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A novel multifunctional metabolic pathway in a marine mollusc leads to unprecedented prostaglandin derivatives (prostaglandin 1,15-lactones)

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The discovery of high levels of prostaglandin (PG) 1,15-lactones of both the E and F series and their co-existence with PGs has been recently described in the opisthobranch mollusc *Tethys fimbria*. The present study was undertaken in order to investigate the biosynthesis of these novel natural PG derivatives *in vivo* using radiolabelled precursors, and to gain a preliminary understanding of their biological role. PGE_2 1,15-lactone was shown to be produced from both PGE_2 and $PGF_{2\alpha}$ in the mollusc mantle and appeared to be quickly transferred to the mollusc dorsal appendices (cerata). The detachment of the latter during the typical defence behaviour of *T. fimbria* was accompanied by the conversion of PGE_2 and PGE_3 1,15-lactones back to the corresponding PGs. Both PGE_2 and PGE_2 1,15-lactone were also shown to be biosynthesized from arachidonic acid. Lactones of the F series were present as 11-acetyl derivatives in *T. fimbria* mantle and as 9- and 11-fatty acyl esters in the mollusc egg-mass and reproductive gland, and their biosynthesis from $PGF_{2\alpha}$ was demonstrated in all of these tissues. A multiple biological role of PG 1,15-lactones in *T. fimbria* defensive behaviour, smooth muscle contraction and egg production/fertilization control is hypothesized. The high amounts of PG derivatives found in *T. fimbria* and the biosynthetic studies described herein indicate that this marine mollusc may be a useful model for future studies on PG biosynthesis.

INTRODUCTION

Prostaglandins (PGs) and other eicosanoids have been detected in several marine and freshwater invertebrate species, and their participation in some aspects of invertebrate physiology, such as regulation of ion fluxes, temperature, hatching, reproduction and cell aggregation, has been suggested (for a review, see Stanley-Samuelson, 1987). After the first report of PGs in an invertebrate organism, the gorgonian coral *Plexaura homomalla* (Weinheimer & Spraggins, 1969), evidence obtained using techniques such as mammalian smooth muscle bioassays, radioimmunoassays and labelled arachidonic acid (AA) incorporation experiments has led to the suggestion that prostanoids are present in, for example, the freshwater mussel *Ligunia subrostrata* (Saintsing *et al.*, 1983), the freshwater snail *Heliosoma durgi* (Kunigelis & Saleuddin, 1986), the mussel *Mytilus californianus* (Morse *et al.*, 1977) and several coelenterates (Morse *et al.*, 1978). Specific PGA_2 binding sites were detected in homogenates of gills, mantle, siphon, adductors, and upper and lower visceral masses from the marine bivalve *Modiolus demissus* (Freas & Grollman, 1981). However, the structures of the eicosanoids involved were elucidated completely in only a few cases, and usually evidence of eicosanoid participation was based on treatment of specimens with a single compound and observation of the response.

We have recently described the isolation and structural characterization of a novel class of PG derivatives, PG 1,15-lactones (Fig. 1), of both the E and F series, from the opisthobranch mollusc *Tethys fimbria* (Cimino *et al.*, 1989, 1991). Studies (Cimino *et al.*, 1991) on the distribution of these compounds and of some of the corresponding PG acids which were also found (but in smaller amounts) showed that the 1,15-lactones of PGE_2, PGE_3, 11-acetyl-PGE_2, 11-acetyl-PGE_3, PGA_2 and PGA_3 were present mainly in the mollusc mucus secretion and dorsal appendices known as cerata, whereas 11-acetyl-$PGF_{2\alpha}$ and 11-acetyl-$PGF_{3\alpha}$ 1,15-lactones were more abundant in the mantle. 9- and 11-fatty acid ester PGF 1,15-lactones, in which the presence of 9- and 11-arachidonyl- and -eicosapentaenoyl-PGF 1,15-lactones is of note, were only found in *T. fimbria* egg masses and reproductive glands (ovotestis).

The structural variety of PG 1,15-lactones found in this marine mollusc, together with the data on their distribution, suggested a range of different biological functions for this interesting novel class of prostanoids and raised intriguing questions about their biosynthesis. The present study was therefore undertaken with the aim of investigating the biogenesis of PG lactones *in vivo* in order to obtain some understanding of the biological role of these metabolites in *T. fimbria*.

MATERIALS AND METHODS

Materials

The molluscs (*Tethys fimbria*, Tethyidae, Dendronotacea, Opisthobranchia) were caught in the bay of Naples and kept in laboratory tanks for up to 4 weeks. PG standards were obtained from Sigma. PGE_2 and $PGF_{2\alpha}$ 1,15-lactones were generously provided by Dr. G. L. Bundy, Upjohn Co., Kalamazoo, MI, U.S.A. H.p.l.c. solvents were purchased from Farmitalia Carlo Erba, Milan, Italy. ^{3}H-labelled PGE_2, $PGF_{2\alpha}$ and AA were from Amersham.

Structural characterization of PG 1,15-lactones

Details of the structural elucidation of *T. fimbria* PG 1,15-lactones have been reported previously (Cimino *et al.*, 1989, 1991). Briefly, the structures of PGE lactones and PGA lactones

Abbreviations used: PG, prostaglandin; AA, arachidonic acid; EPA, eicosapenta-*cis*-5,8,11,14,17-enoic acid; GC, gas chromatography; e.i.m.s., electron-impact mass spectrometry; COSY, two-dimensional correlation spectroscopy.

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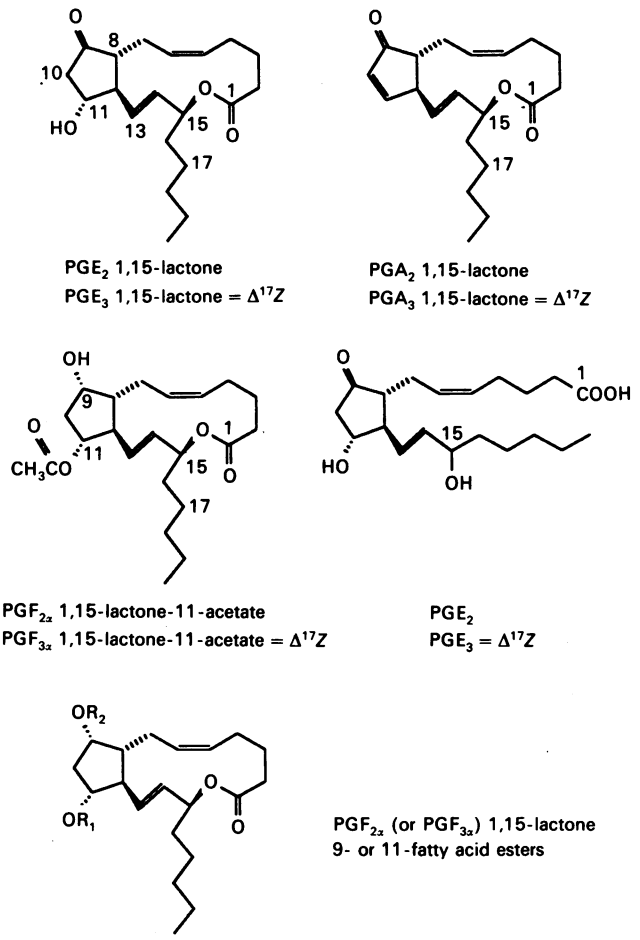

Fig. 1. Structures of PG 1,15-lactones previously isolated from *T. fimbria*

were deduced from both one- and two-dimensional [two-dimensional correlation spectroscopy (COSY) and heteronuclear correlation (HETCOR)] ^{1}H- and ^{13}C-n.m.r. techniques (in C^2HCl_3), and confirmed by electron-impact mass spectroscopy (e.i.m.s.) and by comparison with the e.i.m.s. and n.m.r. data of synthetic PGE_2 1,15-lactone. The absolute configuration of the chiral centres was determined by means of c.d. measurements. Structure elucidation of 11-acetyl-PGF 1,15-lactones was carried out by comparing their ^{1}H-n.m.r. spectra with those of (*a*) synthetic $PGF_{2\alpha}$ 1,15-lactone; (*b*) reduced synthetic PGE_2 1,15-lactone; and (*c*) synthetic $PGF_{2\alpha}$. The stereochemistry of the chiral centres was established as identical with that of synthetic $PGF_{2\alpha}$ 1,15-lactone (and therefore with that of $PGF_{2\alpha}$) by comparing the ^{1}H-n.m.r. spectrum of the 9-acetyl derivative of the natural compound with that of the 9,11-diacetyl derivative of synthetic $PGF_{2\alpha}$ 1,15-lactone. PGF 1,15-lactone 9- and 11-fatty-acid-esters were characterized by means of (*a*) ^{1}H-n.m.r., (*b*) e.i.m.s., and (*c*) methanolysis (see below) followed by g.c.–m.s. of the fatty acid methyl esters and e.i.m.s. of the PGF methyl esters produced.

Incorporation experiments *in vivo*

[^{3}H]PGE_2 or [^{3}H]$PGF_{2\alpha}$ (5 μCi; 200 Ci/mmol) or [^{3}H]AA (20 μCi; 200 Ci/mmol) was injected subcutaneously into the mantle near the upper right gill of a single specimen of *T. fimbria* for each experiment. Specimens of approximately the same size were used and were kept in separate tanks for the duration of the incubation period. After incubations of 1 or 2 days with [^{3}H]PGE_2 (repeated twice), of 6 h, 18 h, 1 day or 2 days with [^{3}H]$PGF_{2\alpha}$, and of 1, 3 or 7 days with [^{3}H]AA, the molluscs were frozen at −80 °C. Mantles and cerata of specimens from radiolabelled PG experiments were separately extracted with acetone and sand (Merck; approx. 1:1, w/w). After evaporation, the aqueous residues were extracted with diethyl ether and purified on SiO_2 columns (eluted with benzene/diethyl ether, 4:1, v/v) and h.p.l.c. Mantles and cerata of specimens from [^{3}H]AA experiments, in which both PGs and PG lactones had to be purified, were separately extracted with 5% (v/v) acetic acid plus 15 μM-indomethacin. After centrifugation of the homogenates at 10000 rev./min for 30 min, the supernatants were pre-purified on Sep-Pak C_{18} cartridges washed with 5% acetic acid and eluted with methanol. Sep-Pak eluates were then submitted to h.p.l.c. H.p.l.c. fractions were counted for radioactivity, and portions of the radioactive fractions corresponding to peaks of PG derivatives were acetylated. Acetylation was performed for 3 h at room temperature in 500 μl of anhydrous pyridine with 100 μl of acetic anhydride. Mono- and di-acetyl derivatives of PG lactones or PGs were again purified by h.p.l.c. Radioactivity counts were determined with 35–50% efficiency in Insta-Fluor II scintillation liquid (Packard).

Egg-masses were laid by the same specimens that had been injected with [^{3}H]$PGF_{2\alpha}$ after 1, 2 and 3 days incubation. Moreover, the reproductive glands (ovotestis) of these specimens could be obtained by dissection. This allowed the measurement of incorporation of radioactivity into the PG lactones egg-mass and ovotestis, which were extracted and purified as described by Cimino *et al.* (1991). Methanolysis of these compounds was conducted for 20 h at room temperature in anhydrous methanol in the presence of a few milligrams of solid Na_2CO_3, and yielded $PGF_{2\alpha}$ and $PGF_{2\alpha}$ methyl esters and a mixture of fatty acid methyl esters. The reaction mixture was loaded on to an analytical SiO_2 t.l.c. plate (Merck) eluted first with light petroleum (b.p. 40–60 °C)/diethyl ether (9:1, v/v) in order to obtain the fatty acid methyl esters, and then with chloroform/methanol (9:1, v/v) to purify PGF methyl esters.

Conversion of PG lactones to the corresponding PGs *in vivo*

Spontaneous detachment of cerata from a few specimens kept in tanks was induced, and groups of three cerata of comparable size and weight were either immediately frozen or kept contracting in sea water at room temperature for 15 min, 45 min or 4 h. In separate experiments, cerata, immediately after their detachment, were injected with 15000 c.p.m. of h.p.l.c.-purified radiolabelled PG lactones from previous incorporation experiments, and were again either immediately frozen or kept contracting for 15 min or 4 h. In both cases the cerata were then extracted with 5% acetic acid + 15 μM-indomethacin and the extracts were treated as described above in order to isolate and/or quantify and/or count the radioactivity in both PGEs and PGE 1,15-lactones. The amounts of PGEs and PGE lactones from cerata at different times after detachment were compared, as percentages of total (PG + corresponding lactone) amounts, using Student's unpaired *t* test, with a level of significance of $P < 0.05$.

H.p.l.c. separation and analysis

Purification by h.p.l.c. was carried out using two different columns and eluting systems, depending on whether only PG lactones or both PG and PG-lactones were to be purified and/or quantified. In biosynthesis experiments with radiolabelled PGs, fractions from SiO_2 column chromatography or from acetylation

of fractions from the first h.p.l.c. run were further purified using a μ-Porasil column (4.5 mm × 30 mm; Waters Associates) eluted isocratically with n-hexane/ethyl acetate at a ratio ranging between 8:2 and 9:1 (v/v), depending on the type of compound, and at a flow rate of 1 ml/min. The eluate was monitored by means of a differential refractometer. In biosynthesis experiments with [^{3}H]AA and in experiments of the conversion of PG lactones by cerata, Sep-Pak eluates were purified on a Spherisorb 5 μm pore size column (ODS-2; 4.5 mm × 25 mm) eluted with a 90 min gradient of 30–70% acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid (flow rate = 1 ml/min). U.v. absorbance was monitored at 205 nm. PG derivatives from all experiments were quantified using this system and using PG standards, commercially available or previously purified from *T. fimbria*, to calibrate the column, with a detection limit of 1 μg. Acquisition, integration and calibration were performed using a Maxima 820 chromatography workstation (Waters Associates). The amounts of PG lactones and PGs were between 2 and 245 and between 20 and 98 μg/g dry wt. of tissue respectively, according to the compound and to tissue type (Cimino *et al.*, 1991).

Determination of *T. fimbria* mantle fatty acid composition

The mollusc mantle was extracted with chloroform/methanol (2:1, v/v). The extract was evaporated under vacuum and the solid residue was treated with water (pH adjusted to 8.5 with NaOH) to wash out salts. The organic phase was then evaporated and the extract was loaded on to preparative SiO_2 t.l.c. plates (Merck) developed first with light petroleum (b.p. 40–60 °C)/diethyl ether (2:3, v/v), to obtain non-esterified fatty acids, and then with chloroform/methanol/water (60:25:4, by vol.) to obtain several phospholipidic fractions. The latter were pooled and submitted to methanolysis as described above, which yielded a mixture of methyl esters of the fatty acids of phospholipid origin. The non-esterified fatty acid fraction was purified further by means of SiO_2 column chromatography and then methylated with diazomethane in methanol for 15 min at room temperature. Samples of the two fractions and saturated and unsaturated fatty acid ester standards (Sigma) were analysed by means of g.c. on a Carlo Erba HRGC 5300 Megaseries chromatograph using a capillary column FS SE-30-CB-0.5 (25 m × 0.32 mm internal diam.).

RESULTS AND DISCUSSION

When *T. fimbria* mantles were injected with 5 μCi of [^{3}H]PGE_2, separate extraction of both the mantles and the cerata after incubations for 1 or 2 days followed by SiO_2 column chromatography and h.p.l.c. purification, led to the isolation of highly labelled PGE_2 1,15-lactone (Table 1). The incorporation ranged between 0.5 and 10% of the injected counts, depending on the incubation time. In order to ascertain that this radioactivity was not due to impurities, samples of the h.p.l.c. fractions containing the lactone were submitted to acetylation and re-purified by means of h.p.l.c., yielding radiolabelled 11-acetyl-PGE_2 1,15-lactone. Incorporation of the label into natural 11-acetyl-PGE_2 1,15-lactone and PGA_2 1,15-lactone was always very low after subtraction of background radioactivity and did not allow the performance of derivatization and/or a second h.p.l.c. purification to assess its authenticity. Thus acetylation of PGE_2 1,15-lactone to its 11-acetyl derivative *in vivo* must be a slow process, as is also suggested by the very low levels of this metabolite found in both the mollusc mantle and cerata (Cimino *et al.*, 1991). As expected, no incorporation was found in h.p.l.c. fractions containing PGE_3 and PGA_3 1,15-lactones or 11-acetyl- $PGF_{3\alpha}$ 1,15-lactone.

Injection of 5 μCi of [^{3}H]$PGF_{2\alpha}$ into *T. fimbria* mantles resulted in the finding, in both the mantle and the cerata, of the labelled PG derivatives shown in Table 2. Again, samples of the h.p.l.c. fractions were used for acetylation which, after a second h.p.l.c. step, confirmed that incorporated radioactivity, retained into the mono- and di-acetylated lactones, was not due to impurities. The specific incorporation observed for 11-acetyl-$PGF_{2\alpha}$ 1,15-lactone (total incorporation was 0.05%–1% of injected counts) seemed to reach a maximum after 2 days. Although no $PGF_{2\alpha}$ 1,15-lactone was ever found in either the mantle or the cerata of *T. fimbria* (Cimino *et al.*, 1991), the mollusc was able to convert [^{3}H]$PGF_{2\alpha}$ into this lactone *in vivo*, with the radioactivity detected in the corresponding h.p.l.c. fractions (0.5–2% of injected counts) being maximal at 1 day after injection. This suggests that $PGF_{2\alpha}$ 1,15-lactone, and probably PGF_3 1,15-lactone, are short-lived intermediates in the biosynthesis of the corresponding 11-acetyl derivatives *in vivo*. Surprisingly, injection of [^{3}H]$PGF_{2\alpha}$ also yielded strongly labelled PGE_2 1,15-lactone (total incorporation 0.3–4% of injected counts), thus indicating the occurrence of an unusual oxidative step whereby PG derivatives of the F series are

Table 1. Biosynthesis experiments *in vivo* with [^{3}H]PGE_2

Total (c.p.m.) and specific (c.p.m./mg of pure compound) radioactivity incorporated into PGE_2 1,15-lactone is shown both in the mantle and the cerata of *T. fimbria* after the injection of 5 μCi of [^{3}H]PGE_2. Amounts of each h.p.l.c.-purified metabolite and the percentage incorporation of total injected radioactivity are also shown. This experiment was repeated twice. The average starting amounts of PGE_2 1,15-lactone in tissue were 221 μg and 99 μg of dry weight respectively in cerata and mantle.

	Incorporation							
	Mantle				Cerata			
Incubation time	(c.p.m.)	(%)	Amount (mg)	(c.p.m./mg)	(c.p.m.)	(%)	Amount (mg)	(c.p.m./mg)
Expt. 1								
1 day	161177	3.0	0.9	179086	89100	1.6	1.0	89100
2 days	47440	0.8	0.6	79077	194 810	3.6	0.80	243513
Expt. 2								
6 h	6120	0.1	0.2	30600	5880	0.,1	0.25	23520
1 day	262380	4.8	0.3	874600	183040	3.4	0.22	832000
2 days	35920	0.6	0.6	64140	516360	9.4	0.58	890276
3 days	85456	1.6	0.7	124937	120136	2.2	0.50	240272

Table 2. Biosynthesis experiments *in vivo* with [^{3}H]$PGF_{2\alpha}$

Total (c.p.m.) and specific (c.p.m./mg of pure compound; in parentheses) radioactivity incorporated in each PG derivative as shown in both the mantle and the cerata of *T. fimbria* after the injection of 5 μCi of [^{3}H]$PGF_{2\alpha}$. The percentage incorporation injected radioactivity is shown in square brackets. The average starting amounts of 11-acetyl-$PGF_{2\alpha}$ 1,15-lactone in mantle and cerata were respectively 60 and 30 μg/g dry wt. of tissue. The amounts found for each metabolite in each experiment are not shown for the sake of clarity, but can be easily calculated from the comparison of total and specific incorporated radioactivity. N.D., not detectable.

	Mantle				Cerata			
Incubation time	$PGF_{2\alpha}$ lactone	11-Acetyl-$PGF_{2\alpha}$ lactone	PGE_2 lactone	PGE_2	$PGF_{2\alpha}$ lactone	11-Acetyl-$PGF_{2\alpha}$ lactone	PGE_2 lactone	PGE_2
6 h	29720	2060	130790	–	4420	N.D.	58050	–
		(19200)	(658870)				(148900)	
	[0.5]	[0.04]	[2.4]		[0.08]		[1.1]	
18 h	–	10650	16050	17900	–	2050	21000	46940
		(87890)	(83010)			(36100)	(48960)	
		[0.2]	[0.3]	[0.3]		[0.04]	[0.4]	[0.9]
1 day	99470	15000	63900	–	N.D.	1410	61170	–
		(150000)	(127000)			(27000)	(120000)	
	[1.8]	[0.3]	[1.2]			[0.03]	[1.1]	
2 days	31650	60000	132020	–	3120	6040	194600	–
		(580263)	(132020)			(110545)	(870000)	
	[0.6]	[1.1]	[2.4]		[0.06]	[0.1]	[3.5]	

converted into ones belonging to the E series. The finding of high radioactivity in PGE_2 at only 18 h after the injection of [^{3}H]$PGF_{2\alpha}$ suggests that this latter PG is quickly oxidized to PGE_2 before lactonization. However, oxidation of $PGF_{2\alpha}$ 1,15-lactone to PGE_2 1,15-lactone is also a possibility.

In both [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ incorporation experiments, the specific radioactivity found in PGE_2 1,15-lactone seemed to decrease in the mantle and increase in the cerata passing from short to long incubation periods (Tables 1 and 2). This might suggest that the lactone is mainly produced in the mantle and then specifically accumulated into the cerata. The high concentration of PGE lactones in the cerata compared with the mantle (Cimino *et al.* 1991) seemed to favour this hypothesis, which was also corroborated by the outcome of the following experiments *in vivo*. First, a 1 day incubation of 5 μCi of [^{3}H]PGE_2 in a specimen without cerata resulted in the production of [^{3}H]PGE_2 lactone (total counts 10200 c.p.m., specific incorporation 51000 c.p.m./mg of pure compound). Secondly, injection of labelled PGE_2 1,15-lactone (25000 c.p.m.), obtained pure from one of the previous incorporation experiments, into the mantle of an intact specimen resulted in the finding, after a 1 day incubation, of 6500 c.p.m. in the lactone from the mantle and 3500 c.p.m. in the lactone from the cerata. Thirdly, 6 h incubations of intact isolated cerata with 5 μCi of [^{3}H]PGE_2 never produced a significant incorporation of radioactivity into the lactone (this experiment was repeated three times): however, a 6 h incubation of 5 μCi of the same labelled precursor with the intact mollusc mantle resulted in the production in the cerata of labelled PGE_2 1,15-lactone (5880 c.p.m.).

From the data presented so far it can be concluded that (1) PG 1,15-lactones are derived from the lactonization of PGs; (2) conversions *in vivo* of both PGE_2 and $PGF_{2\alpha}$ into PGE_2 1,15-lactone and of $PGF_{2\alpha}$ into $PGF_{2\alpha}$ 1,15-lactone occur mainly in the mantle and are complete in 1 day; (3) acetylation of the lactones on the 11-hydroxy group is slow for PGE_2 1,15-lactone and quick for $PGF_{2\alpha}$ 1,15-lactone; the latter therefore cannot be found as an end-product; and (4) the lactones of the E series are specifically transported to and accumulated in the cerata. At this point it seemed interesting to determine the origin of PGs in *T. fimbria*.

When the mollusc mantle was injected with 20 μCi of [^{3}H]AA, significant and authentic incorporation of radioactivity with respect to background levels was only found for PGE_2 and PGE_2 1,15-lactone (Table 3). Consistent with the situation observed with ^{3}H-labelled PGE_2 and $PGF_{2\alpha}$ as precursors, total and specific radioactivity incorporated into PGE_2 1,15-lactone at 3 and 7 days after the injection were higher in the cerata than in the mantle. However, very low counts were detected in this lactone and in PGE_2 after 1 day and 3 day incubations. This may be due to the fact that, as in mammals, the cyclo-oxygenase catalysing the oxidation of AA does not use as a substrate *in vivo* AA of exogenous origin, and that the enzymic release of AA from special pools of cell-membrane phospholipids is the necessary and rate-limiting step of the AA cascade (Samuelsson *et al.*, 1978). Indeed, g.c. analysis of the methyl esters of both free and phospholipid-bound fatty acids revealed the virtual absence of free AA and eicosapentaenoic acid (EPA) in the mollusc mantle, whereas these two fatty acids were very abundant in phospholipidic fractions (Table 4). Therefore, if the requirements of *T. fimbria* AA-oxidizing enzyme are similar to those of the mammalian cyclo-oxygenase, a certain delay before the appearance of labelled PG derivatives was to be expected, since the injected AA had to be incorporated into the membrane phospholipids before being released and converted into PGs and PG lactones. Counts detected in h.p.l.c. fractions corresponding to $PGF_{2\alpha}$ and $PGF_{2\alpha}$ lactones in the [^{3}H]AA incorporation experiments were too low to allow derivatization and/or a second h.p.l.c. purification. This should not necessarily lead to the conclusion that AA is not converted into $PGF_{2\alpha}$ in *T. fimbria*. Indeed, h.p.l.c. u.v. peaks observed at the $PGF_{2\alpha}$ retention time were also too small to permit the isolation and full characterization of this compound, which might be a short-lived intermediate as suggested by its conversion into PGF lactones and by its quick oxidation to PGE_2.

In mammals and in some invertebrates PG_2s are derived from AA via common endoperoxide intermediaries, PGG_2 and PGH_2 (Stanley-Samuelson, 1987). However, it has been suggested that PGA_2 biosynthesis in the marine invertebrate *Plexaura homomella* and other coral species follows a different route, via 8-(*R*)-hydroperoxyeicosapentenoic acid and/or allene oxide inter-

Table 3. Biosynthesis experiments *in vivo* with [^{3}H]AA

Total (c.p.m) and specific (c.p.m./mg of pure compound) radioactivity incorporated into each prostanoid is shown in both the mantle and the cerata of *T. fimbria* after the injection of 20 μCi of [^{3}H]AA. The percentage incorporation of injected radioactivity in the 7 h incubation experiment is shown in parentheses in each case. N.D., not detectable.

	Mantle				Cerata			
	PGE_2		PGE_2 lactone		PGE_2		PGE_2 lactone	
Incubation time (days)	Total (c.p.m.)	Specific (c.p.m./mg)	Total (c.p.m.)	Specific (c.p.m./mg)	Total (c.p.m.)	Specific (c.p.m./mg)	Total (c.p.m.)	Specific (c.p.m./mg)
1	750	35000	N.D.	–	210	5600	N.D.	–
3	560	18000	345	6900	220	5500	655	8188
7	1845 (0.008%)	30750	2820 (0.013%)	51272	1610 (0.007%)	29727	9375 (0.04%)	85227

Table 4. Composition of unbound and phospholipid-bound fatty acids in *T. fimbria* mantle

The mollusc mantle was extracted and fatty acids were purified as described in the Materials and methods section. Non-esterified fatty acids were methylated with CH_2N_2, whereas the methyl esters of phospholipid-bound fatty acids were obtained by methanolysis of phospholipid fractions. The composition of each fraction was determined by g.c. by comparison with both saturated and unsaturated fatty acid ester standards. $C_{20:5}$ is EPA and $C_{22:6}$ is *cis*-docosahex-4,7,10,13,16,19-enoic acid; N.D., not detectable.

	Composition	
Fatty acid	Phospholipid-bound (% of total)	Unbound (% of total)
$C_{14:0}$	2.5	4.4
$C_{16:0}$	21.7	42.2
$C_{16:1}$	6.7	5.6
$C_{18:0}$	12.5	5.6
$C_{18:1}$	9.3	42.2
$C_{20:4}$(AA)	8.0	N.D.
$C_{20:5}$	8.8	N.D.
$C_{22:6}$	31.6	N.D.

mediaries (Corey *et al.*, 1988; Brash, 1989), yielding both 15(*R*)- and 15(*S*)-PGA_2. It is unlikely that this is the case for *T. fimbria*, which (*a*) synthesizes PGE and PGF derivatives as well as PGA derivatives, and (*b*) only produces the 15(*S*) stereoisomers of these derivatives (Fig. 1; see also Cimino *et al.*, 1989).

The accumulation of PGE 1,15-lactones into the cerata, shown by ^{3}H-labelled PGE_2, $PGF_{2\alpha}$ and AA incorporation experiments *in vivo*, raised the question of what, if any, biological role would be played by these metabolites in this tissue. *T. fimbria*, and other opisthobranch molluscs belonging to the family Tethyidae, exhibit a typical defence behaviour when attacked by predators (Thompson & Crampton, 1984). First, they secrete, from both the mantle and cerata, a supposedly defensive mucus and then, if molested further, they release their cerata which carry on contracting and secreting mucus for long periods of time without the occurrence of necrobiosis. In a separate study (A. Marin, G. Cimino & V. Di Marzo, unpublished work) it has been observed that PGE and PGA 1,15-lactones are the main lipophilic components of *T. fimbria* mucus and that, unlike PGF lactones and PGs, they are toxic to fish. Here we describe how, when spontaneous release of cerata from a few specimens of the mollusc was induced and the appendices were kept contracting for different periods of time in sea water at room temperature, the amounts of PGE lactones decreased compared with the basal levels found in cerata frozen immediately after detachment (Table 5). The levels of PGEs increased correspondingly and had already reached a plateau at 15 min after detachment. These newly formed PGs originated from lactones and not from biosynthesis *de novo*, as shown by the following experiment. Radiolabelled PGE_2 1,15-lactone was injected into cerata, which were then either immediately frozen or kept contracting as described above. [^{3}H]PGE_2, as identified by h.p.l.c., was formed during these incubations, whereas the radioactivity associated with PGE_2 lactone decreased correspondingly (Table 6). This conversion was not observed when [^{3}H]lactone was added for 4 h to a heat-inactivated homogenate of cerata, and therefore cannot be due

Table 5. Conversion of PGE_2 and PGE_3 1,15-lactones into the corresponding PGs in *T. fimbria* cerata *in vivo*

Cerata in triplicate were either frozen immediately after spontaneous detachment or left contracting for different periods of time. PG lactones and PGs were quantified as described in the Materials and methods section. Results are expressed as a percentage of the total amounts of PG+PG lactone in each group of cerata, and are means ± S.E.M. of three independent experiments. Total amounts (μg) for each period of time are also reported. Statistical analysis was conducted using Student's unpaired *t* test: $^*P < 0.05$, $^{**}P < 0.005$.

	PGE_2			PGE_3		
Time after detachment	Free acid (%)	1,15-Lactone (%)	Total (μg)	Free acid (%)	1,15-Lactone (%)	Total (μg)
0	13.5±11.1	86.4±11.1	69±24	35.9±10.0	64.1±10.0	39±9
15 min	42.2±5.4*	57.8±5.4*	84±3	67.9±11.0	32.1±11.0	23±3
45 min	48.5±1.0**	51.5±1.0**	87±16	68.6±7.3*	31.3±7.3*	22±5
4 h	46.0±4.1**	53.9±4.1**	70±22	69.3±7.6*	31.7±7.6*	22±4

Table 6. Conversion of labelled PG 1,15-lactones into the corresponding PGs in *T. fimbria* cerata *in vivo*

Labelled lactones (15000 c.p.m.) were injected into cerata (in duplicate) immediately after the spontaneous detachment of the latter. The appendices were then either frozen or left contracting for 15 min or 4 h. The tissue was then extracted and the extracts were purified as described in the Materials and methods section. Values are in c.p.m. and h.p.l.c. background radioactivity has been subtracted.

Time after detachment and injection	Incorporation (c.p.m.)					
	PGE_2 1,15-lactone	PGE_2	$PGF_{2\alpha}$ 1,15-lactone	$PGF_{2\alpha}$	11-Acetyl-$PGF_{2\alpha}$ 1,15-lactone	$PGF_{2\alpha}$
0	5500	540	1157	800	1868	56
15 min	1800	3200	–	–	–	–
4 h	1300	5325	64	836	130	1869

to a mere chemical hydrolysis of the lactone. It is noteworthy that cerata can also open PG lactones which are not usually accumulated into this tissue, e.g. $PGF_{2\alpha}$ 1,15-lactone and 11-acetyl-$PGF_{2\alpha}$ 1,15-lactone, as shown by experiments, analogous to the one described above, conducted with labelled PGF lactones (Table 6). The enzyme(s) responsible for this conversion does not therefore appear to be specific for the lactones of the E series. Indeed, it might be the same enzyme which, with a different subcellular localization and under different physiological circumstances, catalyses the lactonization of PGs, since it has been shown that certain lipases can promote the lactonization *in vitro* of some hydroxy-acids (Makita *et al.*, 1987).

Thus PGE_2 and PGE_3 1,15-lactones are either secreted with the defensive mucus or they are transferred to the cerata, where again they are either secreted or converted back into PGs upon detachment. This PGE lactonization → transfer to cerata → lactone-opening sequence represents the first example of a mechanism by which PGs are stored *in vivo* as structurally related compounds, ready to be released during a physiological stimulus such as a defence behaviour. The purpose of this response might be questioned. A hypothesis is that PG lactone-derived PGs might intervene in the spontaneous and prolonged contractions typical of *T. fimbria* cerata. Histological analyses (A. Marin, G. Cimino & V. Di Marzo, unpublished work) showed that this tissue is rich in contractile fibres which are similar to those in vertebrate smooth muscle, a tissue usually potently relaxed or contracted by PGs. Should this hypothesis be proved correct, *T. fimbria* would have developed a very economical way of exploiting PG lactones for more than one purpose: (*a*) as defence allomones in the mollusc defensive secretion; (*b*) as inactive precursors of bioactive PGs within the cerata (or the mantle, which also contains PGs); and (*c*) as bioactive lactones in tissues other than the cerata and the mantle, as might be the case for PGF lactones.

Apart from being present in the mantle as 11-acetyl derivatives, PGF lactones are actually more abundant in the mollusc ovotestis and egg-mass as the very unusual 9- and, in smaller amounts, 11-fatty acyl esters (Cimino *et al.*, 1991). When the ovotestis and egg-masses of the specimens injected with [^{3}H]$PGF_{2\alpha}$ were extracted and the extracts fractionated by means of t.l.c., incorporation of radioactivity was observed into these metabolites (Table 7). Methanolysis conducted on the mixture of PGF 1,15-lactone 9- and 11-fatty acyl esters yielded non-radioactive saturated and unsaturated fatty acid methyl esters and [^{3}H]$PGF_{2\alpha}$ methyl ester, thus confirming that label incorporation was not due to impurities and that these metabolites actually originate from PGFs. Interestingly, a comparison between Tables 2 and 7 shows that, in the same specimen after a 2 day incubation, more counts were incorporated in the egg-mass lactones than in the mantle PGF lactones (respectively 283385 and 91650 c.p.m.). Moreover, egg-masses laid after a 2 day incubation period exhibited the highest labelling of PGF lactones, and in the ovotestis the incorporation also reached a maximum after 2 days. Hence $PGF_{2\alpha}$ 1,15-lactone, present in the mantle after 1 day (Table 2) might serve as the precursor of these PGF lactones; also, 11-acetyl-$PGF_{2\alpha}$ 1,15-lactone might be an intermediate for the synthesis of the major constituents of the fatty acyl ester mixture, the 9-fatty acyl derivatives, via an acylation step on C-9 followed by loss of an acetyl group from the hydroxy group on C-11. PGF lactones are likely to play a role in the control of oocyte production and/or fertilization, since they are synthesized in a gland, the ovotestis, in which oocytes are

Table 7. Conversion of [^{3}H]$PGF_{2\alpha}$ into PGF 1,15-lactone 9- and 11-fatty acyl esters *in vivo*

Total (c.p.m.) and specific (c.p.m./mg of total mixture) radioactivity incorporated in the mixture of PGF lactone fatty acyl esters are shown in *T. fimbria* egg-masses and ovotestis (hermaphrodite gland). The molluscs used were the same specimens used to obtain the results shown in Table 2.

Incubation time	Egg masses		Ovotestis	
	Total (c.p.m.)	Specific (c.p.m./mg)	Total (c.p.m.)	Specific (c.p.m./mg)
6 h	–	–	2176	19782
18 h	–	–	8225	91389
1 day	123000	180882	24634	82113
2 days	260480	321580	22905	109071
3 days	62380	91735	23741	87929

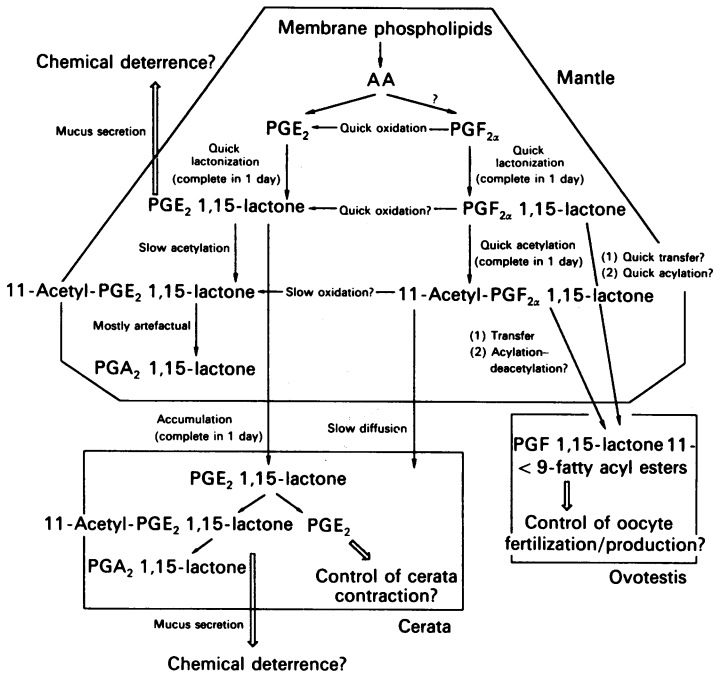

Fig. 2. The PG lactone pathway

produced before being transferred to the anatomical region in which they are fertilized, but are absent from the non-mature ovotestis. A significant activity as anti-fertility agent, albeit in mammals, had indeed been described for $PGF_{2\alpha}$ 1,15-lactone (Bundy *et al.*, 1983), and it has been suggested that PGs in general induce oocyte production (Kunigelis & Salueddin, 1986) and spawning (Morse *et al.*, 1977) in freshwater and marine molluscs.

The novel and rather complex metabolic pathway arising from the data described in this paper is depicted, in its main features, in Fig. 2. Steps which are likely to occur but are not sufficiently supported by our findings are indicated with a question mark. An analogous pathway, starting with EPA, would exist for PGE_3 and $PGF_{3\alpha}$ lactones. A key role is hypothesized for $PGF_{2\alpha}$ 1,15-lactone, an 'invisible' metabolite, which, as shown here, is produced from $PGF_{2\alpha}$ and is not found in *T. fimbria* extracts, probably because it serves as an alternative precursor of PGE_2 1,15-lactone in the mantle, or as an intermediate in the synthesis of 11-acetyl-$PGF_{2\alpha}$ 1,15-lactone in the mantle and of $PGF_{2\alpha}$ 1,15-lactone 9- and 11-fatty acyl esters in the ovotestis and egg-masses. The production of PGA_2 1,15-lactone is described as mostly artefactual, since it has been observed (Cimino *et al.*, 1989) that this metabolite, analogous to PGA_3 1,15-lactone, originates from 11-acetyl-PGE_2 1,15-lactone upon elimination of acetic acid from the purification procedure.

This step is probably the only non-enzymic transformation among those summarized in Fig. 2; long incubations of labelled PGs with heat-inactivated *T. fimbria* mantle homogenates, in fact, never resulted in the production of the lactones, thus showing that the process of lactonization is not likely to be a chemical one. This is also suggested by the fact that PG 1,15-lactones could only be chemically synthesized by a laborious procedure (Bundy *et al.*, 1983) including the activation of both the carboxy and the 15-hydroxy groups. Experiments aimed at isolating the enzyme(s) responsible for the catalysis *in vivo* of the PG-lactonization reaction are required, but some difficulties due to the possible co-existence of both 'lactonizing' and 'hydrolysing' enzymes in cell-free homogenates, in which any subcellular compartmentalization is destroyed, can be predicted.

PGE and PGF lactonization steps are described as 'quick', although the time probably required for their completion (1 day) may be regarded, in comparison with mammalian cells, as actually being relatively long. This is in agreement with the biological roles hypothesized here for PG lactones either as precursors of PGEs (whose formation from PGE-lactones is fast), or as defence allomones, or as molecules involved in oocyte production (which, in opistobranch molluscs, is a slow process). This proposed multiple biological action of PG lactones in *T. fimbria*, however, requires further substantiation by means of pharmacological studies on the effect of PGs on cerata contractile fibres and on the role of PGF lactones in oocyte production and control of fertilization. It will also be interesting to investigate the fascinating possibility of this novel biosynthetic pathway being present, even if only on a smaller scale, in other molluscs and in more developed organisms. The application of more sensitive techniques such as radioimmunoassay using antisera raised against PGLs might also allow their isolation from PG-synthesizing tissues of other animal species. 1,8-Lactones of eicosanoid origin have been isolated from a marine ascidian (Lindquist & Fenical, 1989) and sponge (Niwa *et al.*, 1989), and therefore enzymes catalysing the formation of medium–large-membered ring lactones from AA and EPA must exist and have some relevant physiological action, at least in marine invertebrates. The high expression of the PG branch of the AA cascade in *T. fimbria*, having facilitated the characterization of the structure and biogenesis of PG 1,15-lactones, may now promote the use of this mollusc as a new model for the study of the biochemical and genetic control of PG biosynthesis.

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