities of neutrophil homogenate GTPase incubated with or without chemotactic hexapeptide (100 nM) or phorbol myristate acetate (1 $\mu g \cdot ml^{-1}$) at 37°C

Assay number	1		2		3		4		Mean ± SD	
Peptide	−	+	−	+	−	+	−	+	−	+
Activity (pmol Pi · min^{-1} · 10^6 $cells^{-1}$)	0.377	0.496	0.416	0.476	0.424	0.510	0.391	0.564	0.402 ± 0.019	0.512 ± 0.032 (Stimulation = 27%)
Regression coefficient	0.989	0.989	0.998	0.997	0.997	0.997	0.995	0.989	0.995 ± 0.035	0.0993 ± 0.004 P = NS
Lag time (s)	12		5		16		24		14.3 ± 6.9	
	No additions + PMA				+ Peptide					
Activity (pmol Pi · min^{-1} · 10^6 $cells^{-1}$)	0.302		0.299		0.378					
Regression coefficient	0.997		0.999		0.995					

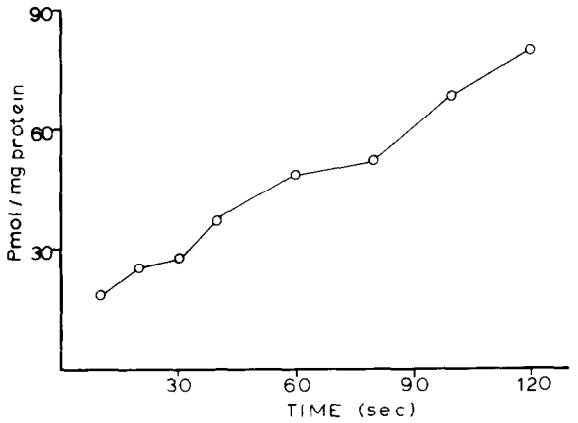

Fig. 2. Kinetics of 0.25 μM GTP hydrolysis by purified plasma membranes from human neutrophils at 37°C.

concluded that there is a low K_m GTPase activity in neutrophil plasma membranes, with a specific activity comparable with other cell types with hormone stimulatable GTPases [6,9,17].

No stimulation of this activity was apparent when the purified membranes were incubated with derivatized hexapeptide (not shown), indicating that the stimulatable activity may not be stable during subfractionation.

Finally, in order to compare cAMP accumulation and GTPase activation by CHO-pep the kinetics of intracellular cAMP activation were examined in experiments parallel to those in table 1. The results of this study are shown in fig. 3. Intracellular cAMP reached a maximum after about 10 s, and gradually declined toward basal values after about 2 min. These kinetics are very representative of steady-state intracellular levels on exposure to the peptide reported in [3] and [2].

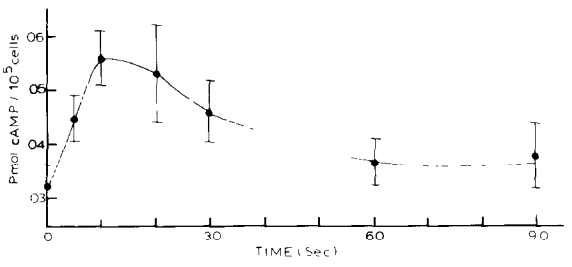

Fig. 3. Time course of intracellular cAMP concentration after exposure of intact human neutrophils to 100 nM chemotactic peptide at 37°C.

4. DISCUSSION

The neutrophil homogenates contain a high affinity GTPase which is stimulatable (+27%) by the addition of the chemotactic hexapeptide. The highly purified plasma membrane GTPase activity did not respond to the presence of CHO-peptide utilizing the subfractionation procedure in [14]. Unfortunately we cannot yet localize the stimulatable GTPase activity to the plasma membrane at this stage. Since the CHO-peptide leads to a rapid and transient elevation in the intracellular levels of cAMP, it is reasonable to assume that this is achieved, at least in part, by activation of the cyclase system, rather than solely by inhibition of phosphodiesterase activity. This notion is supported by the idea that, if anything, the transient elevation of intracellular Ca^{2+} following neutrophil activation is expected to increase PDE activity [18].

High affinity N-binding proteins have been suggested to be intimately associated with the *N*-formyl peptide as well as other receptors, since Gpp(NH)p-binding increases the peptide off-rate from its receptor [9]. If the *N*-formyl-peptide–adenylate cyclase system in human neutrophils is analogous to the myriad of other hormone stimulation adenylate cyclase systems, then the high affinity GTPase activity observed in this study within the neutrophil plasma membrane matrix could possibly serve as the terminating signal for modulation of cyclase activity by N-binding proteins.

The time course of GTPase activation by CHO-peptide is consistent with its role in cyclase modulation. The GTPase activation appears to persist after cAMP elevation following peptide addition to neutrophils. Thus, this GTPase activation would compete against the cyclase activation sequence and serve to modulate the rise in cAMP. PMA is a powerful activator of neutrophil granule release [2] and the respiratory burst [19]. This agent, however, does not elicit a rise in neutrophil intracellular cAMP [2], and is unable to alter neutrophil homogenate GPTase activity (table 1) after preincubation of the homogenate with 1 $\mu g \cdot ml^{-1}$ PMA for 60 s. We suggest that this observation is consistent with the view that GTPase activation occurs by ligand-specific receptor interaction, and is not secondary to neutrophil activation.

Finally, while the statistical analysis of the GTPase activation is consistent with a sustained activation preceded by a lag period, the high basal activity precludes an absolute conclusive discrimination between sustained and transient GTPase activation. A more thorough analysis awaits a purified membrane preparation possessing an activatable GTPase.

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