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REGULATION OF MEMBRANE ASSOCIATED PROTEIN KINASE C ACTIVITY BY GUANINE NUCLEOTIDE IN RABBIT PERITONEAL NEUTROPHILS

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Evidence is shown that protein kinase C is the major kinase which can phosphorylate histone H-1 in a membrane fraction prepared from rabbit peritoneal neutrophils. Addition of phorbol-12-myristate-13-acetate (PMA) (0.1μg/ml) or guanosine-5'-(3-O-thio)triphosphate (GTPγS) (10μM) to the membrane fraction results in an increase of the phosphorylation of histone H-1. To achieve this effect, calcium (20μM) is required for GTPγS but not for PMA. The effect of GTPγS, but not PMA is inhibited in membranes obtained from cells pretreated with pertussis toxin. The kinase activity is also enhanced by treatment of the membrane with 10μM of GppNHp or GTP but not with GDP, GMP, cGMP, ATP, ADP, AMP and cAMP. This is the first direct evidence that a GTP binding protein is involved in the activation of membrane associated protein kinase C.

There is now increasing evidence which indicates that a GTP binding protein (Gp) in the neutrophil is coupled to the chemotactic receptors and plays an important role in mediating many of the biological responses (1-9). Gp can be ADP-ribosylated with pertussis toxin (PT), treatment which results in the inhibition of various biological responses (1-9).

It has been suggested that, upon chemotactic activation, Gp stimulates a membrane associated phospholipase C which causes increases of levels of diacylglycerol (DG) and

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ABBREVIATIONS: DG, diacylglycerol; EGTA, ethylene glycol bis (β-amino ethylether)-N,N,N',N'-tetraacetic acid; Gp, pertussis toxin-sensitive GTP-binding protein in neutrophil; GppNHp, guanosine 5'-(β-imido) triphosphate; GTPγS, guanosine 5'-(3-O-thio) triphosphate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acids; IP_3, inositol triphosphate; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine; PT, pertussis toxin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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inositol triphosphate (IP_3) (8-10). IP_3 is responsible for the release of intracellular calcium (11,12) and DG is responsible for the activation of protein kinase C (13,14). Protein kinase C is an enzyme which requires calcium and phospholipid for its activation. In the presence of DG, the requirement of calcium is diminished. The tumor promoter, phorbol-12-myristate-13-acetate (PMA), is an analogue of DG. It can also activate the neutrophil through the triggering of protein kinase C (13-17). Biological responses induced by PMA are inhibited by a potent protein kinase inhibitor, 1-(5-iso-quinoline-sulfonyl)-2-methylpiperazine (H-7) (18-20). The nonhydrolyzable GTP analogue, guanosine-5'-(3-O-thiotriphosphate) (GTPγS), has recently been used to induce the degranulation of permeabilized neutrophils (21) and platelets (22) and to demonstrate the role of GTP-binding protein in activating phospholipase C in various systems including neutrophil membranes (10), blowfly salivary gland membranes (23) and liver membranes (24). We have been interested in studying the role of protein phosphorylation in neutrophil activation (9,16,17,25). In the neutrophil, protein kinase C appears to be much more active than cAMP-dependent protein kinase and calmodulin-dependent protein kinase (25). The purified protein kinase C uses only ATP but not GTP to phosphorylate its substrate (14). In this paper we test the possibility that GTPγS can activate the membrane associated protein kinase C in neutrophils.

Material and Methods

Partially purified protein kinase C was prepared as described (17). Pertussis toxin was a generous gift from Dr. John Munoz; H-7 from Seikagaku America Inc; Histone H-1 (Type IIIS), PMA, IP_3, phospholipase C (from Clostridium perfringens, P7776), f-Met-Leu-Phe, leupeptin, GppNHp, GTP, GDP, GMP, cGMP, ATP, and cAMP were obtained from Sigma; GTPγS from Boehringer-Mannheim; phosphatidylserine (PS) from Serdary Research Laboratories; [γ-^{32}P] ATP from New England Nuclear; phosphocellulose paper (P-81) and DEAE-52-cellulose from Whatman and the protein standards for SDS-PAGE from Pharmacia. Buffer A contained 10mM HEPES, 1mM EGTA, pH 7.0.

Rabbit peritoneal neutrophils were collected 4-16 hr after the intraperitoneal injection of 200-400 ml 0.1% glycogen in sterile saline. Subcellular fractions were prepared by a slight modification of the method described in (25). In brief, neutrophils ($2-10 \times 10^9$), were washed in Hanks' buffer (137 mM NaCl, 5 mM KCl, 0.7 mM KH_2PO_4, 10mM N-2-hydroxyethyl piperazine- N'-2-ethane sulfonic acid (HEPES), 17 mM $NaHCO_3$, pH 7.2). The cells were treated with diisopropylfluorophosphate (26), centrifuged and suspended in ice-cold washing buffer (11.6% sucrose containing 10mM HEPES, 1mM EGTA, pH 7.2) and washed 4-times with the washing buffer. They were resuspended in the same buffer and homogenized for 3 min with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 x g for 10 min to remove unbroken cells and nuclei and the postnuclear supernatant was centrifuged at 13,000xg for 10 min to remove granules. The post granular supernantant was then centrifuged at 100,000 x g for 1 hr to obtain the cytosol (supernatant) and the membrane (pellet) fractions. The membrane fraction was washed once with Buffer A, resuspended in Buffer A at a protein concentration of 3-5 mg/ml and used immediately.

In some experiments, cells (10^7cell/ml, 50ml) were treated with or without PT (500 ng/ml) at 37°C for 1 hr before being subjected to subcellular fractionation. Aliquots of control and PT-treated cells were tested for granule enzyme secretion induced by the chemotactic factor, f-Met-Leu-Phe as described (1). The PT-treatment completely inhibited granule enzyme secretion induced by f-Met-Leu-Phe, similarly to what has been reported (1). The inhibition is paralleled by ADP-ribosylation of a membrane protein of molecular weight of 41,000 (Gp) (9).

The kinase activity in the membrane fraction was determined by using histone H-1 as the substrate and analyzed by SDS-PAGE. The membrane fractions (60 μg to 180 μg protein) derived from the control and PT-treated cells, were first treated (in 120 μl Buffer A with leupeptin (100 μg/ml)) without or with H-7 (50μM) and (a) free calcium (20 μM) and GTPγS (10 μM or as indicated) or (b) PMA (0.1 μg/ml) with or without calcium (20μM) for 10 min at room temperature and then kept at 4°C for 30 min. Fifteen μl of the samples were transfered and mixed with a reaction mixture (30 μl) which contained 25 mM $MgCl_2$, 20 μM sodium orthovanadate, 5mM p-nitrophenylphosphate, 60 μM [γ-^{32}P] ATP (1.6-3 x 10^3 cpm/pmol) and with or without histone (1 mg/ml). The reaction was stopped by adding 50 μl of "SDS stopping solution" (9% SDS, 6% mercaptoethanol, 15% glycerol and a trace amount of bromophenol blue dye in 0.186 M Tris/HCl (pH 6.7)) and samples were analyzed by gel electrophoresis (15% acrylamide gel for detecting histone phosphorylation and 10% acrylamide gel for endogenous membrane protein phosphorylation) and autoradiography as described (25). The portion of the gel which contained membrane phosphoproteins or [^{32}P]-histone was cut out from the gel added to scintillation fluid and counted as described (25). The percent of [^{32}P]-ATP remaining after the reaction was determined by ascending thin layer chromatography on PEI-cellulose in 0.85M potassium phosphate-HCl pH 3.5 (31). The membrane-containing reaction mixture was stopped by 1 ml of 7% trichloroacetic acid and clarified by centrifugation. Aliquots of the supernatant (5 μl) were diluted with 0.5 ml of 0.85M potassium phosphate (pH 3.5) and 5 μl was spotted on PEI-cellulose plate (E. Merck) and analyzed by ascending chromatography and autoradiography (31). About 60-70% of the [^{32}P]-ATP was recovered and GTP γS (10 μM) had no effect on the preservation of ATP in the phosphorylation reaction (data not shown).

Tryptic fingerprints of phosphorylated histone H-1 were performed as described (28). Dried gel pieces containing histone H-1 were reswollen in 700 μl of 100mM ammonium bicarbonate pH 8.0, 1 mM dithiothreitol containing a trace amount of phenol red and 150 μg/ml of trypsin. Incubation was carried out for 24 hr at 37°C. Then the gel piece was removed, and the eluate, which contained 80% of the original radioactivity, was lyophilized. The residue was dissolved in 30 μl of electrophoresis buffer (15% (v/v) acetic acid/5% (v/v) formic acid, pH 1.9) containing a trace amount of basic fucsin and 20 μl was spotted on a cellulose plate (Eastman). Separation of phosphopeptides was performed with electrophoresis for 90 min at 400 V in the first dimension and ascending chromatography in n-butanol/pyridine/acetic acid/water, 40 : 30 : 6 : 24 (v/v), in the second dimension. Autoradiographs were obtained by using Quanta II intensifying screen at -80°C for 16 hr.

The phospholipase C activity in the membranes was measured by the method described by Smith et al. (8) with slight modification. The membranes (1 mg/ml, 100 μl) were preincubated for 2 min at room temperature in the presence of $MgCl_2$ (5 mM), [γ-^{32}P]ATP and 0.5 mM spermine to form ^{32}P-labeled phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5,-bisphosphate (PIP_2) (8). The membranes were then treated with $CaCl_2$ (20 μM) and GTPγS(10 μM) for 2 min at room temperature. Ten volumes of hexane/isopropanol/concentrated HCl (300:200:4) was added to the reaction mixture and the contents of ^{32}P-labeled PIP, PIP_2 were analyzed as described (9) by thin layer chromatography.

All the calcium concentrations described in this paper refer to the free calcium concentration estimated by using an apparent binding constant for calcium-EGTA of 7.61 x $10^6 M^{-1}$ (29). The location of [^{32}P]-histone in the gel was identified by Coomasie blue staining of the histone band. Protein concentration was determined by Lowry et. al. (30). All the data shown are representatives of two to four independent experiments. Assays were usually done in duplicate with an experimental error within ±7%.

Results and Discussion

In the presence of calcium (20 μM), addition of PMA (0.1 μg/ml) or GTPγ S (1μM to 16μM) to the membrane fraction resulted in an increase (40-70%) of the phosphorylation of several membrane proteins. The effect was most evident with the protein of molecular weight of 40,000 (Fig. 1a). To test whether the protein kinase C activity in the membrane was activated, histone H-1 was used as a substrate for the kinase. Fig. 1b shows that treatment of the membrane with GTPγ S or PMA stimulated phosphorylation of histone H-1 indicating that kinase activity was enhanced. To test whether GTPγ S had any direct effect on the protein kinase C, the effect of GTPγS on a partially purified

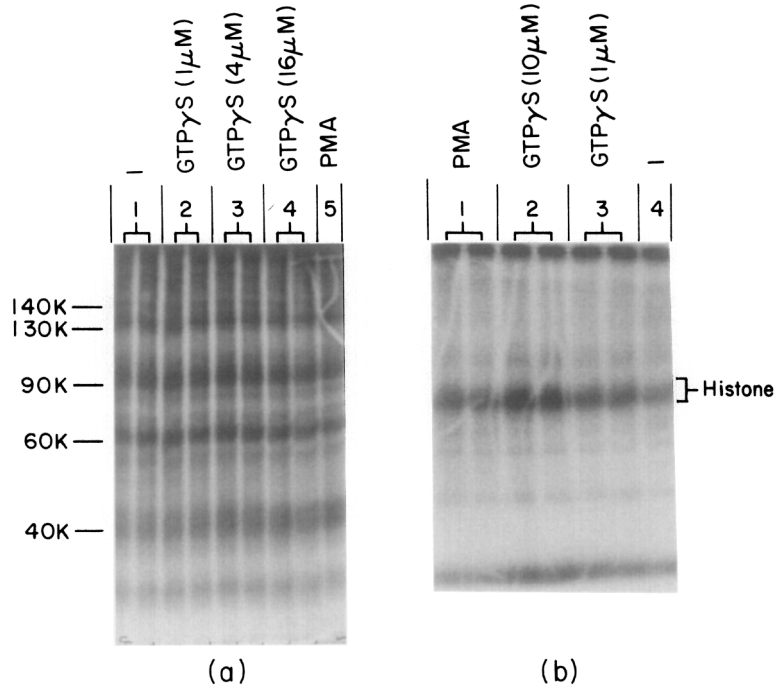

Figure 1. Autoradiographs showing the effect of GTPγ S and PMA on (a) the phosphorylation of membrane proteins (10% gel) and (b) on the phosphorylation of histone H-1 added to the membrane (15% gel).
Neutrophil membranes (1 mg protein/ml) were pretreated with or without GTP γS (various concentrations as indicated in the figure) or PMA (0.1μg/ml) in the presence of 20μM free calcium at room temperature for 10 min and then kept at 4°C for 30 min. Fifteen μl of the samples were transfered and mixed with a reaction mixture (30μl) which contains 25 mM $MgCl_2$, 20μM sodium orthovanadate, 5mM p-nitrophenylphosphate, 60μM [γ-^{32}P] ATP (10^3 cpm/pmol) without (a) or with (b) histone H-1 (1 mg/ml). After 10 min at room temperature, the reaction was stopped by "SDS-stopping solution" and the samples subjected to SDS-PAGE and autoradiography as described in the text. Fig. 1a; lane 1 (control), lanes 2-4 (GTPγ S pretreated, 1 μM, 4 μM and 16 μM respectively), lane 5 (PMA pretreated). Fig. 1b: lane 1 (PMA pretreated), lane 2,3 (GTPγ S pretreated. 10 μM and 1 μM respectively), lane 4 (control).

Table 1

Effects of GTPγS, PMA, calcium and H-7 on the histone H-1 kinase activity in rabbit peritoneal neutrophil membrane

Condition[a]				Membranes from untreated cells	Membranes from PT-treated cells
Ca	GTPγS	PMA	H-7		
-	-	-	-	1.00[b]	1.00[b]
-	-	+	-	1.44 ± 0.03 (p<0.01)	1.30 ± 0.04 (p<0.01)
-	+	-	-	1.17 ± 0.09 (N.S.)[c]	1.00 ± 0.04 (N.S.)
+	-	-	-	1.02 ± 0.07 (N.S.)	0.94 ± 0.04 (N.S.)
+	-	+	-	1.55 ± 0.06 (p<0.01)	1.47 ± 0.07 (p<0.01)
+	+	-	-	1.61 ± 0.03 (p<0.01)	1.08 ± 0.05 (N.S.)
+	-	-	+	0.26 ± 0.04	
+	-	+	+	0.40 ± 0.05	
+	+	-	+	0.27 ± 0.04	

a. Membranes from untreated and PT-treated cells were treated with PMA (0.1 μg/ml) or GTPγS (10μM) in the presence or absence of calcium (20 μM) and H-7 (50 μM) and tested for histone H-1 kinase activity as described in the text.

b. Each value represents the mean ± S.E. of at least three different experiments. The p values were calculated using paired sample t tests by comparing to control values. The control values for membrane from untreated cells and PT-treated cells are 15 ± 3.5 and 17 ± 1.7 pmol/min/mg protein respectively.

c. N.S. not significant

protein kinase C (17) was studied. No effect of GTPγS (up to 20μM) was observed but a higher GTPγS concentration (80 μM) gave a 10% inhibition (not shown). The effects of GTPγS (10 μM), in the presence and absence of calcium (20μM), using membranes from control and PT-treated cells were studied (Table I). The histone H-1 kinase activity was stimulated (61% ± 3%) by GTPγS in the presence of calcium. A similar effect (55% ± 6%) was observed with PMA (0.1 μg/ml). Stimulation of kinase activity by GTPγS but not by PMA required added calcium. The effect of GTPγS but not PMA was inhibited completely in membranes obtained from cells pretreated with PT, suggesting that a PT-sensitive GTP binding protein is involved. Both the basal activity and the stimulated kinase activity are strongly inhibited by H-7 (50μM), a potent inhibitor of cAMP-dependent protein kinase and protein kinase C (20-22) (Table 1).

In the presence of calcium (20μM), the effects of various nucleotides (10μM), IP_3 (10μM) and a phospholipase C preparation (2 unit/ml) on the histone kinase activity was studied. The kinase activity was stimulated by GTPγS (62% ± 5%), GppNHp (45% ± 4%) and GTP (30% ± 3%) but not by GDP, GMP, cGMP, ATP, ADP, and cAMp. Addition of phospholipase C to the membrane also stimulated the kinase activity (54% ± 4%) to about the same extent as GTPγS. IP_3 had no effect on the kinase activity (not shown).

To test whether the stimulation of histone H-1 phosphorylation with GTPγS-treated membrane is localized at similar or different site(s) than the phosphorylation seen with purified protein kinase C, two dimensional separation of phosphopeptides derived from histone H-1 after exhaustive digestion with trypsin was performed (Fig 2). It is clear that in both GTPγS and PMA treated membrane the phosphorylation of a peptide similar to that phosphorylated by an activated protein kinase C is observed. Essentially no phosphorylation of the histone H-1 was observed with unstimulated protein kinase C (not shown).

Histone H-1 is a very good substrate for protein kinase C (14). It can also be phosphorylated by cGMP-dependent or cAMP-dependent protein kinase but is a poor substrate for the various types of calmodulin-dependent protein kinases (31). We believe that the kinase activity we have studied here is not related to the cAMP, cGMP or calmodulin-dependent protein kinases because, cAMP or cGMP or calmodulin does not stimulate the phosphorylation of histone H-1 under our experimental conditions. A heat-stable inhibitor of cAMP-dependent protein kinase also has no effect on the phosphorylation of H-1 (not shown). Histone H-1 is most likely phosphorylated by a membrane associated protein kinase C because (a) protein kinase C is apparently the major histone H-1 kinase in the membrane, as demonstrated by DEAE-cellulose chromatography of Triton X-100 solubilized membrane fraction (not shown); (b) H-7, a potent inhibitor of protein kinase C, strongly inhibits the phosphorylation of histone H-1 (Table 1); (c) addition of phospholipase C to the membrane stimulates the phosphorylation; (d) PMA, an activator of protein kinase C, stimulates the phosphorylation of histone H-1 (Table 1) and (e) tryptic fingerprints of the phsphorylated histone H-1 indicate that a similar phosphopeptide is produced by both the membrane kinase and the partially purified protein kinase C (Fig 2). Apparently, the protein kinase C in the membrane is partially

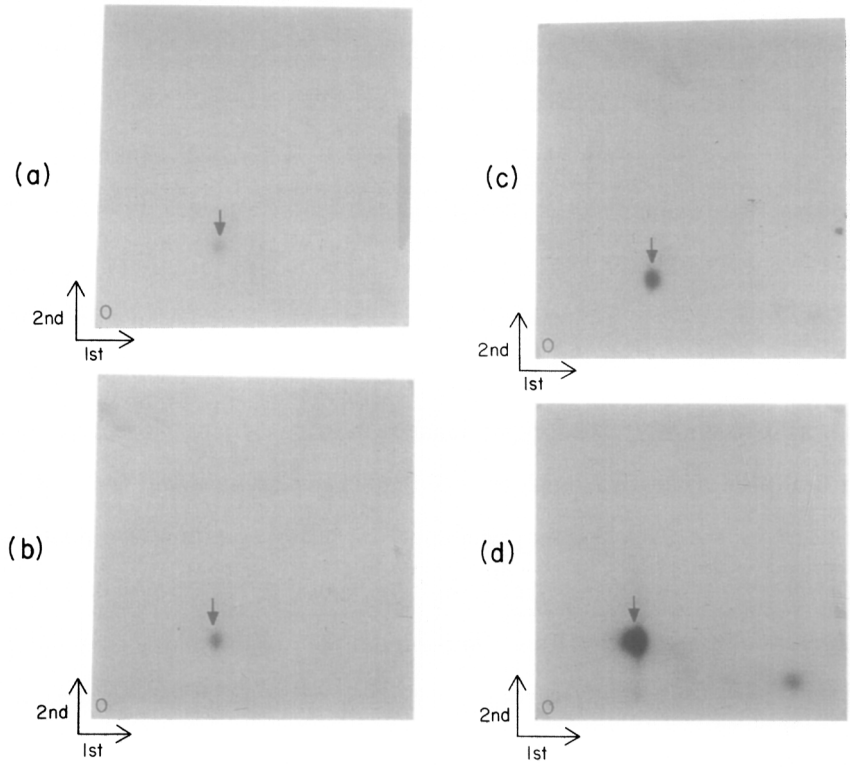

Figure 2. Autoradiographs showing tryptic fingerprints of phosphorylated histone H-1. Histone H-1 was phosphorylated in the presence of [γ-^{32}P] ATP by control membrane (a), GTP γS(10μM)-treated membrane (b), PMA-treated membrane (c) (as described in the legend of Fig 1) or by a partially purified protein kinase C (10μg) (d) prepared from the cytosol and activated by calcium (300 μM), phosphatidylserine (0.1 mg/ml) and PMA (0.1 μg/ml) (17). The samples were analyzed by SDS-PAGE and the [^{32}P]-histone H-1 bands were cut out of the dried gel and subjected to exhaustive digestion by trypsin. Phosphopeptides were spotted on cellulose plate (O designates origin) and were separated in two dimensions, first by electrophoresis in the horizontal dimension (negative pole right) and then by ascending chromatography in the vertical dimension. In (a), (b) and (c), the arrows indicate the major phosphopeptide of histone H-1 phosphorylated by membrane protein kinase corresponds to the one phosphorylated by a partially purified protein kinase C (d). Phosphorylation of this peptide is stimulated in GTPγS (b) or PMA (c) treated membrane when compared with the control (a).

activated. The basal activity is strongly inhibited by H-7 (Table 1). That the protein kinase C is partially activated may be due to the lipid environment of the membrane where the kinase is attached (32). Addition of exogenous PS has no effect on the histone H-1 phosphorylation (not shown). The requirements of micromolar concentration of calcium and GTPγS to stimulate membrane protein kinase C is very similar to the conditions required to stimulate the activity of phospholipase C in other systems, such as human neutrophil membranes (8,10), blowfly salivary gland membrane (23) and liver membrane (24). Phospholipase C activity in the membranes was analyzed by the method of

Table II

PIP_2 and PIP hydrolysis in isolated membranes

Phospholipid	Addition		[^{32}P]phospholipid remaining
	Ca	GTPγS	
PIP_2	-	-	1.00[a, b]
	-	+	1.07 ± 0.04
	+	-	0.81 ± 0.05
	+	+	0.39 ± 0.05
PIP	-	-	1.00[a, b]
	-	+	1.02 ± 0.05
	+	-	1.32 ± 0.18
	+	+	0.69 ± 0.09

[a] Membranes phospholipids were first labeled with [γ $^{-32}$P]ATP and then treated with $CaCl_2$ and GTPγS. The contents of labeled PIP_2 and PIP were analyzed by thin layer chromatography followed by autoradiography and densitometric scanning of the spots of TPI and DPI (8). Details are described in the text.

[b] Each value represents the mean ± S.E. of four different experiments.

Smith et al. (8). Addition of calcium (20 μM) and GTPγS (10 μM) to the membranes resulted in the loss of 61% ± 5% of the labeled PIP_2 and 31% ± 9% of the labeled PIP. (Table II). As has been discussed in reference (8), the decreases of labeled PIP_2 and PIP represent the activation of phospholipase C but not the activation of phospholipase A_2 or a phosphomonoesterase. The results suggest the activation of phospholipase C under the condition of protein kinase C activation described in this paper. Similar results have been reported in human neutrophil membranes (8, 10, 33).

The inhibitory effect of PT treatment on the GTPγS stimulated protein kinase C indicates that Gp is involved in the activation process. Whether Gp is able to interact directly with the membrane protein kinase C or with an inhibitor of protein kinase C is not known. Our data support the hypothesis that the membrane protein kinase C is related to the phospholipase C pathway. Obviously, further analysis of the phospholipid metabolism in the membrane and the use of purified protein components will be required

to elucidate the detailed pathway of the activation process. In neutrophils, PT treatment results in the inhibition of many biological responses stimulated by chemotactic factors. It remains to be shown whether the GTPγS stimulated membrane protein kinase C reaction reported in this paper, can be coupled to the chemotactic factor receptor.

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