Journal of Cellular Biochemistry 35:345–359 (1987)
Signal Transduction in Cytoplasmic Organization
and Cell Motility 31–45

Regulation of Inositol Phospholipid and Inositol Phosphate Metabolism in Chemoattractant-Activated Human Polymorphonuclear Leukocytes

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Binding of chemoattractants to specific cell surface receptors on polymorphonuclear leukocytes (PMNs) initiates a series of biochemical responses leading to cellular activation. A critical early biochemical event in chemoattractant (CTX) receptor-mediated signal transduction is the phosphodiesteric cleavage of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), with concomitant production of the calcium mobilizing inositol-1,4,5-trisphosphate (IP_3) isomer, and the protein kinase C activator, 1,2-diacylglycerol (DAG). The following lines of experimental evidence collectively suggest that CTX receptors are coupled to phospholipase C via a guanine nucleotide binding (G) protein. Receptor-mediated hydrolysis of PIP_2 in PMN plasma membrane preparations requires both fMet-Leu-Phe and GTP, and incubation of intact PMNs with pertussis toxin (which ADP ribosylates and inactivates some G proteins) eliminates the ability of fMet-Leu-Phe plus GTP to promote PIP_2 breakdown in isolated plasma membranes. Studies with both PMN particulate fractions and with partially purified fMet-Leu-Phe receptor preparations indicate that guanine nucleotides regulate CTX receptor affinity. Finally, fMet-Leu-Phe stimulates high-affinity binding of GTPγS to PMN membranes as well as GTPase activity. A Gα subunit has been identified in phagocyte membranes which is different from other Gα subunits on the basis of molecular weight and differential sensitivity to ribosylation by bacterial toxins. Thus, a novel G protein may be involved in coupling CTX receptors to phospholipase C. Studies in intact and sonicated PMNs demonstrate that metabolism of 1,4,5-IP_3 proceeds via two distinct pathways: 1) sequential dephosphorylation to 1,4-IP_2, 4-IP_1 and inositol, or 2) ATP-dependent conversion to inositol 1,3,4,5-tetrakisphosphate (IP_4) followed by sequential dephosphorylation to 1,3,4-IP_3, 3,4-IP_2, 3-IP_1 and inositol. Receptor-mediated hydrolysis of PIP_2 occurs at ambient intracellular Ca^{2+} levels; but metabolism of 1,4,5-IP_3 via the IP_4 pathway

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Received April 6, 1987; revised and accepted June 26, 1987.

requires elevated cytosolic Ca^{2+} levels associated with cellular activation. Thus, the two pathways for 1,4,5-IP_3 metabolism may serve different metabolic functions. Additionally, inositol phosphate production appears to be controlled by protein kinase C, as phorbol myristate acetate (PMA) abrogates PIP_2 hydrolysis by interfering with the ability of the activated G protein to stimulate phospholipase C. This implies a physiologic mechanism for terminating biologic responses via protein kinase C mediated feedback inhibition of PIP_2 hydrolysis.

Key words: inositol phosphates, G proteins, phospholipase C, leukocyte activation

Polymorphonuclear leukocytes (PMNs) function as mediators of host defense by ingesting and degrading microbial agents. A prerequisite for this activity is the migration of circulating PMNs into sites of inflammation in response to locally produced chemoattractants (CTX) [reviewed in 1]. Exposure of PMNs to chemoattractants in vitro stimulates both motility-related changes such as polarization (change in cell shape from round to triangular), cytoskeletal rearrangement, and directed chemotaxis as well as microbicidal activities such as release of lysosomal enzymes and production of superoxide anions [1,2]. The study of the biochemical processes involved in PMN activation was greatly facilitated by the development of synthetic oligopeptide chemoattractants such as N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) [3], which resemble bacterial N-formylated protein chemoattractants [4]. Characterization of the binding of fMet-Leu-[^{3}H]Phe to intact human PMNs demonstrated the presence of specific receptors for this peptide. The average equilibrium dissociation constant (K_D) for the interaction of fMet-Leu-Phe with intact PMNs was 22 nM; however, analysis of fMet-Leu-[^{3}H]Phe binding to PMN membranes showed that the fMet-Leu-Phe receptors exist in both high- (kD = 0.53 nM) and low- (kD = 24 nM) affinity states [5]. Presumably, ongoing metabolic processes in intact cells allow interconversion between high- and low-affinity receptors, thus permitting detection of only a single average-affinity state. Because guanine nucleotides were found to promote interconversion between high- and low-affinity formylpeptide receptors, interaction of this receptor with a guanine nucleotide binding (G) protein was proposed [6]. There is now substantial evidence that there is indeed a leukocyte CTX receptor:G protein complex which activates a polyphosphoinositide-specific phospholipase C [7]. The products formed by CTX receptor hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) result in the release of Ca^{2+} from intracellular stores (by inositol 1,4,5-trisphosphate) [reviewed in 8] and in protein kinase C activation (by 1,2-diacylglycerol) [reviewed in 9]. Synergy between these biochemical signals promotes cell activation [10]. The subsequent metabolism of 1,4,5-IP_3 can result in the formation of additional inositol phosphate products which may also have second messenger function [11]. This review will deal with recent advances in defining the regulation of inositol phospholipid and inositol phosphate metabolism in PMNs.

CHEMOATTRACTANT RECEPTORS ON LEUKOCYTE PLASMA MEMBRANES ARE COUPLED TO A POLYPHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C VIA A GUANINE NUCLEOTIDE REGULATORY PROTEIN

Initial evidence suggesting that the formylpeptide CTX receptor is coupled to a G protein was derived from studies in PMN particulate fractions in which the addition of guanosine di- or triphosphates reduced the percentage of high-affinity fMet-Leu-Phe binding sites [6]. Since the total number of receptors was not affected by GTP or

its analogues, it was deduced that GTP caused conversion of high-affinity receptors to a low-affinity state [6,12]. Furthermore, since studies in both intact PMNs and in PMN membranes provided evidence that the CTX receptor did not directly activate adenylate cyclase, it was postulated that another effector enzyme system was the target of the receptor:G protein complex [13,14]. Experiments in numerous laboratories showed that pretreatment of PMNs or monocytes with pertussis toxin (PT) (which ADP-ribosylates and inactivates certain G proteins) [15] inhibited CTX-induced phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, IP_3 formation, calcium mobilization, arachidonic acid release, and cellular activation [14,16–23]. These studies thus provided evidence that the receptor:G protein complex was coupled to a phospholipase C rather than to adenylate cyclase. This hypothesis was more directly supported by studies with purified PMN plasma membranes in which fMet-Leu-Phe was shown to stimulate phosphodiesteric cleavage of PIP_2 or phosphatidylinositol 4-phosphate (PIP) only in the presence of GTP [24]. Nonhydrolyzable analogues of GTP also stimulated PIP and PIP_2 hydrolysis in PMN membranes [25,26]. Both CTX receptor plus GTP, or GTPγS-mediated PIP_2 hydrolysis occurred at the ambient intracellular calcium concentration ($[Ca^{2+}]_i$) of 100 nM, whereas direct activation of phospholipase C by Ca^{2+} in PMN membranes required concentrations of 0.1–1 mM. It was therefore postulated that CTX receptors activate an associated G protein via the substitution of GTP for GDP. The activated G protein then activates a polyphosphoinositide-specific phospholipase C by enabling the enzyme to hydrolyze PIP_2 at ambient intracellular concentrations of Ca^{2+} (Fig. 1). Additional evidence for a chemoattractant receptor-G protein interaction was provided by studies in which it

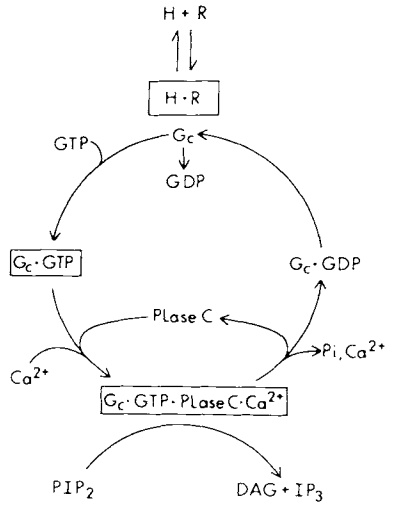

Fig. 1. Model for receptor-mediated stimulation of phospholipase C by an activated G protein complex. The boxed elements represent the activated forms of the indicated components. Binding of Ca^{2+}-mobilizing hormone (H) or chemoattractant to its receptor (R) produces an activated hormone-receptor complex (H·R). This complex induces the exchange of a bound GDP on a CTX receptor-associated protein (G_c), yielding an activated G protein species (G_c·GTP). Our data suggest that activated G_c interacts with polyphosphoinositide-specific phospholipase C (PLase C) enabling it to express activity at physiological Ca^{2+} concentrations. The activated phospholipase cleaves PIP_2 into 1,2-diacylglycerol (DAG) and inositol trisphosphate (IP_3). The (G_c·GTP·PLase C·Ca^{2+}) complex is inactivated by the hydrolysis of the bound GTP, producing the inactive G_c·GDP. (Adapted from Smith et al [25].)

was shown that preincubation with fMet-Leu-Phe enhances the rate of binding of radiolabeled GTP analogues to PMN membranes [27,28] as well as hydrolysis of GTP [28–30]. The GTPγS binding sites in PMN membranes displayed a dissociation constant of 20 nM [27], which is in close agreement with the affinity of purified G proteins for GTPγS [31]. The addition of guanosine, but not the corresponding adenosine di- and triphosphates, blocked GTPγS binding, further suggesting association with a G protein [27]. Recently, direct physical evidence for association of the formylpeptide receptor with a G protein was obtained, when a partially purified receptor preparation from the dibutryl cAMP-differentiated myeloid HL60 cell line was found to contain a 40-kD substrate for ADP ribosylation by PT [32]. Furthermore, addition of GTPγS to liposomes containing the partially purified CTX receptor with noncovalently bound radiolabeled ligand enhanced the rate of ligand dissociation [32]. Therefore, GTP can modulate the affinity of a partially purified CTX receptor for ligand, as had been previously demonstrated in studies using whole PMN particulate fractions [6].

Several lines of evidence now also support the view that the G protein associated with chemoattractant receptors may represent a new addition to the family of previously described G proteins which include G_i, G_s, G_o, transducin, and others [see refs. 15 and 33 for recent reviews]. First, PMN membranes contain a ca 40-kD substrate for both PT and choleratoxin (CT), and the degree of ribosylation by CT is inhibited by increasing concentrations of GTP [34]. In contrast, ribosylation of a 43-kD CT substrate in PMN membranes, presumed to represent G_s, is enhanced by GTP [34]. Experiments designed to study the role of a CT substrate in chemoattractant receptor:G protein mediated PIP_2 hydrolysis have been problematic since increases in cAMP levels can also inhibit PIP_2 hydrolysis and other PMN responses [7,16,35]. Since fMet-Leu-Phe binding was not altered by agents that increase cAMP [36], the role of the CT substrate in receptor affinity was investigated. Treatment of PMNs with CT resulted in a 42 ± 10% reduction of fMet-Leu-Phe binding to membrane high-affinity sites and also reduced by 44 ± 11% the ability of Gpp(NH)p to convert high-affinity to low-affinity sites [34], whereas both of these parameters were more dramatically altered by treatment with PT [29,34]. The relative inefficiency of CT vs PT inhibition of these responses correlates with the fact that only incomplete ribosylation of the CT substrate occurs in whole cells, which is probably due to prohibitively high intracellular concentrations of GDP and/or GTP [34]. These studies thus provide functional evidence that the G protein which is coupled to the fMet-Leu-Phe receptor is a substrate for both pertussis and choleratoxins. Transducin is also ADP ribosylated by both toxins; however, the molecular weight of the alpha subunit of transducin is lower (ca 39 kD) than the substrate in PMNs. The fact that the majority of the PT substrate in PMN membranes is immunochemically distinct from transducin, G_o, and G_i [37] provides further evidence that the PT/CT-ribosylated substrate in PMN membranes represents a unique G protein (referred to as G_c for chemoattractant, phospholipase C related).

Recent studies on G proteins purified from HL60 cells showed that two proteins of ca 40 kD are resolved as closely spaced doublets on SDS-PAGE (Fig. 2). Since both of these proteins are ADP-ribosylated by PT, and the upper band comigrates with G_i, the presence of an additional lower band suggests a previously undescribed G protein, presumably G_c [38]. Additionally, genetic evidence for the presence of a unique G protein has also been obtained in our laboratory. Using a cDNA library

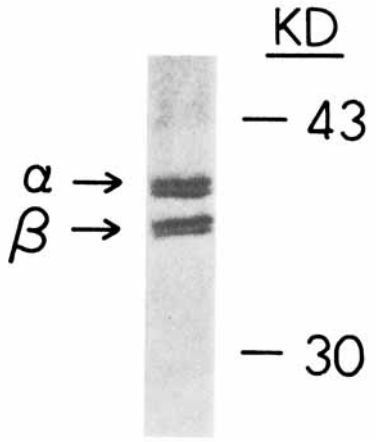

Fig. 2. SDS-PAGE of G proteins purified from HL60 cell membranes. GTP-binding proteins were purified from HL60 cells using chromatography on Ultragel AcA34, Heptylamine-Sepharose, and DEAE-Fractogel. Presented is a Coomassie blue stain of an SDS-polyacrylamide gel of the pooled pertussis toxin-substrates. Both components of the 40-kD doublet are ADP-ribosylated by pertussis toxin (not shown).

prepared from dibutyrl cAMP-differentiated HL60 cells, a cDNA clone was identified which encodes a novel G protein. The derived protein sequence is most closely homologous with the bovine brain G_i α-subunit but contains 5′ and 3′ noncoding regions distinct from all known G α-subunit cDNAs [39]. These data collectively suggest that G_c couples CTX receptors to phospholipase C in myeloid cells. However, a recent report [40] showed that partially purified preparations of G_o and G_i reconstituted CTX receptor-mediated activation of phospholipase C in membranes prepared from HL60 cells which had been inactivated by pretreatment with PT. Although this study showed that G_i or G_o can affect coupling of the CTX receptor to phospholipase C, in the absence of highly purified preparations of G_c and the other G proteins, the relative efficiency of coupling by these G proteins cannot be compared.

Additional studies in the neutrophil have provided evidence that G proteins may also be involved in secretion of azurophilic granule enzymes [41], receptor-coupled, phospholipase A_2-mediated arachidonic acid release [16,18], and activation of the NADPH oxidase enzyme system [42,43]. There is evidence that G proteins are coupled to phospholipase A_2 in some systems [reviewed in 33]; however, the possibility that arachidonate production is secondary to phospholipase C-mediated PIP_2 hydrolysis and Ca^{2+} mobilization in PMNs has not been ruled out [16,18]. The exact locus of the guanine nucleotide effect on NADPH oxidase activation in a cell-free system was not determined [42,43]. This effect could also be secondary to G protein-mediated PIP_2 hydrolysis occurring in the membrane preparation, since the 1,2-diacylglycerol (DAG) formed would be expected to promote activation of protein kinase C [44], which closely correlates with stimulation of the respiratory burst in PMNs [45].

PATHWAYS OF 1,4,5-IP_3 METABOLISM IN PMNs

The products formed via phospholipase C-mediated hydrolysis of PIP_2 include DAG, which activates protein kinase C [reviewed in 9], and 1,4,5-IP_3, which can

mediate the release of Ca^{2+} from intracellular stores in a variety of cell types [reviewed in 8], including PMNs [46]. The levels of 1,4,5-IP_3 generated after fMet-Leu-Phe stimulation of rabbit PMNs are sufficiently high (ca 1 μM) to release intracellular Ca^{2+} [47]. The 1,4,5-IP_3 isomer is degraded in many tissues by the selective action of a 5'phosphomonoesterase to produce 1,4-IP_2 [48–50]. More recently it has been shown that 1,4,5-IP_3 can also be phosphorylated via an ATP-dependent 3'kinase to form inositol 1,3,4,5-tetrakisphosphate (IP_4) [51]. IP_4 is then converted to 1,3,4-IP_3 by a 5-phosphomonoesterase [52–58]. Metabolism of 1,4,5-IP_3 via these pathways probably serves to regulate cytosolic calcium levels in activated cells, since 1,4-IP_2 does not mobilize Ca^{2+} [59], whereas conversion to IP_4 may promote Ca^{2+} influx [60]. With the advent of HPLC anion exchange systems capable of resolving isomeric forms of the inositol phosphates [52,61,62], it has also been recognized that different isomers of IP_2 and IP_1 can be formed in response to hormonal stimulation [56,62–66]. We therefore recently performed a detailed analysis of inositol phosphate metabolism in fMet-Leu-Phe-stimulated PMNs using HPLC systems capable of resolving isomers of IP_1, IP_2, and IP_3. In addition, disrupted cells were used to study metabolic pathways by which each of the IP_3 isomers are converted to free inositol.

In extracts from [^{3}H]-myo-inositol-labeled unstimulated PMNs, the major inositol phosphate products present were IP_1, 1,4-IP_2, and 1,4,5-IP_3 (Fig. 3). Chromatography on a different gradient system designed to resolve the IP_1 isomers [62,65] showed that in resting cells, the majority of the IP_1 eluted in a peak corresponding to 1- and/or 3-IP_1 which co-elute in this system [65]. After stimulation with 1.0 μM fMet-Leu-Phe, 1,4-IP_2, 1,4,5-IP_3, and IP_4 were elevated above background by 5 sec, whereas there was a definite lag (ca 15 sec) before IP_1 (predominantly the 4-IP_1

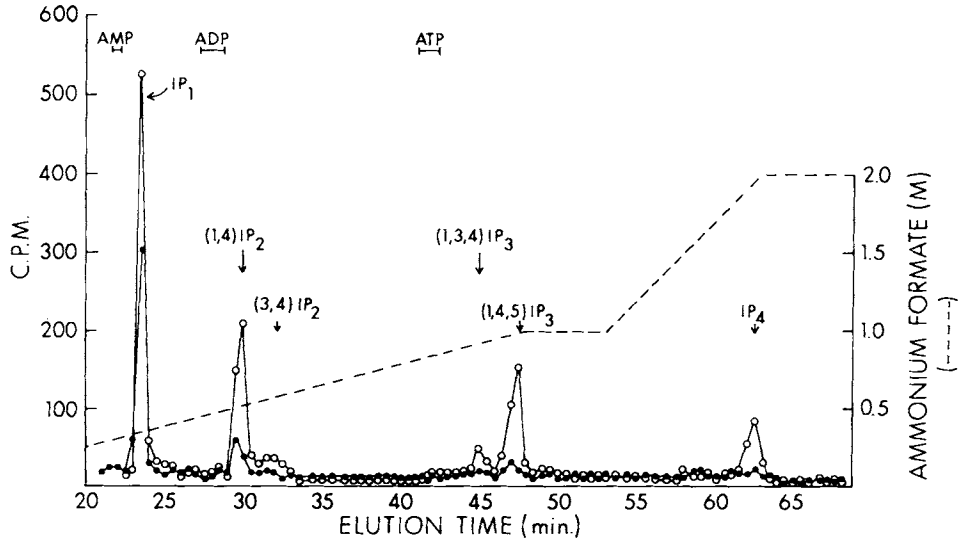

Fig. 3. HPLC analysis of inositol phosphates in human PMNs. Trichloroacetic acid (TCA)-soluble material from ^{3}H-myo-inositol-labeled PMN [25] incubated with buffer (●) or 1.0 μM fMet-Leu-Phe (○) for 15 sec was analyzed by anion-exchange chromatography as described [66]. A mixture of unlabeled adenine nucleotides (standards) was added to each sample; absorbance was monitored at 254 nM.

isomer), 3,4-IP_2, or 1,3,4-IP_3 levels began to increase (Fig. 4). Total IP_2, IP_3, and IP_4 levels remained elevated for ca 60 sec after activation, and then declined to near basal values by 5–10 min. As has been reported previously [18,68], IP_1 production is relatively prolonged and was still elevated at 5 min after stimulation. These studies were done in the presence of $LiCl_2$ (20 mM), which has been reported to slow the degradation of 1-IP_1, 1,4-IP_2, and 1,3,4-IP_3 [49,56,64,69,70] in some cell types; however, the pattern of inositol phosphate isomer formation in PMNs was not markedly different when the cells were stimulated in the absence of $LiCl_2$ (not shown).

The stepwise conversion of 1,4,5-IP_3 to IP_4 and then 1,3,4,-IP_3 which has been shown to occur in vitro is reflected in the order of appearance of these compounds in chemoattractant stimulated myeloid cells; ie, the rise in 1,4,5-IP_3 and IP_4 is most rapid (2–5 sec), whereas 1,3,4-IP_3 appears only after an initial lag period ([47,66,71,72] and Fig. 4). To determine the route of IP_4 and 1,3,4-IP_3 formation in PMNs and to define the pathways of IP_3 degradation, radiolabeled inositol phosphate standards were incubated with cell lysates, and the metabolic products formed were measured by HPLC. Incubation of ^{3}H-IP_4 (0.5 μM) with PMN sonicates (Fig. 5A) resulted in the formation of 1,3,4-IP_3 and an IP_2 isomer which we previously identified as 3,4-IP_2 on the basis of the IP_1 products formed after base hydrolysis [65]. In stimulated PMNs, the 3,4-IP_2 isomer appeared only after an initial lag, concomitant with 1,3,4-IP_3 (Fig. 4). These studies therefore confirm that in PMNs the 1,3,4-IP_3 isomer is derived from IP_4 via a 5-phosphatase [52,73], and further show that the major route of 1,3,4-IP_3 degradation in intact PMNs occurs via a 1-phosphatase to form 3,4-IP_2 [65–66]. Isomers of IP_2 were also formed after hormonal stimulation of liver [56], parotid gland [54], and fibroblasts [63], but the second IP_2 isomer (ie, other than 1,4-IP_2) was not identified. More recently, however, degradation of 1,3,4-IP_3 to 3,4-IP_2 has been demonstrated in extracts from rat liver [74] and calf brain [75].

Incubation of ^{3}H-1,4,5-IP_3 with PMN sonicates in the presence of ATP resulted in formation of both 1,4-IP_2 and IP_4 by 5 min (Fig. 5B); by 30 min after incubation both 1,3,4-IP_3 and 3,4,-IP_2 were also present (Fig. 5C). Thus, in PMNs IP_4 is formed via phosphorylation of 1,4,5-IP_3, as has been shown in other tissues [51]. Dephosphorylation of the 1,4- and 3,4-IP_2 isomers was also studied. After incubating PMN sonicates for 60 min with ^{3}H-1,4-IP_2 (0.5 μM), the major IP_1 isomer formed was 4-

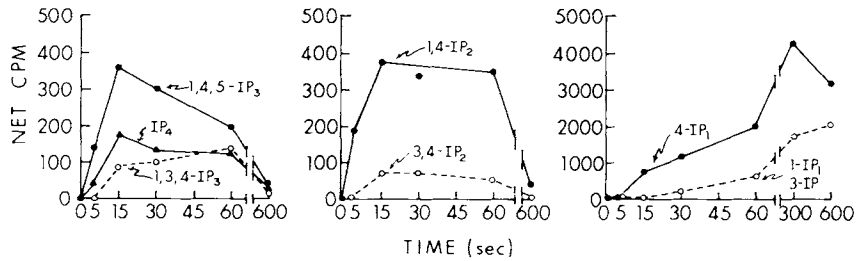

Fig. 4. Kinetics of inositol phosphate isomer formation in PMNs. TCA-soluble material from [^{3}H]-myo-inositol-labeled PMNs stimulated with 0.1 μM fMet-Leu-Phe was analyzed by HPLC using an ammonium formate gradient to separate isomers of IP_2 and IP_3 or an ammonium phosphate gradient to separate isomers of IP_1 [65,66]. Values shown are from an individual donor. Net cpm indicate cpm in stimulated cells minus cpm in control (buffer only) cells.

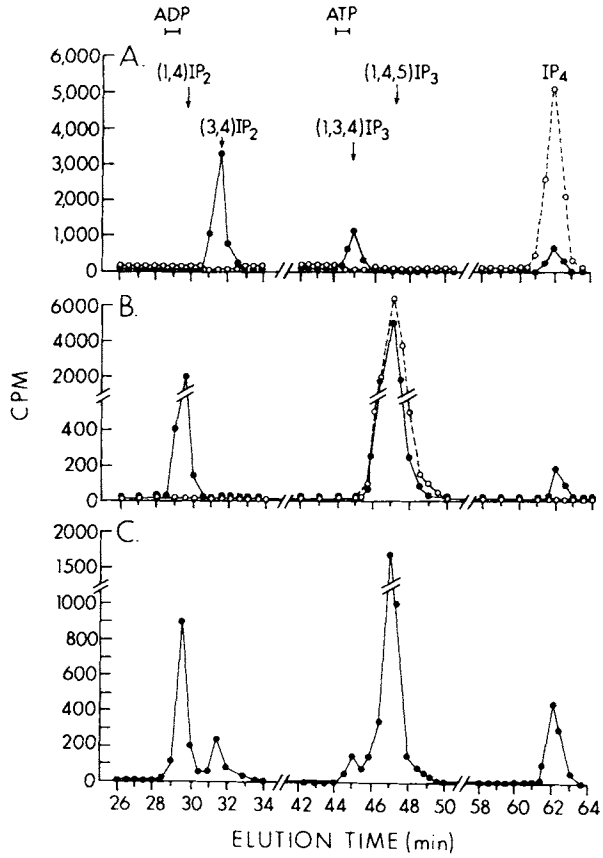

Fig. 5. Metabolism of [^{3}H]-1,4,5-IP_3 and [^{3}H]-IP_4 by PMN sonicates. Sonicates of human PMNs were incubated with 0.5 μM [^{3}H]-IP_4 and reactions were stopped with 10% TCA (final concentration) at time zero (○---○) or 15 min (●---●) **(A)**. In **B** and **C**, PMN sonicates were incubated with 0.5 μM [^{3}H]-1,4,5-IP_3 in Hepes/Tris (pH 7.5) with 10 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 3 mM 2,3-diphosphoglycerate. Reactions were stopped at time zero (○---○), 5 min (●---●) **(B)**, and 15 min (●---●) **(C)** with 10% TCA and analyzed by HPLC as described (see Fig. 3, legend). (Adapted in part from Dillon et al [65].)

IP_1 (ca 99% of total cpm in IP_1 peaks), whereas 3,4-IP_2 was degraded to only the 3-IP_1 isomer [66]. Collectively, these studies provide evidence that the action of 1,3,4, and 5 phosphatases is required for full recycling of the higher inositol phosphates to free inositol in PMNs (Fig. 6). The relative specificity of each of these phosphatases for the different inositol phosphate substrates in PMNs is not known; however, a highly purified platelet 5-phosphomonoesterase hydrolyzes both 1,4,5-IP_3 and IP_4 [73]. Furthermore, a partially purified inositol polyphosphatase activity has been identified in calf brain extracts which removes the 1-phosphate from 1,4-IP_2 and 1,3,4-IP_3, but does not dephosphorylate 1,3,4,5-IP_4, 1,4,5-IP_3, or 1-IP_1 [75]. The inositol polyphosphatase was further distinguished from an inositol monophosphate phosphatase activity, which, unlike the polyphosphatase, was stable to heating at 70°C for 30 min [75]. The inositol monophosphate phosphatase from calf brain dephosphorylates both 1-IP_1 [69,75] and 4-IP_1 [75], but it is not yet clear whether this enzyme can also utilize other inositol monophosphates (ie, 3-IP_1) as substrates.

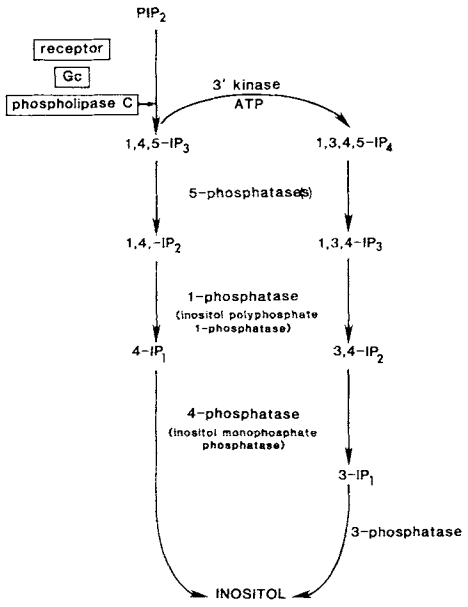

Fig. 6. Pathways of 1,4,5-IP_3 metabolism in PMNs. A PIP_2-specific phospholipase C can be activated via a G protein (G_c) coupled to chemoattractant receptors. The initial products formed are 1,4,5-IP_3 and 1,2-diacylglycerol (not shown). 1,4,5-IP_3 is metabolized via the indicated pathways to free inositol. Substrate specificities for each phosphatase are described in the text (enzyme names in parentheses are from Inhorn et al [75]). (Adapted in part from Dillon et al [66].)

There have been preliminary reports on characterization of an IP_3-specific 5-phosphatase activity from guinea pig [76] or rabbit [77] neutrophil cytosol; however, more detailed information is not yet available. Characterization of the various phosphatases involved in IP_4 and IP_3 degradation in PMNs will be necessary in order to understand how the levels of these second messengers are regulated after activation.

REGULATION OF INOSITOL PHOSPHATE FORMATION IN PMNs

In PMN plasma membranes stimulated with fMet-Leu-Phe and GTP [24,25] or with GTP analogues [25,26], 25–35% of radiolabeled PIP_2 was rapidly hydrolyzed. In intact cells, the extent of PIP_2 hydrolysis is similar to that seen in membranes (ca 20% of total cpm) [14], and the peak PIP_2 loss or IP_3 production occurred by 5–15 sec in both whole cell and membrane systems [14,23,25,67,78]. It appears that PIP_2 hydrolysis is limited in both magnitude and duration of the response, since no further loss of radioactivity is measurable by 30–60 sec [25,67,78]. PIP_2 is regenerated via plasma membrane PI and PIP kinases [reviewed in 79] which are sensitive to Mg^{2+} and polyamine concentrations [80], and may be regulated by protein kinase C [27,81]. Thus, a portion of the PI loss seen in intact fMet-Leu-Phe stimulated cells [67,82] occurs secondary to PIP_2 hydrolysis [reviewed in 58,79]. Collectively, these results imply a tightly regulated system for control of production and metabolism of the phosphoinositides. Some of the control mechanisms which have been delineated are discussed in the following sections.

Role of Protein Kinase C

Studies in several cell types have shown that both PIP_2 hydrolysis [83,84] and IP_3 production [84–89] are attenuated by phorbol myristate acetate (PMA)-induced activation of protein kinase C. Presumably, this effect is also reflected physiologically by increased DAG levels formed via the hydrolysis of phosphoinositides or other membrane phospholipids [90–92] in activated cells. In PMNs, pretreatment with active phorbol esters did not affect fMet-Leu-Phe binding to intact, differentiated HL60 cells [89], or to PMN plasma membrane preparations [27]. Pretreatment of intact PMN with PMA also did not affect fMet-Leu-Phe-stimulated binding of non-hydrolyzable GTP analogues to membrane preparations [27,28], suggesting that G protein:CTX receptor coupling was still intact. However, PMA treatment blocked GTPγS-stimulated phospholipase C activation, suggesting that protein kinase C can act to block the coupling of the activated G protein to phospholipase C (Table I). Although the exact locus of PMA-mediated inhibition of PIP_2 hydrolysis in PMNs remains to be established, one possible mechanism would involve protein kinase C-mediated phosphorylation of the α-subunit of G_c, as has been reported for $G_{i\alpha}$ [93]. Since Ca^{2+}-induced PIP_2 hydrolysis was equivalent in membranes from control or PMA-treated cells [27,94], the phospholipase C itself was probably not affected.

In platelets, PMA can also act to control IP_3 levels by promoting the conversion of cytosolic 1,4,5-IP_3 to IP_2 [95]. The mechanism for enhanced 1,4,5-IP_3 breakdown was elucidated in a recent study by Connolly et al [96], using the 5-phosphomonoesterase purified from platelets. Protein kinase C phosphorylated the 5-phosphatase, with the Vmax of the phosphorylated enzyme approximately threefold that of the native enzyme [96].

Pertussis toxin (PT) acts to block CTX-receptor coupling at a locus different from the PMA inhibition. Pretreatment of cells with PT, which abrogates fMet-Leu-Phe-induced IP_3 formation and biologic responses [7,16–23], did not affect PIP_2 hydrolysis stimulated by GTPγS (Table I). In contrast, PT treatment attenuated fMet-Leu-Phe-induced GTPγS binding to PMN plasma membranes [27], guanine nucleotide regulation of receptor affinity [29,34], and activation of phospholipase C by the CTX receptor (Table I). Pertussis toxin-mediated ADP-ribosylation of G_c therefore appears to prevent efficient coupling of the CTX receptor to G_c.

TABLE I. Effect of Pertussis Toxin (PT) or Phorbol Myristate Acetate (PMA) on G-Protein-Mediated PIP_2 Breakdown*

	Percent [^{32}P]PIP_2 remaining		
Addition	Buffer	PT	PMA
Buffer	100	100	100
10 μM GTP + 0.1 μM fMet-Leu-Phe	70 ± 1	97 ± 2	N.D.[a]
100 μM GTPγS	72 ± 3	76 ± 3	100 ± 5
1 mM $CaCl_2$	60 ± 8	65 ± 3	50 ± 3

*Plasma membranes isolated from PMNs which were pretreated at 37°C with either buffer (HEPES-buffered Hanks' balanced salt solution, pH 7.4), PT (1 μg/ml for 90 min), or PMA (10 ng/ml for 5 min) were incubated with ^{32}P-ATP for 60 sec to label the PIP_2 [24]. Buffer containing 1 μM $CaCl_2$, or buffer plus the indicated additions were then incubated with the labeled membranes for an additional 60 sec. Reactions were stopped with chloroform:methanol and phospholipids were extracted and assayed by affinity chromatography as described [24]. Values represent the mean percentage ± SEM (n = 3–6) of [^{32}P]PIP_2 compared with samples incubated with buffer only. (Adapted from Smith et al [27].)
[a]Not determined.

Role of Ca^{2+}

Purified cytosolic phospholipase C from platelets can utilize both PI and PIP_2 as substrates with hydrolysis of PI being favored at higher Ca^{2+} levels [58]. Unlike PIP_2 hydrolysis, the transient loss of radioactive PI in fMet-Leu-Phe-stimulated intact PMNs is dependent upon extracellular Ca^{2+} [67,82]. Loss of PI may therefore reflect, at least in part, the action of a calcium-sensitive phospholipase C. However, when plasma membranes prepared from [^{3}H]-inositol-labeled PMNs were incubated with 1 mM $CaCl_2$, [^{3}H]-PIP and [^{3}H]-PIP_2, but not [^{3}H]-PI levels, were reduced; and [^{3}H]-IP_3, and [^{3}H]-IP_2, but not [^{3}H]-IP_1 levels, were increased [24,25,40]. These results suggest that the phospholipase C in PMN membranes selectively hydrolyzes polyphosphoinositides. However, a phospholipase C activity in rat peritoneal PMN lysates hydrolyzed both PI and PIP_2 [97]. Together, these results raise the possibility that PI hydrolysis in PMNs may be mediated via a distinct, calcium-sensitive phospholipase C which does not copurify with the plasma membrane fraction. Without chromatographic methods to separate 1-IP_1 from the other IP_1 isomers, it is difficult to determine the proportion of PI loss that results from phospholipase C hydrolysis vs PI kinase-mediated conversion to PIP in intact cells. However, since the 4-IP_1 isomer predominates in stimulated PMNs, most IP_1 must be derived via the sequential dephosphorylation of 1,4,5-IP_3 and 1,4-IP_2 [66].

As already noted, the Ca^{2+} requirement for polyphosphoinositide-specific phospholipase C activation in PMN membranes is lowered to physiologic levels (0.1 μM) via association of the enzyme with an activated G protein (see Fig 1). This model is further supported by studies in intact PMNs [18,66,68] or HL60 cells [72] stimulated by fMet-Leu-Phe, where IP_3 formation occurs even when the associated rise in cytosolic Ca^{2+} is prevented by prior depletion of $[Ca^{2+}]_i$ stores (see Fig. 7). However, there is a minimal Ca^{2+} requirement for phospholipase C activation, since lowering cytosolic Ca^{2+} to $\leqslant$ 10 nM by loading with high concentrations of the

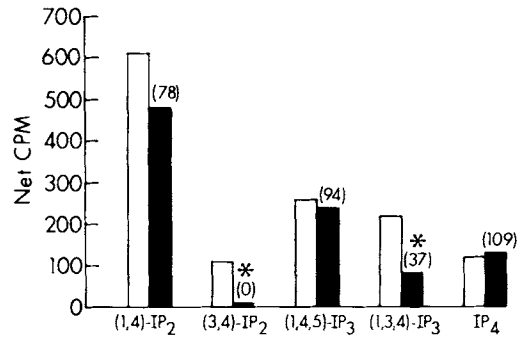

Fig. 7. Production of 1,3,4-IP_3 and 3,4-IP_2 in CTX-stimulated PMNs requires elevated $[Ca^{2+}]_i$. PMNs labeled overnight with ^{3}H-myo-inositol were suspended in buffer containing 1.2 mM calcium (open bars) or in buffer with 2 mM EGTA (shaded bars). Cells were prewarmed for 5 min before adding buffer (open bars) or 0.2 μM ionomycin (shaded bars). After an additional 5 min, the cells were stimulated with buffer or 1.0 μM fMet-Leu-Phe; reactions were then terminated at 30 sec with TCA and analyzed by HPLC as described [66]. Experiments with ^{3}H-inositol-labeled Quin-2-loaded cells confirmed [68,72] that the EGTA/ionomycin pretreatment completely abrogated the fMet-Leu-Phe-induced rise in $[Ca^{2+}]_i$. Values in parentheses show the percent of the control response. One representative experiment of four is shown. Asterisks indicate that mean values in the test group were significantly different ($p \leqslant 0.05$) from control in Student's paired t-test. (Adapted in part from Dillon et al [66].)

calcium binding dye Quin 2 (in the absence of extracellular calcium) completely abrogates fMet-Leu-Phe-induced IP_3 production [72,98]. Increased cytosolic Ca^{2+} levels may act in turn to feedback-regulate PIP_2 hydrolysis, since prior treatment of PMNs with ionomycin reduced 1,4,5-IP_3 formation in response to fMet-Leu-Phe [72]. The exact locus of this calcium-mediated inhibition was not determined; however, one possible mechanism would involve ionophore-mediated production of DAG [90] with activation of protein kinase C [99].

Our studies indicate that the $[Ca^{2+}]_i$ levels required for direct degradation of 1,4,5-IP_3 to 1,4-IP_2 vs metabolism of 1,4,5-IP_3 via the IP_4 pathway (see Fig. 6) differ. In fMet-Leu-Phe-stimulated PMNs, 1,4-IP_2, 1,4,5-IP_3, and IP_4 formation were not significantly altered by preventing the agonist-induced increase in $[Ca^{2+}]_i$, whereas both 1,3,4,-IP_3 and 3,4-IP_2 levels were dramatically reduced by this treatment (Fig. 7). These results could implicate 1,3,4-IP_3 or 3,4-IP_2 as second messengers in Ca^{2+}-dependent PMN activation. Alternatively, if IP_4 acts to promote Ca^{2+} influx in PMNs, calcium may in turn act as a feedback signal to promote conversion of IP_4 to 1,3,4-IP_3, thus attenuating calcium influx. Studies in stimulated HL60 cells [72], adrenal glomerulosa cells [100], and in the insulin-secreting RINm5F cell line [57] also showed a Ca^{2+} dependency for hormone-stimulated formation of the 1,3,4-IP_3 isomer, whereas stimulated rat pancreatic acinar cells did not exhibit this requirement [101]. A role for Ca^{2+} in 1,3,4-IP_3 formation is also suggested by the observation that ionomycin-induced selective production of the 1,3,4-IP_3 isomer in HL60 cells [72]. Although IP_4 formation was not measured in ionomycin-stimulated HL60 cells, a calcium-driven formation of IP_4 and/or conversion of IP_4 to 1,3,4-IP_3 may account for the selective appearance of this isomer. In PMNs, we detected low levels of both IP_3 and IP_4 after ionomycin stimulation [66].

Most in vitro studies of the 5′-phosphomonoesterase, which dephosphorylates 1,4,5-IP_3, inositol 1,2 cyclic 4,5-trisphosphate, and 1,3,4,5-IP_4 [73], have shown that the enzyme requires Mg^{2+} but is not Ca^{2+} dependent [48,50,57], and is in fact inhibited by nonphysiologic (mM) Ca^{2+} concentrations in the presence of Mg^{2+} [48,50]. However, in a cytosolic fraction from coronary artery smooth muscle, the 5′-phosphatase activity was sensitive to 0.1–1 μM Ca^{2+} [102]. Additionally, recent work on the 5′-phosphatase in rat brain cortex suggests that there may be several distinct enzymes which differentially utilize 1,4,5-IP_3 vs IP_4 as substrates [103]. As noted above, 5′-dephosphorylation of both 1,4,5-IP_3 and 1,3,4,5-IP_4 is enhanced by protein kinase C in platelets [73,95,96]. Therefore, elevated cytosolic calcium levels could potentially increase 5-phosphatase activity in vivo by activating protein kinase C. The apparent sensitivity of IP_4, but not 1,4,5-IP_3, to calcium-dependent dephosphorylation in PMNs could therefore reflect the relative sensitivity of each substrate to a protein kinase C-regulated 5′-phosphatase. In several reports on the in vitro activity of the 1,4,5-IP_3 3 kinase [57,104], enzyme activity was enhanced by elevating Ca^{2+} over resting levels (0.1 μM); however, this effect was not seen in all the studies done to date [51]. Furthermore, in the human malignant T cell line Jurkat, IP_3 kinase activity was enhanced over resting levels in cells pretreated with either anti-T cell receptor antibody or PMA [104], suggesting that protein kinase C can also regulate the conversion of 1,4,5-IP_3 to IP_4. Collectively, it appears that although elevated $[Ca^{2+}]_i$ is clearly required for metabolism through the IP_4 pathway in PMNs and most other cells, there appear to be several Ca^{2+} and/or protein kinase C sensitive steps which are not yet clearly defined. Since elevation in intracellular Ca^{2+} is

required for full utilization of this pathway but not for degradation via 1,4-IP_2 in PMNs, it can be reasoned that the two metabolic pathways serve different functions in cellular regulation.

ACKNOWLEDGMENTS

The authors thank Ms. Sharon Goodwin for excellent secretarial assistance. This work was supported by grants DEO3738 and CA29589 from the National Institutes of Health. S.B.D. is supported by NIH Training Grant ST32CA09058. J.J.M. is a recipient of Pfizer Postdoctoral Fellowship and RJR Nabisco Awards.

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