Isolation and Characterization of the Inositol Cyclic Phosphate Products of Phosphoinositide Cleavage by Phospholipase C

METABOLISM IN CELL-FREE EXTRACTS*

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The phosphoinositides are metabolized by phospholipase C in response to hormone or agonist stimulation in many cell types to produce diglyceride and watersoluble inositol phosphates. We have recently shown that the phospholipase C reaction products include cyclic phosphate esters of inositol. One of these, inositol 1, 2-cyclic 4,5-trisphosphate, is active in promoting Ca\(^{2+}\) mobilization in platelets and in inducing changes in conductance in Limulus photoreceptors similar to those produced by light (Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A., Rubin, L. J., and Brown, J. E. (1985) J. Biol. Chem. 260, 13496-13501. In the current study, we have examined the metabolism of the inositol phosphates. We find that both cyclic and non-cyclic inositol triphosphates are metabolized by inositol 1, 4,5-trisphosphate 5-phosphomonoesterase, to inositol 1,2-cyclic bisphosphate and inositol 1,4-bisphosphate, respectively. However, the apparent \(K_m\) of the enzyme for the cyclic substrate is approximately 10-fold higher than for the non-cyclic substrate. These inositol bisphosphates are more slowly degraded to inositol 1,2-cyclic phosphate and inositol 1-phosphate, respectively. Inositol 1,2-cyclic phosphate is then hydrolyzed to inositol 1-phosphate, which in turn is degraded to inositol and inorganic phosphate by inositol 1-phosphate phospha-
tase. The human platelet inositol 1,2-cyclic phosphate hydrolyase enzyme and a similar rat kidney hydrolase do not utilize the cyclic polyphosphate esters of inositol as substrates. These results suggest that the inositol cyclic phosphate phosphatase and the non-cyclic inositol phosphates are metabolized separately by phospha-
tases to cyclic and non-cyclic inositol mono-
ophosphates. The cyclic monophosphate is then con-
verted to inositol 1-phosphate by a cyclic hydrolase. We suggest that the enzymes that metabolize the inositol phosphates may serve to regulate cellular re-
sponses to these compounds.

Hormones and other agonists which promote Ca\(^{2+}\) mobilization also effect the increased metabolism of the phosphoinositides (1) phosphatidylinositol, PtdIns; phosphatidylinositol 4-phosphate, PtdIns-4-P; and phosphatidylinositol 4,5-bisphosphate, PtdIns-4,5-P\(_2\). This accelerated phosphoinositide metabolism is catalyzed by a phospholipase C enzyme. The reaction products of PtdIns, PtdIns-4-P, and PtdIns-4,5-
P\(_2\) breakdown are diglyceride and the water-soluble inositol phosphates: IP\(_1\), IP\(_2\), and IP\(_3\), respectively. IP\(_3\) has been suggested to be a "second messenger" (2). Studies of both permeabilized cells and cellular microsomal fractions have shown that this inositol phosphate can promote Ca\(^{2+}\) mobilization from non-mitochondrial cellular stores (Ref. 3, reviewed in Ref. 4). Further evidence of a role for IP\(_3\) in cellular responsiveness was demonstrated in studies in which injection of IP\(_3\) into intact cells of Limulus ventral photoreceptors (5, 6), seaurchin eggs (7), Xenopus oocytes (8), and salamander rods (9) in each case produced a physiological response similar to that promoted by the natural stimulus.

Dawson et al. (10) observed that the products of phospholipase C-mediated breakdown of PtdIns included both IP\(_1\) and inositol 1,2-cyclic phosphate, the latter containing a cyclic phosphate ester between the 1 and 2 carbons of the inositol ring. Wilson et al. (11) have shown that a single phospholipase C from ram seminal vesicles cleaves all three phosphoinositi-

des to yield both the non-cyclic IP\(_1\) and IP\(_2\), and the corre-
sponding inositol cyclic phosphates, cIP\(_3\) and cIP\(_3\) (12). We have recently isolated the inositol cyclic phosphates from the reaction products of phospholipase C-mediated cleavage of the polyphosphoinositides (13). We have shown that cIP\(_3\) is active in \textit{in vitro} assays; it mobilizes Ca\(^{2+}\) from saponin-
permeabilized human platelets and it evokes a response similar to that produced by light when injected into \textit{Limulus} photoreceptors (13). These results suggest that cIP\(_3\) is likely to be involved in cellular responses \textit{in vivo}.

If IP\(_3\) and cIP\(_3\) are mediators of cellular function, their metabolism could be a signal terminating step. Mechanisms for metabolism of non-cyclic inositol phosphates have been demonstrated in several tissues. The conversion of IP\(_3\) to IP\(_2\) has been shown in crude homogenates of the salivary blow fly (14), erythrocytes (15, 16), and rat liver (17-19), which also metabolizes IP\(_3\) to a lesser extent (17-19). We have recently isolated a soluble enzyme from human platelets that specifically removes the 5-phosphate from IP\(_3\) to form IP\(_2\), an

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1 The abbreviations used are: PtdIns, phosphatidylinositol; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-4,5-P\(_2\), phosphatidylinositol 4,5-bisphosphate; IP\(_3\), myo-inositol 1-phosphate; IP\(_2\), myo-inositol 1,4-bisphosphate; IP\(_1\), myo-inositol 1,4,5-trisphosphate; cIP\(_3\), myo-inositol 1,2-cyclic phosphate; cIP\(_2\), myo-inositol 1,2-cyclic phosphate; 4,5-
trisphosphate; HPLC, high performance liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid.
apparently inert compound (20). IP₃, breakdown to inositol and P₃, has been demonstrated in a number of different tissues. The IP₃-phosphatase involved is inhibited by lithium ions (21). Finally, Dawson and Clarke (22) demonstrated that crude extracts from many tissues can convert inositol 1,2-cyclic phosphate to IP₃. Thus, a potential pathway for the degradation of the inositol cyclic polyphosphates also exists.

We now describe the metabolism of the inositol cyclic and non-cyclic phosphates by human platelets and rat kidney. We find that only cIP₃ is metabolized to its corresponding non-cyclic inositol phosphate. The other inositol cyclic phosphates as well as the non-cyclic inositol phosphates are metabolized by phosphomonoesterases.

**EXPERIMENTAL PROCEDURES**

**Materials—**myo-2-[3H]inositol, [³²P]phosphoric acid, and [³H]phosphatidylinositol 4-phosphate were from New England Nuclear. The Partisil SAX HPLC column, silica acid, and Partisil SAX resins were from Whatman. Inositol 1,2-cyclic phosphate was provided by Merck Sharp and Dohme, Rahway, N.J. All other materials are as listed or from Sigma or Fisher.

**Preparation of Radiolabeled Inositol Phosphates and Inositol Phosphatases—**[³²P]-tritium-labeled phosphoinositides and the various cyclic and non-cyclic inositol phosphates, prepared from reaction mixtures of phosphatidic C  with [³²P]- or tritium-labeled phosphoinositides, were prepared as described previously (13). The isolated inositol phosphates were desalted by lyophilization or Sephadex G-10 chromatography and assayed for phosphorus (23) as previously described (13). We now use a 4 × 250-mm precolumn of silicic acid (mounted before the injector loop) and a 4 × 50-mm Partisil SAX guard column which are changed after every 6 runs to prevent degradation of the column by the high concentrations of ammonium formate used to elute the inositol phosphates.

**Human Platelet Sonicate—**Fresh human platelets were obtained from normal donors as previously described (24). The platelets were washed (24) and suspended at 5 × 10^9 platelets/ml in 50 mM Tris, pH 7.8, 10 mM 2-mercaptoethanol, and 200 mM NaCl, and in the same buffer without 2-mercaptoethanol and at 8°C. The platelet-soluble fraction was prepared from reaction mixtures by HPLC. No evidence for a cyclic hydrolase which uses cIP₃ as substrate. No inositol 1,2-cyclic hydrolase activity acting on cIP₃ was detected using undialyzed soluble fraction or in incubations using a platelet membrane fraction. Thus, we found no evidence that platelets convert cIP₃ to IP₃, rather it appears that cIP₃ is metabolized to cIP₂ and P₃.

We next examined whether or not cIP₃, the cyclic inositol product of phospholipase C-mediated cleavage of PtdIns-4-P, is metabolized by a platelet phosphomonoesterase or by an inositol 1,2-cyclic phosphate hydrolase. We incubated [³H]cIP₃ with the platelet-soluble fraction and again fractionated the reaction mixture by HPLC. No evidence for an inositol 1,2-cyclic hydrolase was detected. [³H]IP₃ was formed, which would result from the action of the 5-phosphomonoesterase on any IP₃ that was formed. When the platelet-soluble fraction (enzyme) concentration was increased 10-fold and the incubation time was lengthened 4-fold, no inositol 1,2-cyclic hydrolase activity that acts on cIP₃ was detected (data not shown). No inositol 1,2-cyclic hydrolase activity acting on cIP₃ was detected using undialyzed soluble fraction or in incubations using a platelet membrane fraction. Thus, we found no evidence that platelets convert cIP₃ to IP₃, rather it appears that cIP₃ is metabolized to cIP₂ and P₃.

**RESULTS**

**Metabolism of cIP₃, cIP₂, cIP₁, IP₃, and IP₆, by Human Platelet Extracts**—We used HPLC to separate the various inositol phosphates as described previously (13). We incubated [³²P]cIP₃ with the soluble fraction from human platelets and subsequently fractionated the reaction mixture by HPLC. We found three separate radioactive compounds, corresponding to unmetabolized cIP₂ and reaction products, [³²P]cIP₁ and [³²P]IP₆ (Fig. 1B), demonstrating that cIP₂ was metabolized by a 5-phosphomonoesterase. When [³²P]IP₂ was incubated with the same soluble platelet fraction, phosphatase activity was also observed in agreement with our previous studies on the platelet IP₃ 5-phosphomonoesterase (20). HPLC of this reaction mixture showed three compounds, corresponding to the substrate, IP₃, and the products of a 5-phosphomonoesterase reaction, IP₂ and P₃ (Fig. 1A). In these experiments on inositol triphosphate metabolism we found no evidence for a cyclic hydrolase which uses cIP₃ as substrate. Thus, there was no conversion of cIP₃ to IP₃ (Fig. 1B) and there was also no IP₆ formed, which would result from the action of the 5-phosphomonoesterase on any IP₃ that was formed. When the platelet-soluble fraction (enzyme) concentration was increased 10-fold and the incubation time was lengthened 4-fold, no inositol 1,2-cyclic hydrolase activity that acts on cIP₃ was detected (data not shown). No inositol 1,2-cyclic hydrolase activity acting on cIP₃ was detected using undialyzed soluble fraction or in incubations using a platelet membrane fraction. Thus, we found no evidence that platelets convert cIP₃ to IP₃, rather it appears that cIP₃ is metabolized to cIP₂ and P₃.

**Fig. 1.** Partisil SAX HPLC chromatography of reaction mixture of the human platelet-soluble fraction with inositol phosphates. The radioactively labeled inositol phosphates were incubated with crude platelet extract in a 25-μl final volume as described under "Experimental Procedures." The substrate concentration, incubation times, and protein content, respectively, were the following: 270 μM [³²P]IP₂ for 15 min, 13 μg (A); 220 μM [³²P]IP₁ for 15 min, 13 μg (B); 120 μM [³H]IP₃ for 30 min, 24 μg (C); 160 μM [³H]IP₆ for 60 min, 95 μg (D); 50 μM [³H]IPₖ for 15 min, 24 μg (E); and 20 μM [³H]IP₆ for 60 min, 11 μg (F). The elution positions for standard inositol phosphates are shown in A and B and are applicable for all panels.
The phosphatase-mediated breakdown of IP3 was confirmed in studies in which \(^{[32P]}IP_3\) was the substrate and \(^{[32P]}IP_2\) released was measured. The amount of IP3 hydrolyzed by the platelet supernatants was less than that for IP3, 0.9 \textit{versus} 20 nmol/min/mg protein, respectively. Similarly, the amount of cIP3 hydrolyzed by the platelet supernatants was less than for cIP3, 0.4 \textit{versus} 3.4 nmol/min/mg, respectively. The dialyzed soluble fraction and dialyzed and undialyzed membranes showed no inositol 1,2-cyclic hydrolysis activity which used cIP3 as a substrate. Indeed, dialysis markedly diminished the IP3 phosphatase activity in the soluble fraction.

When \(^{[3H]}cIP_1\) was incubated with the dialyzed platelet-soluble fraction, its breakdown by both a hydrolase and a phosphatase was detected. HPLC chromatography of the reaction mixture showed three compounds: cIP3, the substrate; IP3, the product of hydrolase activity; and inositol, the product of phosphomonoesterase hydrolysis of IP1 (Fig. 1F). The activity of the platelet inositol 1,2-cyclic hydrolase enzyme was 1.91 nM cIP1 hydrolyzed per min/mg of protein. Use of less enzyme or shorter incubation time showed only cIP1 and IP1 in the reaction mixtures suggesting that the hydrolase converts cIP1 to produce IP1, as described by Dawson and Clarke (22), which is the substrate for the IP1 phosphatase. Incubation of \(^{[3H]}IP_1\) with this nondialyzed platelet-soluble fraction resulted in very little hydrolysis to inositol while incubation of \(^{[3H]}IP_1\) with the dialyzed soluble fraction resulted in its breakdown to inositol by a phosphatase as shown in Fig. 1E. When cIP1 was incubated with the platelet-soluble fraction dialyzed against Tris buffer, pH 7.8, more inositol was formed than when cIP1 was incubated with the nondialyzed soluble platelet fraction (data not shown). These results suggest that dialysis of the platelet-soluble fraction yields a more active inositol 1-phosphate phosphomonoesterase. Incubation of either IP1 or cIP1 with the platelet membrane fraction resulted in no apparent metabolism of either substrate (data not shown).

**Metabolism of cIP3 by Platelet IP3 5-phosphomonoesterase**—The metabolism of cIP3 to cIP2, as shown in Fig. 1B, could be an important regulatory step in platelets whereby the active cIP3 is inactivated by its conversion to cIP2. We next incubated the isolated platelet IP3 5-phosphomonoesterase, that removes the 5-phosphate from IP3, with cIP3. HPLC chromatographs of the reaction mixtures of the purified 5-phosphomonoesterase with IP3 and cIP3 are shown in Fig. 2, A and B, respectively. As expected, IP3 was metabolized to IP2 and P1. The compound eluting in fraction 33 is an unknown component that was present in the IP3 substrate. cIP3 was metabolized by this phosphatase to cIP2 and P1. Previous examination of substrates hydrolyzed by the 5-phosphomonoesterase showed that only IP3 was hydrolyzed (20). We next examined the rate of IP3 hydrolysis by the 5-phosphomonoesterase in comparison to that for IP3. The dependence of hydrolysis rate on IP3 or cIP3 concentration is shown in Fig. 3, A and B, respectively. The \(K_m\) of the enzyme for IP3 was 75 \(\mu M\) and for cIP3 was approximately 1000 \(\mu M\). A more thorough evaluation of the enzyme activity towards cIP3 was not possible due to the limited availability of radiolabeled substrate \(^{[32P]}IP_3\). These results, however, suggest that the cyclic inositol trisphosphate is hydrolyzed by the platelet 5-phosphomonoesterase at a much slower rate than that for the corresponding non-cyclic compound.

**Metabolism of cIP1, cIP2, and cIP3 by Rat Kidney Cyclic Hydrolase**—We were surprised that only cIP1 was a substrate for the platelet inositol 1,2-cyclic hydrolase. Thus, we examined the ability of rat kidney cIP1 hydrolase to metabolize the inositol cyclic phosphates. Fig. 4A shows the HPLC chromatogram of the reaction mixture of the rat kidney hydrolase incubated with \(^{[32P]}cIP_3\). Although more enzyme
was used than required to breakdown cIP3, no hydrolase activity towards [32P]cIP2 was observed as no [32P]IP2 was formed. Under these conditions, a small amount of 5-phosphomonoesterase activity was demonstrated as [32P]cIP2 was formed. No [32P]cIP3 was observed in standard [32P]cIP3 or in the reaction mixture when less hydrolase was used (data not shown). When we incubated [32P]cIP3 with the hydrolyase, again no [32P]IP3 was formed, suggesting that cIP3 is also not a substrate for the kidney inositol 1,2-cyclic hydrolase. As shown in the HPLC chromatograph of Fig. 4C, incubation of [3H]cIP3, with the hydrolase formed two products, [3H]IP3, and [3H]inositol. The enzyme activity we observed, 370 nmol/min/mg, was comparable to that found by Dawson and Clarke (26). When we incubated cIP3 under conditions comparable to those used to examine hydrolase activity on cIP2, and cIP3, (10-fold more enzyme and 4-fold longer incubation), the substrate was completely converted to free inositol. Thus, we conclude that the inositol 1,2-cyclic hydrolase does not utilize cIP2 or cIP3, as we could have detected reaction products if their rate of breakdown was as little as 0.25% that of cIP3.

**DISCUSSION**

The recent discovery that IP3 may be involved in the signal transduction of those hormones which are associated with Ca2+ mobilization (4), has focused attention on the breakdown of the polyphosphoinositides and on the generation of their metabolites, the water-soluble inositol phosphates. While the hormone specific changes in the phospholipid compounds have been extensively studied, much less is known about the formation and subsequent metabolism of the inositol phosphates. We have examined the metabolism of the inositol phosphates formed by the phospholipase C-mediated breakdown of the three phosphoinositides, PtdIns, PtdIns-4,5-P2, and PtdIns-4,5-P3.

Dawson et al. (10) first reported that PtdIns-specific phospholipase C converts PtdIns to both IP2 and inositol 1,2-cyclic phosphate. Wilson et al. (12) later showed that phospholipase metabolism of the polyphosphoinositides also resulted in the formation of the inositol cyclic phosphopolipids, cIP2 and cIP3. Recently we demonstrated (13) that these inositol cyclic phosphates can be isolated by HPLC chromatography, yielding sufficient quantities (albeit limited) to examine their physiological properties. cIP3 injection into Limulus photoreceptors evoked a physiological response similar to that produced by light (13). It is more potent than its counterpart non-cyclic inositol phosphate, IP3, in promoting this physiological response. cIP2 is inactive as is IP3, suggesting the necessity of the trisphosphate structure for biological activity. cIP3 as well as IP3 also stimulates 45Ca mobilization from saponin-permeabilized human platelets. Thus, the formation and physiological activity of these two water-soluble products of PtdIns-4,5-P2 breakdown can be demonstrated in vitro. It is likely these compounds mediate cellular functions in vivo.

If inositol 1,2-cyclic trisphosphate is a cellular second messenger, its enzymatic breakdown to inositol 1,2-cyclic 4-bisphosphate, to non-cyclic inositol trisphosphate could be important regulatory steps. Also, the further breakdown of the inositol phosphates to yield free inositol is necessary to provide a supply of free inositol to resynthesize phosphoinositides. A summary of our findings on the metabolism of cIP2 and the other inositol phosphates is shown in Fig. 5. We find that cIP3 is metabolized to cIP2 which is further metabolized to cIP1, by soluble enzymes from human platelets. While cIP1 is broken down by a platelet, inositol 1,2-cyclic hydrolase, to IP2, and IP3, cIP2 and cIP3 are not metabolized by this enzyme. We also used a preparation of inositol 1,2-cyclic hydrolase from rat kidney to demonstrate that the enzyme metabolized cIP1, but not cIP2 or cIP3. The non-cyclic inositol phosphates are metabolized by a series of soluble phosphomonoesterases to sequentially remove phosphates to convert IP3, to IP2, to IP1. The inositol cyclic phosphates are metabolized independently of the non-cyclic inositol phosphates. When IP1 is formed from cIP1, the pathways of metabolism of the cyclic and non-cyclic inositol phosphates converge.

The fact that a specific enzyme, IP3 5-phosphomonoesterase (20), exists that can metabolize cIP3 to cIP2 supports the hypothesis that cIP3 is formed in vivo. In previous studies we found that this enzyme could only metabolize IP3 (20). Because the amount of [32P]cIP3 available was limited, we could not thoroughly examine the properties of its metabolism by the purified IP3 5-phosphomonoesterase. Thus, we do not know if its metabolism is enhanced by other assay conditions. An increase in assay pH to 7.8, as well as a decrease to 5.5, did not greatly change the rate of cIP3 hydrolysis as compared to that for IP3.

The current results are not in conflict with the rapidly growing evidence that IP3 is involved in cellular activation (4). A rapid decrease in PtdIns-4,5-P2 in response to hormone stimulation has been documented in human platelets by several investigators (26-31), as well as the appearance of IP3.
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(30, 31). In these studies the inositol cyclic phosphates would not have been detected as the platelets were extracted under acidic conditions, which converts the cyclic compounds to their corresponding non-cyclic forms (12, 13). Also, although the mass of PtdIns-4,5-P₂ is much less than that of PtdIns-4-P and PtdIns in human platelets (11), interestingly platelets contain 40- to 60-fold more phosphatase activity for the inositol trisphosphates as compared to phosphatases for the inositol diphosphates and the inositol monophosphates. Thus, the regulation of cellular IP₃ and cIP₃ levels may be critical to normal cell function and awaits further examination.

Finally, the existence of a pathway for the metabolism of the cyclic inositol phosphates in platelets supports the hypothesis that they are formed in vivo. Further studies will determine the proportion of cyclic inositol phosphates formed in vivo.

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REFERENCES