Enzymic coupling of acylhydrolase and prostaglandin synthase activities in subcellular fractions from rabbit renal medulla

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(Received 29 October 1981/Accepted 12 November 1981)

We have recently shown that mitochondrial and plasma-membrane fractions from kidney medulla possess Ca^{2+} -stimulated acylhydrolase and prostaglandin synthase activities. The nature of the enzymic coupling between the Ca^{2+} -stimulated arachidonic acid release and its subsequent conversion into prostaglandins was investigated in subcellular fractions from rabbit kidney medulla. Plasma-membrane, mitochondrial and microsomal fractions were found to have similar apparent K_m values for conversion of added exogenous arachidonate into prostaglandins. The rate of prostaglandin biosynthesis ($V_{\max.}$) from added arachidonic acid in the microsomal fraction was approx. 2-fold higher than in the other subcellular fractions. In contrast, prostaglandin E_2 synthesis from endogenous arachidonate in plasma-membrane and mitochondrial fractions was 3–4-fold higher than in microsomes. Furthermore, Ca^{2+} stimulated endogenous arachidonate deacylation and prostaglandin E_2 generation in the former two fractions but not in microsomes. In mitochondrial or crude plasma-membrane fractions, in which prostaglandin biosynthesis was inhibited with aspirin, arachidonate released from these fractions was converted into prostaglandins by the microsomal prostaglandin synthase. Thus an intracellular prostaglandin generation process that involves inter-fraction transfer of arachidonic acid can operate. Prostaglandin generation by such an inter-fraction process is, however, less efficient than by an intra-fraction process, where arachidonic acid released by mitochondria or crude plasma membranes is converted into prostaglandins by prostaglandin synthase present in the same fraction. This demonstrates the presence of a tight intra-fraction enzymic coupling between Ca^{2+} -stimulated acylhydrolase and prostaglandin synthase enzyme systems in both mitochondrial and plasma-membrane fractions.

Previous studies have shown the cellular prostaglandin biosynthetic activity to reside mainly in the microsomal fraction of tissues (Hamberg & Samuelsson, 1967; Anggard *et al.*, 1972), although in rabbit kidney medulla activity was also associated with the mitochondria and plasma-membrane fractions (Bohman & Larsson, 1975). Recently we demonstrated the presence and characterized three distinct prostaglandin synthase activities in the plasma-membrane, mitochondrial and microsomal fractions of rabbit kidney medulla (Erman & Raz, 1981). In the plasma membrane and mitochondrial fractions, but not in microsomes, Ca^{2+} stimulated the hydrolysis of arachidonic acid and linoleic acid from lipids with concomitant conversion of arachidonic acid into prostaglandin E_2 (Erman & Raz, 1981).

In the present report we investigated the nature of

the enzymic coupling between the Ca^{2+} -stimulated arachidonate release and its subsequent conversion into prostaglandin products. The aims of the study were to: (1) characterize the arachidonate-releasing acylhydrolase activities in renal medulla subcellular fractions; (2) contrast the three subcellular fractions with regard to prostaglandin E_2 biosynthesis from exogenous added arachidonate versus endogenous esterified arachidonic acid; (3) determine the feasibility of arachidonate transfer between different subcellular fractions, i.e. the ability of arachidonate released by one subcellular fraction to be utilized by the prostaglandin synthase of a different fraction; (4) assess the overall efficiency of esterified arachidonic acid conversion into prostaglandin products when the process is carried out within one subcellular fraction (intra-fraction process) in comparison with

the efficiency of prostaglandin formation when the participation of two subcellular fractions is required (inter-fraction process).

Methods and materials

Incorporation of radioactive arachidonic acid into cellular lipids

Medulla slices (5–6 g), prepared as described previously (Erman & Raz, 1979), were incubated with 7.0 μ Ci of [1- 14 C]arachidonic acid for 30 min at 37°C with shaking. Control slices were similarly incubated without added arachidonate. After the incubation the medium was discarded and the slices were rinsed twice with 0.1 M-Tris/HCl buffer (pH 8.0) containing bovine serum albumin (2 mg/ml).

Preparation of medullary subcellular fractions

Medulla slices, pre-incubated with or without [1- 14 C]arachidonate, were homogenized (5 ml of buffer/g of tissue) in 0.1 M-Tris/HCl buffer (pH 8.0) for 20 s at 4°C in a Sorvall Omnimixer. In some experiments part of the homogenate was incubated with aspirin (2 mM final concentration) for 10 min at 37°C to inhibit the prostaglandin synthase. The medullary homogenate was centrifuged at 4500 g for zero time (centrifugal force brought to 4500 g and centrifugation immediately stopped) and the pellet was discarded. The supernatant was centrifuged at 1500 g for 15 min to obtain a crude plasma-membrane fraction. The 1500 g supernatant was centrifuged at 10000 g for 15 min to yield a mitochondrial pellet. Microsomes were isolated by centrifugation of mitochondrial supernatant at 140000 g for 60 min. Electron micrographs and enzyme markers have shown the pellets obtained at 1500 g, 10000 g and 140000 g to be crude plasma-membrane, mitochondrial and microsomal fractions respectively (Erman & Raz, 1981). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurement of prostaglandin formation in medullary subcellular fractions

Subcellular fractions (1.0–2.0 mg of protein) were incubated in 1 ml of 0.1 M-Tris/HCl buffer (pH 8.0) for 3–30 min at 37°C in tubes (open to the air) with shaking. In some experiments incubations were done in the presence of [1- 14 C]arachidonic acid (6–66 μ M, 10^5 d.p.m.) (Figs. 1 and 2), whereas in others CaCl_2 was added (1–5 mM final concentrations) (Fig. 3, Table 1). Incubations were terminated by addition of 20 vol. of chloroform/methanol (2:1, v/v), extracted as described previously (Erman & Raz, 1981) and analysed for prostaglandins and non-esterified fatty acids. The lipid extract was separated by t.l.c. on silica-gel G plates using the AIX

chromatography system (Hamberg & Samuelsson, 1966), which allows separation of 6-oxoprostaglandin $\text{F}_{1\alpha}$, prostaglandin $\text{F}_{2\alpha}$, E_2 and D_2 . Each prostaglandin zone was scraped and, depending on the experiment, either counted for radioactivity or extracted with 10 ml of methanol. The methanol extract was evaporated to dryness, dissolved in Tris buffer, and the content of prostaglandin E_2 was determined by bioassay on rat stomach previously calibrated with prostaglandin E_2 standard. The overall recovery of unlabelled generated prostaglandins in the course of the extraction, t.l.c. separation and extraction from t.l.c. plates was 45–55% as determined by addition of [^3H]prostaglandin E_2 to the incubation medium before extraction. The fatty-acid t.l.c. zones were extracted and methyl esters were prepared and quantitatively determined by g.l.c. (Erman & Raz, 1979).

The major prostaglandin products (radioactive and non-radioactive) in the incubations were prostaglandins E_2 and $\text{F}_{2\alpha}$ (molar ratio 2:1–1.5:1). The content of 6-oxoprostaglandin $\text{F}_{1\alpha}$ produced was negligible (2–4% of the total prostaglandin products) (Erman & Raz, 1981).

Materials

Prostaglandins $\text{F}_{2\alpha}$, E_2 , D_2 and A_2 were kindly supplied by Dr. U. Axen and Dr. J. E. Pike of Upjohn Co. (Kalamazoo, MI, U.S.A.). [1- 14 C]-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.). [5,6,8,11,12,14,15(n)- ^3H]Prostaglandin E_2 (sp. activity 86 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Aspirin was obtained from Assia Pharmaceuticals, Petah-Tikva, Israel. Fatty acid-poor bovine serum albumin was obtained from Calbiochem (San Diego, CA, U.S.A.). Fatty acid standards were obtained from Supelco (Bellefonte, PA, U.S.A.). Other reagents were analytical grade.

Results and discussion

Prostaglandin biosynthesis in kidney medulla subcellular fractions: exogenous non-esterified arachidonate versus endogenous esterified arachidonate as substrates for prostaglandin E_2 generation

Plasma membranes, mitochondria and microsomes synthesized prostaglandins from added exogenous arachidonic acid with major products being prostaglandins E_2 and $\text{F}_{2\alpha}$ (Erman & Raz, 1981). Prostaglandin generation (measured as prostaglandin E_2) in all fractions was dependent on incubation time (Fig. 1a), the amount of fraction employed (Fig. 1b), and unaffected by CaCl_2 concentration (0–10 mM) in the incubation medium.

Fig. 1. Effect of incubation time and amount of membrane fraction on biosynthesis of prostaglandin E₂ from added arachidonic acid by kidney medulla subcellular fractions

Fractions (1–2 mg of protein) were incubated with arachidonic acid (66 μ M, 100000 d.p.m.) in 0.1 M-Tris buffer at 37°C for various times (a). In other experiments various amounts of membrane fractions were incubated for 6 min (b). The membrane fractions were: 1500 g, $\bullet$; mitochondria, $\blacksquare$; microsomes, $\blacktriangle$. Values plotted are means of four experiments with S.E.M. being less than 7%.

Lineweaver–Burk plots (Fig. 2) revealed identical apparent K_m ($K_{m,app.}$) values of approx. 14 μ M for the three subcellular fractions. Although these $K_{m,app.}$ values are for the combined reactions of prostaglandin endoperoxide synthase (converts arachidonate into prostaglandin G₂ or H₂) and prostaglandin endoperoxidase isomerase (converts prostaglandin H₂ into prostaglandin E₂), the fact that all three fractions have the same $K_{m,app.}$ values strongly suggests that they contain endoperoxide synthase and endoperoxide isomerase having very similar or identical properties. The $V_{max.}$ value for prostaglandin E₂ synthesis from exogenous arachidonate is highest in the microsomes 2.0 (nmol of prostaglandin E₂/mg of protein per 6 min) with mitochondria and plasma membranes having values of 1.2 and 0.9 respectively. Thus, the rate of prostaglandin E₂ formation from exogenous arachidonate is approx. 2-fold higher in microsomes in comparison with the other fractions. In marked contrast, basal endogenous prostaglandin E₂ generation from lipid-esterified arachidonate is 3–4 times higher in the plasma-membrane and mitochondrial fractions when compared with microsomes (Table 1). Furthermore, the former two fractions showed stimulation of prostaglandin E₂ formation by Ca²⁺. This Ca²⁺ effect was due to stimulation of Ca²⁺-dependent acylhydrolase activity in mitochondrial and plasma-membrane fractions (but not in microsomes) with the resulting increased release of arachidonic, linoleic and, to a lesser extent, also oleic acids (Table 1).

Fig. 2. Effect of different substrate concentrations on the activity of prostaglandin synthase in renal medulla subcellular fractions

Results are presented as a Lineweaver–Burk plot. The subcellular fractions (1–2 mg of protein) were incubated at 37°C for 6 min with different concentrations of arachidonic acid (6–66 μ M) and prostaglandin E₂ was determined. Membrane fractions were: 1500 g, $\bullet$; mitochondria, $\blacksquare$; microsomes, $\blacktriangle$. Values plotted are means of three experiments with S.E.M. being less than 10%.

Inter-fraction transfer of arachidonic acid and concomitant prostaglandin E₂ biosynthesis

The fact that medullary microsomes are fully active in converting added arachidonic acid into

prostaglandins yet they do not contain significant acylhydrolase activity raised the possibility that microsomal generation of prostaglandins may be enhanced by transfer of endogenous arachidonate from the plasma-membrane or mitochondrial frac-

Table 1. *Release of fatty acids and prostaglandin E₂ from kidney medulla subcellular fractions*

Subcellular fractions were incubated at 37°C for 30 min in 0.1 M-Tris buffer (pH 8.0). Ca²⁺ (2 mM final concentration) was added where indicated. After incubation the media were analysed for prostaglandin E₂ and fatty acids as described in the Methods and materials section. Values given are means of four experiments. Standard errors of the mean were less than 7%. In the 1500 g and mitochondrial fractions, values for release of linoleic acid and arachidonic acid and of prostaglandin E₂ in the absence or presence of CaCl₂ were significantly different ($P < 0.05$) from each other.

Fraction	Addition to incubation medium	Prostaglandin E ₂ released (μg/mg of protein)	Fatty acids released (μg/mg of protein)			
			C _{16:0} + C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}
1500 g	—	0.20	11.5	4.20	3.45	0.56
	2 mM-CaCl ₂	0.51	12.5	5.70	5.45	2.30
Mitochondria	—	0.16	12.3	5.20	4.10	0.30
	2 mM-CaCl ₂	0.27	13.5	6.60	6.00	0.96
Microsomes	—	0.05	13.3	4.40	2.60	0.15
	2 mM-CaCl ₂	0.05	14.7	5.10	3.40	0.23

tions to the microsomes. To test the possibility of such inter-fraction transfer of arachidonate, medulla slices were first pre-incubated with [¹⁴C]arachidonic acid to label the medullary lipids. During the incubation approx. 40–45% of added [¹⁴C]arachidonate was incorporated into cellular lipids, mostly phospholipids (30–35%) and neutral lipids (9–11%). Approx. 13–15% of the radioactive acid was converted into prostaglandins E₂ and F_{2α} and 38–43% of the acid was recovered from the medium. Medulla homogenates were prepared from pre-labelled and unlabelled slices. The homogenates were divided into equal portions, with one portion being incubated with aspirin to inhibit prostaglandin biosynthesis and the other portion similarly incubated without aspirin. After that, the homogenates were subfractionated to obtain crude plasma-membrane, mitochondrial and microsomal fractions. Aspirin at the concentration used did not affect basal or Ca²⁺-stimulated release of arachidonate (or other fatty acids) from medulla subcellular fractions. Hence in ¹⁴C-labelled crude plasma-membrane and mitochondrial fractions, which were treated with aspirin, prostaglandin production was inhibited by 90–95%, whereas [¹⁴C]arachidonate release was unaffected. We thus had fractions of crude plasma membrane and mitochondria that possessed lipase activity but no prostaglandin synthase activity and fractions that possessed both activities. Recombination experiments were performed in which aspirin-treated [¹⁴C]arachidonate-labelled crude plasma-membrane or mitochondrial fractions were incubated with unlabelled microsomal fraction and formation of [¹⁴C]prostaglandins was measured. No significant radioactive prostaglandin formation was seen in incubations of aspirin-treated plasma-membrane or mitochondrial fractions (Table 2). Addition of untreated microsomes stimulated severalfold the formation of radioactive prostaglandins, where-

as addition of aspirin-treated microsomes was without effect (Table 2). These data strongly suggested the presence of an inter-fraction transfer process in which [¹⁴C]arachidonic acid released from plasma-membrane or mitochondrial fractions is utilized as substrate by the microsomal prostaglandin synthase.

The data in Table 1 showing Ca²⁺ stimulation of fatty acid release and prostaglandin E₂ production in plasma-membrane and mitochondrial fractions, but not in the microsomal fraction, provided us with an appropriate tool with which we could augment endogenous arachidonate release from specific fractions and measure the extent of the inter-fraction arachidonate transfer to the microsomes, and subsequent prostaglandin biosynthesis. Plasma-membrane or mitochondrial fractions isolated from aspirin-treated homogenate showed no detectable prostaglandin synthase activity, although release of arachidonate from cellular phospholipids is greatly augmented by increasing Ca²⁺ concentrations (Erman & Raz, 1981). When, however, the same fractions were incubated with untreated microsomal fraction, significant prostaglandin E₂ biosynthesis was observed. Since neither arachidonate release nor prostaglandin E₂ production in the microsomal fraction is enhanced by Ca²⁺ (Table 1), these results demonstrate that arachidonic acid that was released from the crude plasma membranes (Fig. 3a) or mitochondria (Fig. 3b) was converted into prostaglandin E₂ by the microsomal prostaglandin synthase. Hence, a Ca²⁺-stimulated inter-fraction process can operate in which arachidonic acid released after acylhydrolase activation in one subcellular fraction would be utilized as substrate for prostaglandin biosynthesis in another subcellular fraction.

The overall formation of prostaglandin E₂ by this inter-fraction transfer of arachidonate is, however,

Table 2. *Radioactive prostaglandin production in renal medulla subcellular fractions*

Medulla slices were incubated with [$1\text{-}^{14}\text{C}$]arachidonate ($7\text{ }\mu\text{Ci}$ per 8 g of slices) to prelabel the medullary lipids. The subsequently isolated homogenate was incubated with or without aspirin (ASA) and subcellular fractions were prepared. The fractions (1.5 mg of protein each) were incubated in 0.1 M -Tris/HCl buffer ($\text{pH } 8.0$) for 30 min at 37°C with shaking. After the incubation the media were extracted and the extract subjected to t.l.c. for separation of prostaglandins. For details see the Methods and materials section. Values given are means $\pm$ S.E.M. of three experiments for combined radioactivity in prostaglandin $\text{R}_{2\alpha}$ and E_2 zones. All values except those marked a or b are significantly different from each other ($P < 0.05$). * indicates fractions from slices prelabelled with [^{14}C]arachidonic acid.

Subcellular fractions	Prostaglandins production (c.p.m.)
$1500\text{ g}^*\text{-ASA}\dagger$ (no incubation)	170 ± 15
$1500\text{ g}^*\text{-ASA}$	370 ± 20^a
$1500\text{ g}^*\text{-ASA} + \text{microsomes-ASA}$	380 ± 20^a
$1500\text{ g}^*\text{-ASA} + \text{microsomes}$	1130 ± 40
Mitochondria $^*\text{-ASA}\ddagger$ (no incubation)	140 ± 15
Mitochondria $^*\text{-ASA}$	350 ± 20^b
Mitochondria $^*\text{-ASA} + \text{microsomes-ASA}$	330 ± 20^b
Mitochondria $^*\text{-ASA} + \text{microsomes}$	820 ± 30

$\dagger$ Fraction contained $10\,350 \pm 300\text{ c.p.m./mg}$ of protein.

$\ddagger$ Fraction contained $11\,160 \pm 280\text{ c.p.m./mg}$ of protein.

Fig. 3. *Endogenous prostaglandin biosynthesis in subcellular fractions treated or untreated with aspirin (ASA)*

Medullary homogenate was incubated (10 min , 37°C) in the absence or presence of aspirin. Membrane fractions were subsequently prepared and incubated in 0.1 M -Tris buffer ($\text{pH } 8.0$) at 37°C for 30 min with shaking in the absence or presence of different concentrations of Ca^{2+} . Results of prostaglandin E_2 biosynthesis are given for a representative experiment. Similar results were obtained in five additional experiments. The differences in prostaglandin E_2 formation in incubations of $1500\text{ g} + \text{microsomal fractions}$ versus 1500 g fraction alone (a) or in incubations of mitochondria + microsomes versus mitochondria alone (b) were approx. equal to prostaglandin E_2 generation by the microsomes. Protein concentration of the various fractions in the incubations were: microsomes, 1.1 mg/ml ; 1500 g-ASA , 2.3 mg/ml ; 1500 g control, 2.3 mg/ml ; mitochondria-ASA, 2.0 mg/ml ; mitochondria control, 2.0 mg/ml . (a) $1500\text{ g-ASA} + \text{microsomes-ASA}$, $\bullet$; microsomes, $\square$; $1500\text{ g-ASA} + \text{microsomes}$, $\blacktriangle$; 1500 g , $\circ$; $1500\text{ g} + \text{microsomes}$, $\blacksquare$. (b) Mitochondria-ASA + microsomes-ASA, $\circ$; mitochondria-ASA + microsomes, $\triangle$; mitochondria, $\blacktriangle$; mitochondria + microsomes, $\bullet$.

less efficient than by an intra-fraction process. Thus when comparing the prostaglandin biosynthetic activities of aspirin-treated plasma-membrane and mitochondrial fractions against the activities of the untreated fractions, the latter fractions appear to convert more arachidonate into prostaglandin E_2 (Fig. 3). Furthermore, the presence of additional microsomal prostaglandin synthase activity in the incubation did not yield additional net increase in prostaglandin E_2 biosynthesis, indicating no apparent release of non-esterified arachidonate from these fractions. It thus appears that in both crude plasma membranes and mitochondria there is a tight enzymic coupling between the Ca^{2+} -stimulated acylhydrolase activity and the prostaglandin synthase activity, leading to effective intra-fraction lipolysis and prostaglandin formation.

The apparent absence of demonstrable Ca^{2+} -stimulated phospholipase A_2 activity in medullary microsomes is intriguing. This subcellular fraction may indeed contain very little phospholipase A_2 enzyme. On the other hand, studies in platelets (Wong & Cheung, 1979) have shown that calmodulin augments Ca^{2+} -stimulated phospholipase A_2 activity and also enhances the conversion of [^{14}C]arachidonic acid into thromboxane A_2 . It is possible that during our preparation of the medullary microsomes dilution or partial removal of bound calmodulin has occurred, thereby markedly limiting the activity of Ca^{2+} -stimulated phospholipase A_2 in the microsomes. The effect of calmodulin on this lipolytic reaction and on prostaglandin biosynthesis in kidney medulla subcellular fractions requires further investigation.

This work was supported by a grant from the Israel

Commission for Basic Research of the Israel National Academy of Sciences, and by a grant from the U.S.-Israel Binational Science Foundation (BSF), Jerusalem, Israel.

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