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IDENTIFICATION OF A NOVEL INOSITOL BISPHOSPHATE ISOMER FORMED IN CHEMOATTRACTANT STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES[1]

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SUMMARY: Analysis of inositol phosphate formation in chemoattractant-stimulated human polymorphonuclear leukocytes demonstrated the production of inositol 1,4,5-trisphosphate, inositol 1,3,4-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, inositol 1,4-bisphosphate and another inositol bisphosphate isomer not detected in unstimulated cells. Studies in cell sonicates provided evidence that the previously unidentified inositol bisphosphate isomer is produced via the degradation of inositol 1,3,4-trisphosphate. This unidentified inositol bisphosphate peak was purified by high pressure liquid chromatography, and base hydrolyzed to form a mixture of inositol monophosphate isomers. Based on these studies, the unidentified peak was identified as inositol 3,4-bisphosphate. Identification of this isomer defines a new metabolic product derived from the initial inositol 1,4,5-trisphosphate formation, and also suggests another substrate for the inositol 1-phosphatase.

Activation of polymorphonuclear leukocytes (PMNs) by chemoattractants is triggered by the rapid hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate by a phospholipase C (1-7). The products thus formed include 1,2-diacylglycerol, which binds to protein kinase C (rev. in 8) and inositol 1,4,5-trisphosphate (IP_3) which mediates the release of Ca^{2+} from intracellular stores (rev. in 9). Elevated cytosolic Ca^{2+} and diacylglycerol synergize to promote protein kinase C activation and translocation to the plasma membrane (10), thus promoting cellular responses. The 1,4,5-IP_3 isomer can be degraded via a 5'-phosphomonoesterase to form inositol 1,4-bisphosphate (IP_2) (11-13), or

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Abbreviations: PMNs, polymorphonuclear leukocytes; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; fMet-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine; HPLC, high pressure liquid chromatography.

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can be phosphorylated via an ATP dependent 3'-kinase to form inositol 1,3,4,5-tetrakisphosphate (IP_4) (14). IP_4 is then converted to 1,3,4-IP_3 by a 5'-phosphomonoesterase (15-21). The stepwise conversion of 1,4,5-IP_3 to IP_4 and then 1,3,4-IP_3 is reflected in the order of appearance of these compounds in stimulated cells, i.e. the rise in 1,4,5-IP_3 is most rapid (2-5 sec), whereas 1,3,4-IP_3 appears only after an initial lag period (10-15 sec) (22,23). It is not yet known if 1,3,4-IP_3 or IP_4 have second messenger functions in PMNs; however, recent studies in other cell systems have provided evidence that these compounds can act to mobilize calcium from intracellular stores (1,3,4-IP_3) (24) or from the external medium (IP_4) (25). In order to study the potential relationship of the IP_3 isomers and IP_4 to cellular activation in PMNs, we analyzed, by high pressure liquid chromatography (HPLC), the inositol phosphate compounds formed after stimulation with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe). During the course of these studies we noted that in addition to 1,4-IP_2, a later eluting IP_2 peak (IP_2-X) was detectable only in stimulated cells. From studies in disrupted PMNs, it was determined that IP_2-X is formed via degradation of 1,3,4-IP_3. Since this IP_2 isomer did not coelute with 1,4-IP_2, it could represent either 1,3- or 3,4-IP_2. In the present communication, we report the identification of this compound as 3,4-IP_2 by analysis of the IP_1 products formed by base hydrolysis.

MATERIALS AND METHODS: fMet-Leu-Phe, 1-phenylmethylsulfonylfluoride (PMSF) and dithiothreotol (DTT) were obtained from Sigma Chemical Co; ammonium formate was from Aldrich Chemical Co. fMet-Leu-Phe was stored as a 0.01 M stock solution in dimethylsulfoxide (DMSO) at -20°C. [2-^{3}H]-myo-inositol-1-P (8.4 Ci/mmole) and [2-^{3}H]-myo-inositol 1,4-P_2 (2 Ci/mmole) were from New England Nuclear; [2-^{3}H]-myo-inositol 1,4,5-P_3 (15 Ci/mmole) was from Amersham Corp. [2-^{3}H]-myo-inositol (15 Ci/mmole) was from American Radiolabeled Chemicals, Inc.; [2-^{3}H]myo-inositol 1,3,4,5-P_4 (1 Ci/mmole) was provided by New England Nuclear.

PMNs ($\geq$95% purity) were isolated as described previously (26) from heparinized (10 U/ml) blood collected from healthy volunteers. The cells were labeled with [^{3}H]-myo-inositol as described (7). Prior to assay, the PMNs were washed twice and then resuspended in assay buffer (Hepes-buffered Hanks balanced salt solution; pH 7.4) with 20 mM $LiCl_2$. Aliquots of prewarmed cells (10^7/0.18 ml) were stimulated with 0.02 ml fMet-Leu-Phe (1 μM), or assay buffer control with an equivalent concentration of DMSO. Reactions were terminated with ice cold 10% trichloroacetic acid (TCA) (final concentration) and ether extracted as described (22). A mixture of unlabeled ATP, ADP and AMP was added to each sample and the samples were passed through a 0.45 μM HV filter (Millipore Corp., Milford, MA) prior to HPLC analysis.

Inositol phosphates were separated on a Whatman Partisal SAX 10 column (0.46 x 25 cm) with a Guard-Pak silica precolumn (Millipore Corp.) and column inlet filter (Beckman Instruments, Berkeley, CA) using a slight modification (Dillon, S.B. et al., in preparation) of the gradient described by Irvine et al. (15,22). The flow rate was 1.25 ml/minute throughout and 0.5 min fractions were collected. Fractions were diluted

with 0.5 ml H_2O and mixed with 3.5 ml Lefkofluor scintillation fluid (Research Products International Corp., Mount Prospect, Ill.) for scintillation counting. Isomers of IP_1 were resolved using the same column described above, with an ammonium phosphate gradient system (27) as follows: 0.04 to 0.075M ammonium phosphate (pH 3.8) over 65 min to resolve IP_1 isomers (see text); 0.075 to 0.15M over 30 min to elute 1,4-IP_2; 0.15 to 0.54M over 25 min to elute IP_2-X (see below), and 0.54 to 0.75M over 5 min to resolve IP_3 isomers. The flow rate was 1 ml/min throughout. The column effluent was mixed with Tru-count scintillation fluid (5:1 ratio) (IN-US Corp., Fairfield, N.J.) and radioactivity was monitored by an on line radioactive detector (Ramona LS-4, IN-US Corp.) equipped with a Roland DG computer and software version 5.3e for peak integration. The counting efficiency was set at 60%. Sonicated PMNs were prepared by suspending the cells (2×10^8/ml) in ice cold buffer containing 0.32M sucrose, 25 mM Hepes/Tris, 1 mM EGTA, 2 mM DTT, 2 mM $MgCl_2$ and 1 mM PMSF, pH 7.5. Cells in an ice slurry were sonicated six times for 10 sec at a setting of 35% with an Artek sonic 300 dismembranator (Artek Systems Corp., Farmingdale, NY). Nuclei and nondisrupted cells were removed by centrifugation (250 x g for 5 min), and PMN sonicates were frozen at -70°C until use. To analyze the products formed from [^{3}H]-IP_4 degradation, cell sonicates (75 µl) were prewarmed for 10 min in the presence of 10 µM $CaCl_2$ (final concentration). Reactions were started with 25 µl 4X assay buffer containing 440 mM KCl, 40 mM NaCl, 4 mM KH_2PO_4, 12 mM $MgCl_2$, 80 mM K^+ Hepes, pH 7.4 and 2.0 µM [^{3}H]-IP_4 (1 Ci/mmole), and terminated with ice cold TCA.

For purification of 1,3,4-IP_3 and IP_2-X, PMN sonicates were incubated with [^{3}H]-IP_4 (15 min) as described above, except that the incubation mixture was scaled up tenfold. HPLC purified 1,3,4-IP_3 and IP_2-X peaks were neutralized, reapplied to 0.5 ml columns of Biorad AG1-X8 resin (200-400 mesh; formate form) and eluted with appropriate concentrations of ammonium formate/formic acid as described (28). The samples were then dried and base hydrolyzed in conc. NH_4OH at 95°C for 12 hr. The samples were dried under N_2 and resuspended in H_2O. Unlabeled AMP and ADP were added to samples before analysis by HPLC.

RESULTS: Inositol Phosphate Isomer Formation in Stimulated PMNs. Extracts from resting vs fMet-Leu-Phe (0.1uM) stimulated [^{3}H]-myo-inositol labelled PMN were analyzed by HPLC (Fig 1A). In resting PMN, the major inositol phosphate products were IP_1 (not shown); 1,4-IP_2; and 1,4,5-IP_3. By 60 sec. after stimulation, six [^{3}H]-inositol containing compounds were elevated over resting levels; these included IP_1 (not shown); 1,4-IP_2; 1,3,4-IP_3; 1,4,5-IP_3; IP_4; and an unidentified peak eluting at 31.5 min, immediately following the 1,4-IP_2 isomer (29.5 min). Because of its close proximity to 1,4-IP_2, this peak was assumed to represent a second IP_2 isomer.

Evidence that IP_2-X is Derived From 1,3,4-IP_3. In a detailed study on the kinetics of inositol phosphate formation in human PMNs, we found that after fMet-Leu-Phe stimulation, IP_2-X was formed after an initial lag, concomitant with 1,3,4-IP_3 (Dillon,S.B., et. al., in preparation). We therefore reasoned that this IP_2 isomer was derived via metabolic breakdown of 1,3,4-IP_3. This hypothesis was tested by analyzing the metabolic products formed when [^{3}H]-IP_4 was added to disrupted PMN. The data in Fig 1B show that incubation of sonicated PMN with [^{3}H]-IP_4 (0.5 µM) resulted in

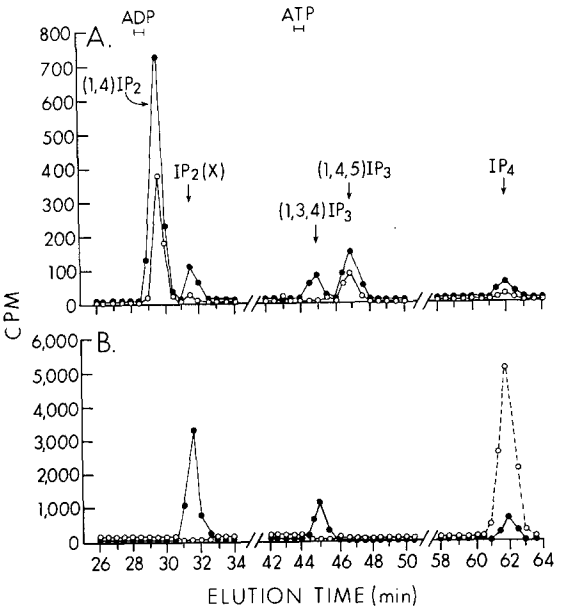

Figure 1. Production of a Novel IP_2 Isomer (IP_2-X) in PMN. In (A), extracts from 10^7 [^{3}H]-myo-inositol labelled PMNs incubated with assay buffer (o—o) or 0.1uM fMet-Leu-Phe (•—•) for 60 sec were analyzed by HPLC (ammonium formate gradient). In (B), chromatographs of extracts from PMN sonicates incubated with 0.5uM [^{3}H]-IP_4 (described in Materials and Methods) are shown. Reactions were stopped at time zero (o---o) or 15 min (•—•). Position of [^{3}H]-1,4-IP_2, [^{3}H]-1,4,5-IP_3 and [^{3}H]-IP_4 standards as well as peaks eluting in 1,3,4-IP_3 and IP_2-X positions are noted (arrows). Unlabelled ADP and ATP were monitored by absorbance (254nm).

the formation of 1,3,4-IP_3 and an IP_2 isomer which had the same retention time as IP_2-X produced in fMet-Leu-Phe-stimulated PMNs.

Identification of IP_2-X. The IP_2-X isomer derived from 1,3,4-IP_3 in stimulated PMN did not coelute with 1,4-IP_2, and could therefore be either 1,3 or 3,4-IP_2. To distinguish between these possibilities, IP_2-X was purified by HPLC (see methods), and base hydrolyzed to determine which IP_1 isomers were formed. The elution pattern of the 1-, 3- and 4-IP_1 isomers was first determined by HPLC analysis of base hydrolyzed [^{3}H]-1,4-IP_2, or [^{3}H]-1,3,4-IP_3. Two IP_1 peaks were detected after base hydrolysis of the [^{3}H]-1,4-IP_2 standard; the early peak coeluted with AMP (Fig 2A) and an [^{3}H]-1-IP_1 standard (not shown). The second peak eluted ca. 3 min after AMP and was identified as 4-IP_1. Base hydrolysis of 1,3,4-IP_3 produced two IP_1 peaks which coeluted with 1-IP_1 and 4-IP_1; however, since the early peak contained twice the radioactivity we reasoned that 3-IP_1 also eluted at this position (Fig 2B). Three IP_2 isomers were formed from base hydrolysis of 1,3,4-IP_3; these coeluted with 1,4-IP_2, the IP_2-X isomer formed in stimulated PMN, and an earlier eluting peak (i.e., before 1,4-IP_2) which is referred to here as IP_2-X^1. Base hydrolyzed IP_2-X

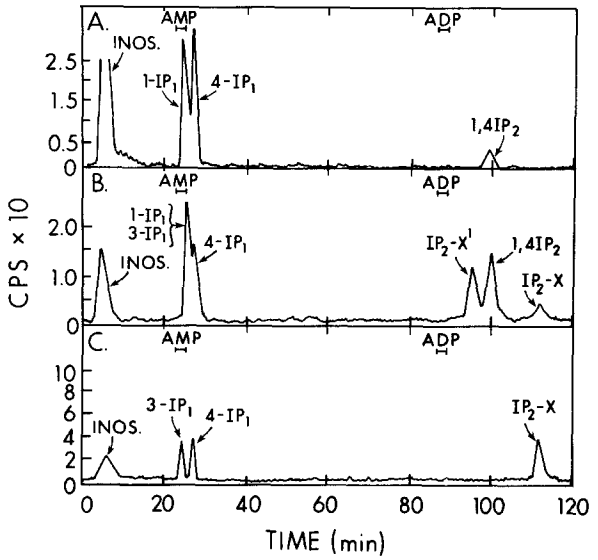

Figure 2. IP_1 products formed via base hydrolysis of 1,4-IP_2, 1,3,4-IP_3 or IP_2-X. Base hydrolyzed [^{3}H]-1,4-IP_2 (A), [^{3}H]-1,3,4-IP_3 (B), or [^{3}H]-IP_2-X (C) were analyzed by HPLC with an ammonium phosphate gradient (see "Materials and Methods"). Data shown represent tracings of radioactivity measured with the radioactive detector. Unlabeled AMP and ADP were detected as described in legend to Fig 1. The basis for identification of tritiated compounds is discussed in the text.

produced two IP_1 peaks, coeluting with 1- or 3-IP_1 and 4-IP_1 (Fig 2C). We therefore conclude that the IP_2-X isomer eluting after 1,4-IP_2 is 3,4-IP_2; and IP_2-X^1 is 1,3-IP_2.

DISCUSSION: Phospholipase C mediated hydrolysis of PIP_2 is centrally important for receptor mediated activation of numerous cell types, since the products formed promote protein kinase C activation (1,2-diacylglycerol) and raise cytosolic Ca^{2+} levels (1,4,5-IP_3). Inositol 1,4,5-trisphosphate is degraded in many tissues by the selective action of a 5'-phosphomonoesterase (11-13;21) to produce 1,4-IP_2 which does not mobilize Ca^{2+} (29); degradation via this pathway could therefore serve to attenuate the calcium rise. However, it is now recognized that 1,4,5-IP_3 can be metabolized via stepwise conversion to IP_4 and 1,3,4-IP_3 (15-21), both of which have recently been shown to have second messenger functions (24,25). Definition of the routes of formation and degradation of each of these products is therefore necessary for understanding the mechanisms by which the inositol phosphate products regulate cellular activation. HPLC analysis of the inositol phosphates in PMNs revealed that after chemoattractant stimulation, two IP_2 isomers were present; whereas only 1,4-IP_2 was detected in resting cells. The second IP_2 isomer is identified here as 3,4-IP_2 on the basis of the IP_1 products formed after base

hydrolysis. Since 3,4-IP_2 but not 1,3-IP_2 was present in fMet-Leu-Phe stimulated PMN, it appears that 1,3,4-IP_3 is preferentially degraded by a 1-phosphatase to form 3,4-IP_2 in intact cells. However, in experiments with PMN lysates scaled up tenfold for purification of 1,3,4-IP_3 and 3,4-IP_2 (from stepwise degradation of [^{3}H]-IP_4 by disrupted PMN), both 3,4-IP_2 and 1,3,IP_2 were produced, comprising 99% vs. 1% of the total IP_2, respectively (not shown). Therefore it appears that 1,3,4-IP_3 can also be hydrolyzed via a 4-phosphatase present in PMNs, although this reaction proceeds less efficiently.

Other recent studies in parotid gland (17), liver (19), or 3T3 cells (30) also showed that two isomeric forms of IP_2 were formed after hormonal stimulation, and suggested that 1,3,4-IP_3 degradation resulted in the formation of a second IP_2 isomer, but the isomeric form of this IP_2 was not determined. Although the identity of this unidentified IP_2 isomer which eluted after 1,4-IP_2 was tentatively assumed to be 1,3-IP_2 in one study (19), we suggest that it is rather 3,4-IP_2 based on the results presented here.

In platelets, it has been shown that degradation of the calcium mobilizing 1,4,5-IP_3 isomer to 1,4-IP_2 can be regulated via protein kinase C mediated activation of the IP_3 5-phosphatase (31-32). Similarly, degradation of 1,3,4-IP_3, which also has calcium mobilizing activity in some cells (24), could also lead to attenuation of the calcium signal. Identification of the 3,4-IP_2 isomer is therefore important, since this implies that an IP_3-1-phosphatase may also serve as a regulatory enzyme in activated cells. Although it is not yet known if the 1-phosphatase that converts 1,3,4-IP_3 to 3,4-IP_2 is specific for this inositol polyphosphate, $LiCl_2$ treatment, which results in elevated 1,3,4-IP_3 levels (19,23,33), also inhibits the degradation of 1-IP_1 via a 1-phosphatase (34-35). Therefore the lithium sensitive IP_1 1-phosphatase may also dephosphorylate 1,3,4-IP_3 to form 3,4-IP_2. Further study of the 5-IP_3- vs. 1-IP_3-phosphatase will clarify whether these two enzymes play a role in the regulation of cellular activation in PMNs.

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