Prostaglandin biosynthesis and lipolysis in subcellular fractions from rabbit kidney medulla

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Three separate prostaglandin-generating activities are associated with plasma membranes, mitochondria and microsomal fractions from rabbit kidney medulla. In the plasma membranes and mitochondria, but not in microsomal fractions, Ca\(^{2+}\) ions stimulate the activity of phospholipase A\(_2\), yielding selective release of arachidonic acid and linoleic acid and concomitant increase in prostaglandin E\(_2\) formation.

We have previously demonstrated that in kidney medulla slices incubated in vitro Ca\(^{2+}\) ions stimulate prostaglandin biosynthesis and a lipolytic process in which there is a selective release of fatty acids (arachidonate and linoleate) into the extracellular medium (Erman & Raz, 1979). As both fatty acids predominate in the 2-position of phospholipids (Morgan et al., 1963) and since Ca\(^{2+}\) ions stimulate phospholipase A\(_2\) activity (Wells, 1972), we suggested that Ca\(^{2+}\) stimulation of prostaglandin biosynthesis in kidney medulla is mediated mainly by stimulation of phospholipase A\(_2\) activity (Erman & Raz, 1979). This tentative conclusion was, however, somewhat contradictory to the fact that in several tissues the microsomal fraction, generally believed to contain the bulk of the cellular prostaglandin-synthesizing activity (Hamberg & Samuelsson, 1967; Anggard et al., 1972; Bohman et al., 1975), contains very little, if any, phospholipase A\(_2\) activity (Waite & Van Deenen, 1967; Vignais et al., 1976). We therefore prepared and characterized several purified subcellular fractions from rabbit kidney medulla and compared their capacity to synthesize prostaglandin E\(_2\) from endogenous and from exogenously added arachidonate. We also compared the effect of Ca\(^{2+}\) ions on lipolysis and prostaglandin E\(_2\) generation in the medulla subcellular fractions.

Methods and materials

Preparation of membrane fractions

Kidneys from rabbits (male, 2.5–3.0kg) were removed as described elsewhere (Erman & Raz, 1979), and the medulla was homogenized in buffer (0.3 M-sucrose/10mM-Tris/HCl, pH 7.4; 5ml/g of tissue). The medulla homogenate was fractionated by a combination of differential centrifugation and isopycnic gradient centrifugation. Details of the differential-centrifugation fractionation procedure appear in Scheme 1. In some experiments, fraction I was suspended in 0.5 M-sucrose and fractionated by

\[
\text{Homogenate} \xrightarrow{4500\text{g}} \text{Supernatant} \xrightarrow{1500\text{g}} \text{Pellet} \xrightarrow{15\text{min}} \text{Resuspended} \xrightarrow{4500\text{g}} \text{Supernatant} \xrightarrow{15\text{min}} \text{Pellet (fraction I)}
\]

\[
\text{Supernatant} \xrightarrow{1500\text{g}} \text{Pellet} \xrightarrow{15\text{min}} \text{Supernatant} \xrightarrow{10000\text{g}} \text{Pellet} \xrightarrow{15\text{min}} \text{Supernatant}
\]

\[
\text{Supernatant} \xrightarrow{140000\text{g}} \text{Supernatant (cytosol)} \xrightarrow{60\text{min}} \text{Pellet (fraction III)}
\]

\[
\text{Supernatant} \xrightarrow{1500\text{g}} \text{Pellet} \xrightarrow{15\text{min}} \text{Supernatant (fraction II)}
\]

Scheme 1. Procedure for isolation of subcellular fractions from rabbit renal medulla

The homogenates were centrifuged at 4500g for zero time (i.e. centrifuge accelerated to 4500g and immediately stopped with brake). For other details see the Methods and materials section. Inset shows further fractionation of fraction I.

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layering the suspension on a discontinuous gradient consisting of 4 ml each of 1.7 M and 1.4 M sucrose (Aronson & Sactor, 1975). The tubes were centrifuged at 90,000 g for 60 min in a Beckman SW-40 swinging-bucket rotor. The bands resolved are shown in Scheme 1 (inset). The bands were isolated by careful aspiration from the top. Membrane fractions from the gradient were diluted 2-fold with 0.1 M-Tris/HCl (pH 8.0) and centrifuged at 45 min at 140,000 g at 4°C. All membrane fractions were resuspended in 0.1 M-Tris/HCl buffer (pH 8.0).

Membrane-enzyme assays

Succinate dehydrogenase activity was determined by the method of Arrigoni & Singer (1962). NADPH-cytochrome c reductase activity was determined by the method of Omura & Takesue (1970). (Na+/K+)-ATPase was determined by the method of Medzihansky et al. (1971) in the presence of 1 mM-ouabain.

5'-Nucleotidase was assayed at 37°C with 5 mM-5'-AMP in 0.1 M-Tris/HCl buffer (pH 8.0) containing 10 mM-MgCl₂ (Aronson & Touster, 1974). Liberated phosphate was determined as described by Fiske & Subba Row (1925). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Measurement of prostaglandin formation

Membrane fractions (0.5–2.5 mg of protein) were incubated in 1 ml of 0.1 M-Tris/HCl buffer (pH 8.0). In some experiments prostaglandin biosynthesis from exogenous arachidonic acid (Fig. 2) was measured by addition of [¹⁴C]arachidonate (110,000 d.p.m., 2 μg) to the incubation medium. In other incubations Ca²⁺-stimulated prostaglandin generation from endogenous arachidonic acid was determined in the absence or presence of CaCl₂. Incubations were carried out at 37°C for 30 min with shaking and terminated by the addition of 20 vol. of chloroform/methanol (2:1, v/v). The medium was acidified to pH 2.8 with 0.025 M-H₂SO₄, and the organic layer washed with ⅓ vol. of water. The final extract was analysed for prostaglandin E₂ and non-esterified fatty acids as described previously (Erman & Raz, 1979). Briefly, the lipid extract was separated by t.l.c. on silica-gel G plates by using the A IX chromatography system (Hamberg & Samuelsson, 1966). The prostaglandins contents in prostaglandins F₂α, E₂ and D₂ t.l.c. zones were determined by bioassay on rat stomach previously calibrated with the appropriate prostaglandin standard, or counted for radioactivity. The overall recovery of prostaglandins (determined by addition of [³H]prostaglandin E₂ to the incubation medium) was 45–55%. The fatty acids t.l.c. zone was extracted, and methyl esters were prepared and quantitatively determined by g.l.c. (Erman & Raz, 1979).

Materials

Prostaglandins E₂, D₂, F₂α and A₂ were kindly supplied by Dr. U. Axen and Dr. J. E. Pike of Upjohn Co. (Kalamazoo, MI, U.S.A.). Fatty acids standards were obtained from Supelco, Bellefonte, PA, U.S.A. [¹-¹⁴C]Arachidonic acid (sp. radioactivity 55 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Arachidonic acid was obtained from Nu-check (Elysian, MN, U.S.A.). Other reagents were analytical grade.

Results

Characterization of kidney medulla subcellular fractions

The ultrastructural appearance of the membrane fractions I, II, III, IV and V obtained by differential centrifugation and isopycnic-gradient centrifugation (Scheme 1) was analysed by electron microscopy (results not shown). Fraction I is composed mainly of mitochondria and small vesicles with or without ribosomes. Fractions II and V consist almost exclusively of mitochondria. Fraction III was morphologically a microsomal fraction with or without ribosomes attached. Fraction IV is the lightest fraction aspirated from the sucrose gradient and consists mainly of closed smooth-membrane vesicles 0.2–0.4 μm in diameter. This fraction has been previously designated as plasma-membrane fraction (Kempson & Price, 1978).

Distribution and specific activities of marker enzymes in the subcellular fractions are summarized in Table 1. The specific activities of (Na⁺/K⁺)-ATPase and 5'-nucleotidase markers of the plasma-membrane fraction are at least 2.5 times as high in fraction IV as in any other fraction examined. The specific activity of NADPH-cytochrome c reductase, a microsomal marker enzyme, in fraction III is approx. 3-fold that in the plasma-membrane fraction (fraction IV). Succinate dehydrogenase, a mitochondrial marker enzyme, had the highest specific activity in fraction II, which consists mainly of mitochondria.

Prostaglandin biosynthesis in kidney subcellular fractions

The endogenous biosynthesis of prostaglandins is a measure of the combined activities of the lipase(s), which release arachidonic acid from esterified lipids, and the prostaglandin synthase enzymes, which convert this acid into prostaglandin products. To determine first the activities of only the prostaglandin synthase enzymes in the subcellular fractions, we determined the profile of prostaglandins
Table 1. Specific activities of enzyme markers in various subcellular fractions from rabbit kidney medulla
For details see the Methods and materials section. The values are means from at least three preparations; S.E.M. values were less than 5%. Specific activity of succinate dehydrogenase is expressed in nmol of succinate oxidized/min per mg of protein, that of NADPH-cytochrome c reductase is in nmol of cytochrome c reduced/min per mg of protein, and those of (Na⁺/K⁺)-ATPase and 5′-nucleotidase are in nmol of Pi formed/min per mg of protein.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Homogenate</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)*</td>
<td>100</td>
<td>7.8</td>
<td>9.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>103.3</td>
<td>708.3</td>
<td>8.3</td>
<td>53.3</td>
</tr>
<tr>
<td>(Na⁺/K⁺)-ATPase</td>
<td>26.7</td>
<td>25.0</td>
<td>26.5</td>
<td>78.3</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>2.0</td>
<td>1.2</td>
<td>4.5</td>
<td>11.0</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>1.8</td>
<td>1.0</td>
<td>11.2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* Some 60–65% of homogenate protein is associated with the pellet obtained in the first centrifugation (4500g, zero time). This material was discarded.

![Radiochromatogram scan of prostaglandin (PG) products obtained from added [14C]arachidonic acid (AA) in incubations with crude plasma-membrane fraction (a; 1.2 mg of protein), mitochondria (b; 1.2 mg of protein) and microsomal fraction (c; 1.3 mg of protein)](Image)

synthesized from exogenous added arachidonate (Fig. 1). In the three fractions tested (crude plasma membranes, mitochondria and microsomal), the products profiles were similar, with prostaglandin E₂ being the major product. The ratio of prostaglandin E₂ to prostaglandin F₂α in all fractions was 2:1–1.5:1. Significant formation of prostaglandin D₂ (8–10% of product radioactivity) was seen only in the microsomal fraction. 6-Oxoprostaglandin F₁α is formed only in trace amounts, comprising 2–4% of the total radioactivity. Addition of Ca²⁺ ions did not affect the pattern of radioactive prostaglandin products.

We next compared renal subcellular fractions for their capacity to synthesize prostaglandin E₂ from added exogenous arachidonate. The results (Table 2) showed the specific activities of the prostaglandin-synthesizing system for net synthesis of prostaglandin E₂ from added arachidonate to be essentially the same in the mitochondrial and microsomal fractions.

Prostaglandin biosynthesis from endogenous arachidonate was determined in crude plasma membrane, mitochondrial and microsomal fractions. The fractions were incubated without added arachidonate and in the absence or presence of Ca²⁺, and the generation of prostaglandin E₂ was determined (Table 3). In the absence of Ca²⁺, basal release of prostaglandins consisted of prostaglandins E₂ and F₂α (molar ratio approx. 2:1–4:1; see Table 3). Ca²⁺ ions stimulated the selective formation of prostaglandin E₂, having no significant effect on prostaglandin F₂α generation (Table 3). A clear differentiation was observed between the plasma membrane (fraction IV) and mitochondrial fractions (fractions II, V) on the one hand, and the microsomal fraction (fraction III) on the other. First, the specific activities for prostaglandin E₂ biosynthesis in the plasma membrane and mitochondrial fractions are considerably higher than that of the microsomal fractions. Secondly, in the first two fractions prostaglandin E₂ generation was stimulated by Ca²⁺ ions, whereas synthesis in the microsomal fraction was unaffected by Ca²⁺. Thus, compared with the other subcellular fractions, the
Table 2. Prostaglandin E₂ biosynthesis in subcellular fractions from rabbit kidney medulla

Membrane fractions were incubated with various amounts of added arachidonic acid for 30 min at 37°C with shaking and the media were analysed for prostaglandin E₂ content. For details see the Methods and materials section. Values in the Table are differences between prostaglandin E₂ content at the end of incubation and the corresponding zero-time values, and are means from six experiments (S.E.M. values were less than 5%). Zero-time values (pmol/min per mg of protein) of prostaglandin E₂ were: fraction I, 6; fraction II, 8; fraction III, 6; fraction IV, 8.

<table>
<thead>
<tr>
<th>Arachidonate added (µg)</th>
<th>Fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td></td>
<td>21</td>
<td>14</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>45</td>
<td>47</td>
<td>45</td>
<td>55</td>
</tr>
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<td>20</td>
<td></td>
<td>63</td>
<td>84</td>
<td>68</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 3. Prostaglandin E₂ biosynthesis in kidney-medulla purified subcellular fractions

The fractions were incubated in the absence or presence of 2 mM-Ca²⁺ and prostaglandin E₂ synthesis was determined. For details see the Methods and materials section. Values are means ± S.E.M. for seven experiments. Prostaglandin F₂₀ formation (pmol/min per mg of protein) in the six fractions was: I. 6.5–8.0; II. 3.5–5.0; III. 3.0–4.0; IV. 9.5–12.5; V. 4.5–6.5; VI. 4.5–7.0. Within each experiment, addition of 2 mM-CaCl₂ (final concn.) to any of the fractions increased prostaglandin F₂₀ by only 5–15%.

<table>
<thead>
<tr>
<th>Prostaglandin E₂ biosynthesis (pmol/min per mg of protein)</th>
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<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
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<tr>
<td>IV</td>
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<tr>
<td>V</td>
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<tr>
<td>VI</td>
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</table>

Discussion

The results of this work demonstrate the existence of three separate prostaglandin-generating activities in three subcellular fractions from rabbit kidney medulla. These activities are associated with the plasma membrane, mitochondrial and microsomal fractions. The prostaglandin-generating systems in these three fractions convert arachidonic acid mainly into prostaglandins E₂ (major product) and F₂₀. The specific activities of prostaglandin E₂ biosynthesis from added non-esterified arachidonate are essentially the same in the plasma membrane, mitochondrial and microsomal fractions (Table 2), indicating that cellular prostaglandin endoperoxide synthase activity is not selectively associated with the microsomal fraction. The potent capacity of the plasma membrane and mitochondrial fractions to synthesize prostaglandin E₂ is even more clearly seen when measuring prostaglandin generation from endogenous, lipid-esterified, arachidonate. Under this assay condition, renal prostaglandin biosynthesis activities in the mitochondria and plasma-membrane fractions are respectively 2.5- and 3.0-fold higher than the microsomal activity (Table 3). The biosynthetic activity of the mitochondria and plasma-membrane fractions (but not the microsomal fraction) exhibits stimulation by Ca²⁺ ions (Table 3). This Ca²⁺-dependent increase in prosta-
Lipolysis and prostaglandin generation in kidney

Prostaglandin production is the result of phospholipase A₂ activation, which leads to increased release of arachidonic acid and linoleic acid. Ca²⁺ stimulated selectively the formation of prostaglandin E₂, suggesting a tight coupling between the Ca²⁺-stimulated phospholipase A₂ and prostaglandin endoperoxide synthase/prostaglandin H₂ isomerase enzymes.

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References

Wells, M. A. (1972) Biochemistry 11, 1030–1041