Biochemical actions of vasoactive peptide hormones

Time-synchronized activation of lipolysis and decreased fatty-acid release by bradykinin and angiotensin in the perfused rabbit kidney

Michal SCHWARTZMAN and Amiram RAZ

Department of Biochemistry, The George S. Wise Center of Life Sciences, Tel-Aviv University,
Ramat-Aviv, Tel-Aviv, Israel

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Bradykinin and angiotensin administered to the isolated perfused rabbit kidney activate two sequential processes: (1) a selective release of the prostaglandin precursor arachidonate with concomitant partial conversion of the arachidonate into prostaglandin E₂; (2) activation of a process that leads to decreased release of all fatty acids in the perfusate. There is a time lag of approx. 1 min between the initial activation of the arachidonate-specific deacylation reaction that is coupled to prostaglandin generation, and the subsequent decrease in the release of all fatty acids. This synchronized cycle provides for instant generation of required amounts of prostaglandins and at the same time serves to conserve cellular arachidonate.

The vasoactive peptide hormones bradykinin and angiotensin evoke the release of prostaglandins from the perfused kidney, heart and other tissues (McGiff *et al.*, 1972; Needleman *et al.*, 1975*a,b*; Blumberg *et al.*, 1977). Studies in which the tissue lipids were prelabelled with radioactive arachidonate (the precursor molecule of prostaglandins) indicated that bradykinin or angiotensin administration leads to release of radioactive prostaglandins but not of precursor arachidonate (Isakson *et al.*, 1977). Subsequent studies (Isakson *et al.*, 1977; Hsueh *et al.*, 1977) have shown that release of arachidonate is seen only when the organ is perfused with serum albumin, which traps the released fatty acids. These studies (Isakson *et al.*, 1977; Hsueh *et al.*, 1977) also provide direct evidence that bradykinin and angiotensin stimulate intracellular lipolysis. The properties of this unique hormone-mediated lipolysis are as follows. (1) There is a selective release of only arachidonate. (2) The amount of arachidonate being released is 2–5-fold in excess of the amount converted into prostaglandins (Isakson *et al.*, 1977; Hsueh *et al.*, 1977; Schwartzman & Raz, 1979). (3) The entire hormone-induced event is a tightly coupled process in which a certain percentage of the released arachidonate is converted into prostaglandins. The amount of prostaglandins produced is dependent on the dose of the administered hormone and is essentially unaffected by extracellular factors (e.g. serum albumin) (Schwartzman & Raz, 1979).

(4) In the absence of albumin in the perfusate, excess arachidonate released by the stimulating hormones but not converted into prostaglandins is apparently re-acylated into tissue lipids (Schwartzman & Raz, 1979). As part of our study on the chain of biochemical reactions initiated by the hormones, this investigation was undertaken to determine whether the hormones' effects are limited to the selective hydrolysis of esterified arachidonate, or whether the hormones also affect the deacylation–acylation balance of the other cellular fatty acids.

Materials and methods

Perfusion of kidneys

Ureter-obstructed rabbit kidneys, previously shown (Nishikawa *et al.*, 1977) to be supersensitive to the release of prostaglandins by bradykinin and angiotensin, were prepared and perfused with Krebs–Henseleit buffer, pH 7.4 (bubbled with O₂/CO₂, 19:1), at the rate of 15 ml/min (Nishikawa *et al.*, 1977). The kidney effluent superfused a battery of smooth-muscle assay organs (rat stomach and rabbit aorta). A mixture of antagonists was infused into the kidneys effluent rendering the assay organs insensitive to acetylcholine, histamine, serotonin and catecholamines (Isakson *et al.*, 1977). The kidneys were perfused for 3–4 h with periodic alternate injections of bradykinin (0.5 µg) and angiotensin (1 µg) before initiation of the experiments. A

minimum of 30 min was allowed between injection of different hormones and 60 min between injections of the same hormone. An experimental set consisted of collection of control (pre-hormone) effluent followed by bolus injections of the hormones (in 0.1 ml of saline) and collection of post-hormone effluent. The initial amount of release of both prostaglandin E_2 and fatty acids is quite variable (2–3-fold) between individual kidneys. Furthermore the onset and time course for the development of supersensitivity to hormone-stimulated release of prostaglandins and arachidonate is also quite variable between individual ureter-obstructed kidneys. Thus, some kidneys begin to show significant increase (i.e. 50% or more) in basal and hormone-mediated release after 2.5 h of perfusion, whereas other kidneys require 4 h of perfusion for similar response. Yet, despite these variations between individual kidneys the same pattern of changes and quantitative differences were observed in all experiments of the same type.

Analysis of kidney effluent

Effluent samples were acidified to pH 3.5 with 2 M-formic acid and extracted twice with 100 ml of ethyl acetate. The combined extract was dried over Na_2SO_4 and portions (15% of extract) were separated by t.l.c. (silica gel G plates; Merck) with either the A-9 system (organic phase of ethyl acetate/iso-octane (2,2,4-trimethylpentane)/acetic acid/water; 11:5:2:10, by vol.) (Hamberg & Samuelsson, 1966) or system C (chloroform/methanol/acetic acid/water; 90:8:1:0.8, by vol.) (Nugteren & Hazelhof, 1973) as solvents. In agreement with previous data (Blumberg *et al.*, 1977) prostaglandin E_2 was the main (>90%) prostaglandin released after stimulation with either hormone. The prostaglandin E_2 zone from each chromatograph was extracted with chloroform/methanol (2:1, v/v), the extract evaporated to dryness, dissolved in 0.1 M-Tris buffer, pH 8.0, and the prostaglandin E_2 content was determined by bioassay on strips of rat stomach and chick rectum (Isakson *et al.*, 1977). The remainder of each extract was separated by t.l.c. with light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (35:15:1, by vol.) as the developing solvent. The non-esterified-fatty-acids zone was similarly extracted and evaporated to dryness. The fatty acids were methylated with freshly prepared diazomethane and analysed by g.l.c. (Packard Instruments; model 417, equipped with flame-ionization detector). The column (2 m × 6 mm) was packed with Supelco SP 2340. Separation was done with temperature programming from 175°C to 235°C. Recovery of prostaglandin E_2 and fatty acids was determined by addition to each effluent sample of [3H]prostaglandin E_2 (10^5 c.p.m.) and 25 µg of $C_{22:5}$ fatty acid respectively. The overall recovery for prostaglandin E_2 was 50–60% and for fatty acid

methyl esters 28–42%. The latter value is composed of the following recoveries in the several stages of preparation: (1) acidic ethyl acetate extraction, 75–85%; (2) drying and concentration by rotary evaporation, 82–88%; (3) isolation from thin-layer plates after chromatography, 63–70%; (4) concentration transfer to methylation tubes, methylation; 80–85%. In each sample the overall percentage recovery was the same for all individual fatty acids. Despite the somewhat low overall recovery, the gas-chromatographic analysis of appropriate portions gave sharp peaks of one-fifth to full-scale height (at suitable sensitivity), thus enabling accurate calculation of fatty-acid composition.

Incorporation of radioactive linoleate into kidney lipids

[1- ^{14}C]Linoleate (100 µg; 4×10^7 d.p.m.) was dissolved in 6 ml of 0.1 M-Tris/HCl buffer, pH 8.2, and infused (0.15 ml/min) into the perfusion medium as it enters the kidney. Afterwards, the kidneys were perfused for 15 min with media containing 1 mg of bovine serum albumin/ml. After an equilibrium period of 4 h the kidneys were stimulated with either bradykinin (0.5 µg) or angiotensin (1 µg) and removed from the perfusion apparatus after 3 min. The kidneys were immediately sliced longitudinally in half and frozen in liquid air. The frozen kidneys were quickly minced, homogenized in 20 ml of Tris/HCl buffer (0.1 M, pH 8.0) and extracted for total lipids by the Folch extraction method (Folch & Lees, 1957). A portion of the lipid extract was analysed by t.l.c. (light petroleum/diethyl ether/acetic acid, 35:15:1, by vol.), and the radioactivity in the phospholipids and fatty-acids zones was determined.

Materials

Male rabbits (local strain weighing 2–2.5 kg) were employed. Angiotensin was obtained from CIBA (Summit, NJ, U.S.A.), and bradykinin from Sigma (St. Louis, MO, U.S.A.). [3H]Prostaglandin E_2 (sp. radioactivity 86 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.) and [1- ^{14}C]linoleate (sp. radioactivity 55.5 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Prostaglandins $F_{2\alpha}$, F_2 , D_2 and A_2 were kindly supplied by Dr. J. E. Pike of the Upjohn Co. (Kalamazoo, MI, U.S.A.). Bovine serum albumin (fatty-acid-poor) was from Calbiochem. Docosa-7,10,13,16,19-pentaenoate ($C_{22:5}$) was from Supelco (Bellafonte, PA, U.S.A.).

Results and discussion

Stimulation by bradykinin (Fig. 1a) or angiotensin (Fig. 1b) resulted in a 10–20-fold increase in prostaglandin E_2 release; no change or a very small

Fig. 1. Release of prostaglandin E_2 , arachidonate and total fatty acids from perfused ureter-obstructed rabbit kidneys

Kidney effluent samples collected for 4 min before (control) and immediately after bolus injection of bradykinin ($0.5 \mu\text{g}$) or angiotensin ($1 \mu\text{g}$). Although there are variations between individual kidneys, the same pattern of changes and quantitative differences as seen in the Figure were observed in all experiments (total of eight kidneys, each receiving the experimental protocol as outlined). Results in each experiment were normalized so that a value of 1 was assigned to the pre-hormone level of release. The percentage change after hormone stimulation was calculated for each parameter, and these values were averaged and analysed by Student's t test. * Indicates post-hormone values that are significantly different ($P < 0.05$) from control values. In general, experiments were started 3–4 h after initiation of kidney perfusion and performed in the sequence: (1) hormonal stimulation without albumin infusion (*a* and *b*); (2) bovine serum albumin infusion (1 mg/ml) and hormone stimulation during infusion through the kidney (*c* and *d*). Experiments were also conducted in which experiments in (*c*) and (*d*) preceded those in (*a*) and (*b*), and the results were essentially identical. Basal release of fatty acids after 3–4 h of perfusion (control values in *a* and *b*) was: $C_{14:0}$ 11–15 μg ; $C_{16:0}$ 32–43 μg ; $C_{16:1}$ 2–3 μg ; $C_{18:0}$ 15–22 μg ; $C_{18:1}$ 25–33 μg ; $C_{18:2}$ 9–14 μg ; $C_{20:3}$ 0.3–0.5 μg ; $C_{20:4}$ (arachidonate), 1.4–2.0 μg . A gradual increase in the release of all fatty acids was seen with perfusion time, reaching a 50–75% higher level after 7–8 h of perfusion. Total release of fatty acids during albumin infusion (control values in *c* and *d*) was approx. 2-fold higher than the basal release. The fatty-acid composition in the effluent, after 3–4 h of perfusion and during albumin infusion was: $C_{14:0}$ 9–14 μg ; $C_{16:0}$ 50–68 μg ; $C_{16:1}$ 7–9 μg ; $C_{18:0}$ 25–32 μg ; $C_{18:1}$ 60–73 μg ; $C_{18:2}$ 50–65 μg ; $C_{20:3}$ 0.8–1.1 μg ; $C_{20:4}$ 3–5 μg .

increase (10–15%) in arachidonate release was observed. In contrast there was a pronounced decrease of 35–50% in the release of total fatty acids after administration of bradykinin or angiotensin.

Experiments in which fatty-acid-free bovine serum albumin was added to the perfusing media provide clear differentiation between the hormone-induced changes in prostaglandin E_2 and arachidonate release and the changes in total fatty-acid release. Albumin increases approx. 2-fold the basal release of all fatty acids (cf. left-hand columns of Figs. 1*c* and 1*d* with left-hand columns of Figs. 1*a* and *b*). Bolus injections of bradykinin or angiotensin produce a 2–2.5-fold increase in the release of arachidonate and a concomitant decrease of 25–30% in the release of total fatty acids. On a weight basis, the increase in arachidonate release of approx. 3–5 μg is accompanied by a decrease of 40–60 μg in total fatty acid release. Although the results shown (Fig. 1) were obtained with ureter-obstructed kidneys, the same pattern of results was also seen in experiments with normal (unobstructed) rabbit kidneys, except that both basal and hormone-stimulated release of prostaglandin E_2 and arachidonate were considerably lower (by 85–95%) as observed previously (Nishikawa *et al.*, 1977). Total fatty-acid release in normal kidneys was also decreased to 20–25% of that seen in the ureter-obstructed kidneys (results not shown). Thus, despite differences between ureter-obstructed and normal kidneys with regard to responsiveness to hormonal stimulation, both types of kidneys exhibit the same hormone-induced biochemical responses: increased release of arachidonate and decreased release of other fatty acids.

Experiments were performed to differentiate qualitatively and quantitatively between the time course for activation of the lipolytic reaction releasing arachidonate and decreased release of other fatty acids. Experimental design was as described above (Fig. 1), but effluent collections were made for 1-min periods. During the first minute after bolus injection of bradykinin or angiotensin there was an increase in both prostaglandin E_2 and arachidonate release with no change in the release of other fatty acids (Fig. 2). During the subsequent 3 min there was a progressive decline in the release of prostaglandin E_2 , arachidonate and total fatty acids. At 5–7 min after hormone injection, a gradual increase in the release of both arachidonate and fatty acids was seen, the prestimulatory level of release being attained 10–12 min after stimulation. Integrating the area under the arachidonate curve in Fig. 2 between the times 0 and 4 min yields a value that is (depending on the particular experiment) equal to or slightly higher than the value obtained during a 4 min control collection, as was seen in Fig. 1(*a*).

The hormone-induced decrease in non-esterified-fatty-acid efflux from the tissue could be mediated by two basic mechanisms: (*a*) reduction in the pool size of free fatty acids; this could result from either inhibition of the acylhydrolase(s) or by stimulation

of enzymes that participate in the acylation of non-esterified fatty acids into cellular lipid; (b) by inhibiting the release of intracellular non-esterified fatty acids into the extracellular fluid.

To differentiate between these possible mechanisms we measured the effects of the hormones on the ratio of non-esterified/esterified linoleate in the kidney. Kidney lipids were prelabelled with [1-¹⁴C]linoleate as described previously (Isakson *et al.*, 1977). After an equilibrium period of 4 h the kidneys were stimulated with either bradykinin

(2.5 µg), angiotensin (5 µg) or saline only (control) and removed from the perfusion apparatus after 3 min. The kidneys were immediately frozen, extracted for lipids and the lipids separated by t.l.c. and their radioactivity was determined. The ratio of radioactivity in the fatty-acids zone to that in the phospholipids zone was higher in control kidneys compared with kidneys after stimulation with 2.5 µg of bradykinin or 5 µg of angiotensin (Table 1). Thus, the two peptide hormones do appear to induce a significant decrease in the concentration of intracellular non-esterified fatty acids, thus supporting the preposition that the hormones affect the rate of either deacylation or acylation.

Differentiation between the possible hormone effects on decreased deacylation versus increased acylation is a rather complex problem which requires investigation. In preliminary experiments we found that the amount of [¹⁴C]arachidonate, injected as a bolus, that is incorporated into the perfused-kidney lipids was higher immediately after bradykinin or angiotensin administration, indicating that the hormones induce an increased acylation of non-esterified fatty acids into cellular lipids.

The time lag that exists between the activation of arachidonate release and the subsequent decrease in fatty-acid release (Fig. 2) is probably crucial for providing sufficient time for arachidonate to be available as substrate for the prostaglandin cyclooxygenase. The mode of control of this synchronized process is an intriguing question, especially in whole kidney, which is composed of a heterogeneous population of cells. The hormone effects on increased acylation or decreased deacylation may be mediated by the hormones themselves (directly or indirectly via an unknown 'messenger'). Another possible means of control involves arachidonate, which may modulate the increased activity of

Fig. 2. Changes in the rate of release of prostaglandin E₂, arachidonate and total fatty acids from perfused rabbit kidney after stimulation by bradykinin (0.5 µg). Experimental details were the same as in Figs. 1(a) and 1(b) and are given in the text and the legend to Fig. 1. After collection of control effluent for 1 min, bradykinin was administered (indicated by the arrow) and 1 min effluent collections were continued for 12 min. Prostaglandin E₂ and fatty-acid analysis was done as described in the Materials and methods section. Values were plotted for the middle of each collection period, i.e. at 0.5 min for the period 0–1 min etc.

Table 1. Relative distribution of radioactive linoleate in phospholipid and non-esterified-fatty acid fractions. Ureter-obstructed kidneys were prelabelled with [1-¹⁴C]linoleate and then injected with either saline solution (0.1 ml) or saline containing bradykinin (0.5 µg) or angiotensin (1 µg). For other details see the text. Results are given for three experiments in each treatment.

Hormone stimulation	Radioactivity (c.p.m./g of kidney)		Ratio of radioactivity in non-esterified-fatty-acid fraction/phospholipid fraction
	Phospholipid fraction	Non-esterified-fatty-acid fraction	
None	1.730 × 10 ⁶	9.47 × 10 ⁴	0.055
	1.595 × 10 ⁶	8.02 × 10 ⁴	0.050
	1.290 × 10 ⁶	6.95 × 10 ⁴	0.054
Bradykinin	1.750 × 10 ⁶	7.88 × 10 ⁴	0.045
	1.645 × 10 ⁶	6.74 × 10 ⁴	0.041
	1.355 × 10 ⁶	6.01 × 10 ⁴	0.043
Angiotensin	1.735 × 10 ⁶	7.81 × 10 ⁴	0.045
	1.605 × 10 ⁶	6.42 × 10 ⁴	0.040
	1.350 × 10 ⁶	5.54 × 10 ⁴	0.041

* Mean values significantly different (*P* < 0.05) from the mean obtained in the absence of hormone stimulation.

acylation enzymes (for which it is a substrate) or the decreased activity of the deacylation enzymes (for which it is a product). In fact, increased concentrations of arachidonate in the microenvironment of the lipase-cyclo-oxygenase system may serve to both deactivate the hormone-induced lipolytic process and activate the acylation process.

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