Degradation of the Cyclic AMP Antagonist Prostaglandylinositol Cyclic Phosphate (Cyclic PIP) by Dephosphorylation*

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* Dedicated to Prof. Dr. Hans Reinauer on occasion of his 65th birthday
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The cyclic AMP antagonist, prostaglandylinositol cyclic phosphate (cyclic PIP), is synthesized from prostaglandin E and activated inositol phosphate. From various tissues only that amount of cyclic PIP can be isolated that constitutes the difference between synthesis and degradation. In order to overcome this drawback, the cyclic PIP degrading enzyme or enzymes had to be characterized prior to searching for inhibitors. Cyclic PIP degrading activities have been found in all rat tissues tested, and are lowest in brain (380 pmol x min^-1 x g^-1 wet weight) and highest in liver (1460 pmol x min^-1 x g^-1 wet weight). They are associated primarily with particulate structures of the cells, but not with the plasma membrane. There appear to be at least two different enzymatic activities involved in the degradation of cyclic PIP, because there are two pH-optima, one between pH 7 and 8 and another between pH 4 and 5. It is assumed that these activities are located in microsomes and lysosomes. Because prostaglandylinositol is the final product obtained in the degradation of cyclic PIP, a phosphodiesterase and a phosphatase should be involved, which could not yet be identified individually. Like alkaline phosphatase, cyclic PIP-degrading enzymes require Mg^2+ and they are inhibited by heavy metal ions such as mercuric and copper chloride, by sodium fluoride and interestingly, by prostaglandins.

Key words: Cyclic AMP antagonist / Cyclic PIP / Phosphatases / Prostaglandylinositol / Prostaglandylinositol cyclic phosphate.

The functional cyclic AMP antagonist, cyclic PIP, first isolated from hepatocytes (Wasner, 1981) has been detected in all rat organs so far tested (Wasner et al., 1993). The cAMP antagonist is also present in the yeast Saccharomyces cerevisiae (Wasner and Salge, 1987) and in the slime mould Dictyostelium discoideum (Kaiser et al., 1998). Hormones like insulin and noradrenaline (a-adrenergic action) stimulate the synthesis of cyclic PIP (Wasner, 1981; Wasner et al., 1993), whose regulatory properties are activation of protein serine/threonine phosphatases and inhibition of protein kinase A (Wasner, 1975). Cyclic PIP is composed of prostaglandin E (PGE) and inositol phosphate, i.e. the inositol phosphate is bound most likely by the C4-hydroxy group to the C15-hydroxy group of PGE (Wasner et al., 1992, 1993, 1996). A plasma membrane-bound enzyme synthesizes cyclic PIP (Wasner et al., 1996) from PGE, ATP and activated inositol phosphate (n-IP), which is most likely a guanosine diphosphate inositol phosphate (Gypakis and Wasner, 1996). Upon isolation and purification of cyclic PIP labeled with [3H]PGE, it was found that in addition to cyclic PIP, the phosphomonoester prostaglandylinositol phosphate (PIP) and the phosphophorylated compound prostaglandylinositol (PI) could also be isolated (Wasner et al., 1993). By reversed phase HPLC, PI could be further resolved into prostaglandylinositol itself and dinor prostaglandylinositol (Wasner et al., 1992), a result of β-oxidation of the PGE component (Hamberg and Samuelsson, 1971). It is not certain whether the occurrence of the phosphomonoester PIP is solely the result of instability of the phosphodiester because of its very labile, 5-membered ring structure (Robison et al., 1971; Wilson et al., 1985). However, the presence of PI is the result of inactivation of cyclic PIP by dephosphorylation (Wasner et al., 1992; 1993).

Sutherland and Rall have been able to isolate cyclic AMP from liver by blocking the degradation of cyclic AMP with cyclic AMP phosphodiesterase-inhibiting chemicals, such as methylxanthines (Sutherland and Rall, 1958). Presently, the isolation of cyclic PIP is only possible in the absence of agents, that stop the degradation of cyclic PIP. The amount of extractable PI is close to 10-fold higher than the amount of isolated cyclic PIP (Wasner et al., 1992, 1993), indicating that in rat liver degradation of cyclic PIP is nearly as rapid as its synthesis. The time course of cyclic PIP synthesis peaks within 3 min after hormonal stimulation and cyclic PIP levels decrease to nearly basal levels within 10 min thereafter (Wasner, 1981), this may also indicate that degradation of cyclic PIP is rapid. Therefore the degrading enzyme or enzymes of cyclic PIP had to be systematically studied prior to the search for inhibitors. Based on the characterization of the products isolated (Wasner et al., 1992), it is assumed that cyclic PIP is de-
graded primarily by dephosphorylation and the responsible enzyme or enzymes are expected to be phosphodiesterases and phosphatases.

The degradation of cyclic PIP is determined as follows: a given amount of cyclic PIP is incubated with various subcellular fractions for a fixed time. Thereafter enzymatic activity is denatured and the residual cyclic PIP is determined by its action to inhibit protein kinase A (Wasner et al., 1993). The rat organs brain, heart, intestine, kidney, liver, lung, skeletal muscle, spleen and testis were separately homogenized in Tris buffer of pH 7.5. The homogenates obtained were separated into sediment and supernatant fractions and their ability to inactivate cyclic PIP was determined. As shown in Table 1, the degrading activity of cyclic PIP is lowest in brain and about 4 times higher in liver. Furthermore, this activity is primarily present in all tissues in the pellet fraction, indicating that this enzyme should be predominantly membrane-bound. The following experiment supports this conclusion: resuspension in fresh buffer of the sediment fraction and repeated (5 times) resedimentation showed that cyclic PIP-degrading activity remained quantitatively associated with the particulate fraction (data not shown). In contrast, the cyclic AMP phosphodiesterase activity is predominantly cytosolic and only a small fraction is membrane-bound, and its activity is increased by the action of insulin (Loten and Sneyd, 1970). To assess whether cyclic PIP degradation might be subject to hormonal regulation as well, livers were perfused for 5 min either with glucagon, or with insulin, or without hormone. Thereafter, cyclic PIP inactivation was measured (Figure 1). The time-dependent degradation by the sediment and supernatant fractions showed that the cyclic PIP-degrading activity appears not to be subject to regulation by hormones.

It is of interest to know with which subcellular structure cyclic PIP-degrading activity is associated. The sediment fraction of a rat liver homogenate was layered on a linear sucrose gradient and centrifuged. The most prominent zone in the sucrose gradient at a density of approximately 1.22 g/ml, containing subcellular particles like mitochondria and microsomes, showed cyclic PIP-degrading activity. Above this zone there is a smaller one with a density of approximately 1.16 g/ml, containing the plasma membranes. Nearly no cyclic PIP-degrading activity (Table 2) was detectable in this fraction, thus excluding the plasma membrane as the site of this enzyme. Using a standard procedure for cell fractionation by differential centrifugation (Mahler and Cordes, 1966; Fleischer and Kervina, 1974), nuclei and major cell debris were separated by centrifugation at 700 g for 10 min; mitochondria at 24,000 g for 10 min and microsomes at 105,000 g for 60 min. The enzymes succinic acid dehydrogenase, thiamine pyrophosphatase, glucose-6-phosphatase and lactate dehydrogenase were used as marker enzymes for mitochondria, lysosomes, golgi, microsomes and the cytosol (Bergmeyer, 1974). The cyclic PIP-degrading activity in these different fractions did not correspond to the

| Table 1 Rates of Cyclic PIP Degradation in Supernatant and Sediment Fractions of Different Rat Organs. |
| Organ | Degradation rate of the | sediment | supernatant |
|       | (pmol × min⁻¹ × g⁻¹ tissue wet weight) |
| Brain | 380 | 0 |
| Testis | 425 | 0 |
| Kidney | 435 | 70 |
| Spleen | 445 | 195 |
| Muscle | 485 | 385 |
| Lung | 905 | 125 |
| Heart | 800 | 240 |
| Intestine | 745 | 450 |
| Liver | 1235 | 225 |

The organs (1 g) listed were homogenized in 5 ml Tris/HCl buffer (pH 7.5) and separated into sediment and supernatant fractions by centrifugation (16,300 g for 10 min). Tissue was homogenized with an Ultra Turrax (3 times for 20 s in 30 s intervals). Because of the low levels of cyclic PIP in the assays and the resulting non-linearity of the time courses of cyclic PIP degradation, the initial velocity (1 and 2 min) was determined and under these conditions less than 60% of cyclic PIP was degraded. Cyclic PIP was prepared and purified as described (Wasner et al., 1993).
activity profile of a given marker enzyme (data not shown), indicating that an attachment of cyclic PIP-degrading activity to a specific subcellular structure is unlikely. However, when comparing cyclic PIP-degrading activities of the fractions crude mitochondria, mitochondria and micro-

Table 2 Activity of Cyclic PIP Degradation in Subcellular Particle Fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cyclic PIP degradation rate (pmol x min⁻¹ x g⁻¹ tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Sucrose gradient</td>
<td></td>
</tr>
<tr>
<td>subcellular particles</td>
<td>1257</td>
</tr>
<tr>
<td>plasma membrane</td>
<td>16</td>
</tr>
<tr>
<td>nuclei</td>
<td>29</td>
</tr>
<tr>
<td>b) Differential centrifugation</td>
<td></td>
</tr>
<tr>
<td>crude mitochondria</td>
<td>342</td>
</tr>
<tr>
<td>mitochondria</td>
<td>221</td>
</tr>
<tr>
<td>microsomes</td>
<td>749</td>
</tr>
</tbody>
</table>

a) The sediment fraction obtained by centrifugation (16 300 g for 10 min) was layered on a linear sucrose gradient (20 – 60 % w/w sucrose) and centrifuged (105 000 g for 90 min). The indicated bands were aspirated, diluted (1:4) with 10 mM Tris/HCl buffer of pH 7.5, sedimented and resuspended in fresh Tris/HCl buffer prior to the assay. b) Homogenate (1 part tissue and 2 parts of 0.32 M sucrose) and centrifuged (700 g for 10 min). The supernatant was then centrifuged at 7000 g for 10 min to obtain a crude mitochondrial fraction, which was resuspended and spun down at 24 000 g for 10 min giving the mitochondria-containing fraction. Centrifugation of the combined supernatants at 105 000 g for 60 min sedimented the microsomes. The fractions obtained were assayed for cyclic PIP-degrading activities as described in the legend of Figure 1.

![Fig. 2](Image) Effect of pH on Cyclic PIP-Degrading Activity. 10 mM citrate/HCl buffer was applied in the pH-range 1 – 5, 10 mM Bis Tris Propane buffer in the pH-range 6 – 8 and 10 mM glycine/NaOH buffer in the pH-range 9 – 12. Degradation of cyclic PIP was measured as described in Figure 1, with the difference that in a total volume of 50 μl, 30 pmol cyclic PIP and 10 μl sediment fraction were incubated and the reaction was stopped by the addition of 300 μl chloroform/methanol (2:1). Prior to the determination of non-degraded cyclic PIP, the assay solutions were pH adjusted. To assess a possible loss of cyclic PIP because of extreme pH values, cyclic PIP was incubated in the buffer solutions in the absence of degrading enzymes and thereafter the amount of cyclic PIP remaining was determined. The loss in these controls was negligible.

Some enzymes, the highest cyclic PIP-degrading activity is found in the microsomal fraction (Table 2).

We then measured the dependence of cyclic PIP-degrading activity on the pH of the assay. As shown in Figure 2, a rather broad range of activity is found, with a maximum between pH 7 – 8 and a second, lower activity having a maximum at pH 4 – 5, supporting the idea that at least two different degrading enzymes exist. These findings indicate that the prevalent cyclic PIP-degrading enzymes appear to be associated with microsomes and the smaller activity may be associated with lysosomes because of the pH optimum in the acidic pH-range. With respect to the existence of the many different cyclic AMP phosphodiesterases (Manganiello et al., 1995), the presence of more than one cyclic PIP-degrading enzyme is easily conceivable.

Inactivation of cyclic AMP by cyclic AMP phosphodiesterase leads to AMP (Robison et al., 1971). The prevalent product of cyclic PIP degradation is dephosphorylated cyclic PIP, prostaglandininostol (PI), which has been characterized by mass spectrometry, and which can be obtained on incubation of cyclic PIP with phosphodiesterase and phosphatase (Wasner et al., 1992, 1993). In order to show that the degradative enzymes described in this report inactivate cyclic PIP accordingly by dephosphorylation, [3H]cyclic PIP was used as a substrate in the degradation assay, and after removal of protein the sample was subjected to anion exchange chromatography. The chromatography revealed a decrease in the [3H]cyclic PIP peak concomitant with an increase of [3H]labeled material eluting at a lower salt concentration in the range of PI (Figure 3). Also, on successive C18-reversed phase chromatography (data not shown), this [3H]-labeled material eluted in the range of PI elution (Wasner et al., 1992), indicating that the enzymatic activities described here degrade cyclic PIP by dephosphorylation. Normally, a phosphodiester is hydrolyzed to a phosphomonoester prior to dephosphorylation. As cyclic PIP is known to have a phosphodiester structure (Wasner et al., 1991), dephosphorylation would require two enzymes, which, due to their particular nature, have not yet been separated.

As addition of 1 mM NEM (N-ethyl-maleimide) to the assay does not inhibit cyclic PIP-degrading enzymes, SH-groups appear not to be essential for their activity. Addition of 10 mM EDTA had no effect, but addition of 10 mM EGTA led to complete loss of enzymatic activity. In the presence of 20 mM Mg²⁺, but not Zn²⁺, the inhibition by EDTA was overcome, indicating that cyclic PIP-degrading enzymes are dependent on a divalent cation like Mg²⁺, as is known for alkaline phosphatase (Bergmeier, 1974). In the presence of 1 mM citrate, a 30% inhibition is observed, which may be related with its chelator action comparable to EDTA. Enzymatic degradation of cyclic PIP was characterized in order to search for its inhibition. In addition to the inhibitory effect of EDTA, only known, but unsuitable, phosphatase inhibitors were found thus far: heavy metal ions, such as mercuric or copper chloride, and also fluoride completely inhibit the enzyme (Figure 4); 10 mM lithium chloride showed a 30% inhibition. It is interesting that
In a total volume of 50 μl of rat liver microsomal fraction and [3H]cyclic PIP (50 000 cpm) were incubated in a total volume of 500 μl at 30 °C for 10 min. The reaction was stopped by denaturation of the proteins (see legend to Figure 1). After centrifugation and removal of organic solvents, the water phase was applied to a QAE-Sephadex A-25 column (50 ml; 1.5 × 30 cm). Elution was performed by applying a linear gradient from 20 – 220 mM Tris/HCl buffer (pH 7.6) of 200 ml each. 100 fractions of 4 ml were collected. Elution of PGE1 peaks in fractions 21 – 22; PI elutes not completely resolved thereafter; cyclic PIP elutes in the fractions 60 – 68 and its phosphomonoester in the fractions 68 – 80.

10 μM PGE caused a 60% inhibition, though the applied concentration is above physiological levels. This effect may, however, indicate that cyclic PIP-degrading enzymes have a specificity for prostaglandin-containing compounds and they may be specific for cyclic PIP as are cyclic AMP phosphodiesterases for cyclic AMP. Various inositolphosphates (inositol 1-, or 2-, or 4-monophosphate), applied in 0.1 – 0.4 mM concentrations, showed no effect, requiring further searching for an appropriate inhibitor.

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**References**


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**Fig. 3** Chromatographic Characterization of the Product of Enzymatic Degradation of Cyclic PIP. Zero minute incubation (open circles), 10 min incubation (closed circles). 100 μl of rat liver microsomal fraction and [3H]cyclic PIP (50 000 cpm) were incubated in a total volume of 500 μl at 30 °C for 10 min. The reaction was stopped by denaturation of the proteins (see legend to Figure 1). After centrifugation and removal of organic solvents, the water phase was applied to a QAE-Sephadex column (50 ml; 1.5 × 30 cm). Elution was performed by applying a linear gradient from 20 – 220 mM Tris/HCl buffer (pH 7.6) of 200 ml each. 100 fractions of 4 ml were collected. Elution of PGE1 peaks in fractions 21 – 22; PI elutes not completely resolved thereafter; cyclic PIP elutes in the fractions 60 – 68 and its phosphomonoester in the fractions 68 – 80.

**Fig. 4** Inhibition of Cyclic PIP-Degrading Activities by Mercuric Chloride (Closed Circles) and Sodium Fluoride (Open Squares). In a total volume of 50 μl, 30 pmol cyclic PIP, 25 μl sediment fraction and the indicated amount of either fluoride or mercuric chloride were incubated (for further details see legend to Figure 1).