*Discussion*

The salt extraction experiments showed that the H1 histone of *Tetrahymena* is bound less firmly than the bovine H1 histone. A hydrophoby comparison between *Tetrahymena* H1 and human H1b has suggested that the *Tetrahymena* C-terminal region (residues 51-165) corresponds to the human C-terminal region (residues 108-218)[4]. Because the net charge difference between these proteins is large in this region ( + 31 for *Tetrahymena*, + 44 for human), the difference in salt extractability between *Tetrahymena* and bovine H1 histones may be due to the difference in charge density in the C-terminal regions of these proteins if the modes of association of histone H1 are similar.

As expected from the lack of the globular region in the *Tetrahymena* H1 histone[4, 5], micrococcal nuclease digestion failed to give chromatosomes. Two interpretations are possible for this, i.e. 1) histone H1 is present at the DNA exit point on the nucleosome, but nevertheless does not provide resistance to micrococcal nuclease trimming there, or 2) it does not exist at the DNA exit point, i.e. *Tetrahymena* histone H1 is not a linker histone. At present, data are not available to discriminate between these possibilities. If the latter is the case it follows that in at least three unicellular eukaryotes, i.e. *Tetrahymena, Trypanosoma*[3] and yeast, the presence of typical linker histones and chromatosomes is questionable.

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## A marine mollusc provides the first example of in vivo storage of prostaglandins: Prostaglandin-1,15-lactones

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*Summary.* Prostaglandin- (PG) 1,15-lactones and, in smaller amounts, free acids, were isolated from both the mantle and the dorso-lateral appendices of the opisthobranch mollusc *Tethys fimbria.* In vivo conversion of PGs into the corresponding lactones and accumulation of $PGE_2$- and $PGE_3$-1,15-lactones in the appendages were shown. The detachment of these appendages from the molested mollusc caused the in vivo conversion of $PGE_2$- and $PGE_3$-lactones back to $PGE_2$ and $PGE_3$ respectively, thus providing the first example of a mechanism by which prostaglandins can be stored and, when needed, released.
*Key words.* Prostaglandins; prostaglandin-1,15-lactones; prostaglandin biosynthesis; marine eicosanoids; opisthobranch molluscs.

Prostaglandins are well-known mediators of several biological responses in both vertebrate and invertebrate organisms[1]. They are not pre-formed mediators and their biosynthesis from a common precursor like arachidonic acid generally occurs de novo following chemical, immunological or mechanical stimulation[1]. However, during our study of the chemical defenses of opisthobranch molluscs[2], we came across novel natural prostaglandin (PG) derivatives whose structure suggests a role as PG precursors.

The species belonging to the opisthobranch molluscan family Tethyidae, when molested, release a possibly defensive mucous secretion and easily detach their dorso-lateral appendages (cerata), which then contract and continue to release mucus for many hours without decomposing[3]. From the mantle and the cerata of one of these species, *Tethys fimbria*[4], we isolated a series of PG-1,15-lactones (fig. 1)[5, 6]. High pressure liquid chromatography (HPLC) analysis of extracts from both the mantle and the cerata of the mollusc also revealed the presence of peaks co-eluting with $PGE_2$ and $PGE_3$ standards (fig. 2, table 1), and proton nuclear magnetic resonance (NMR) analysis of these components confirmed this finding (unpublished results). The co-existence of PG-1,15-lactones and PG free acids raised questions regarding: 1) their biogenetic relationship, and 2) which of the two classes of compounds, if any, has a biological role in *T. fimbria.*

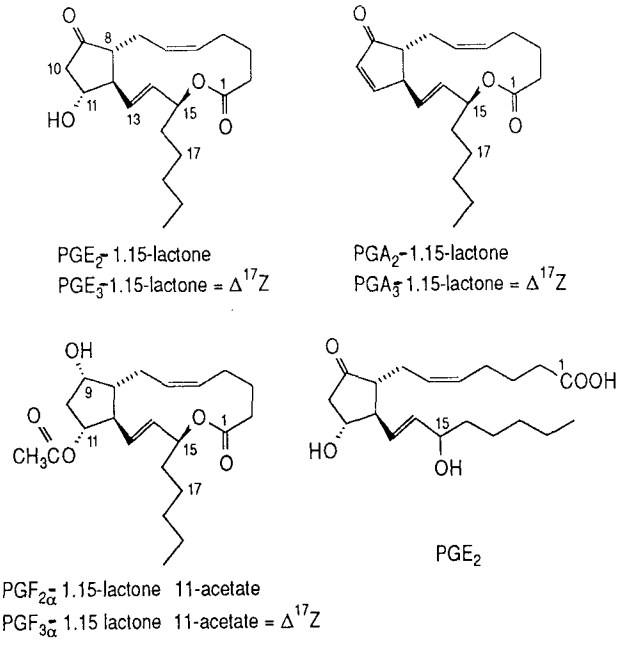


Figure 1. Prostaglandin derivatives present in *T. fimbria*.

*Materials and methods*

The molluscs were caught in the Bay of Naples. Biosynthesis experiments were conducted in vivo in *T. fimbria* by injecting 5 μCi [$^3$H]-$PGE_2$ or -$PGF_{2\alpha}$ (200 Ci/mmol, Amersham) subcutaneously into the mantle near the upper right gill of a single specimen for each experiment. Specimens of approximately the same size were used. After the different incubation periods, the molluscs were frozen at − 80 °C and subsequently extracted with acetone and sand (Merck, approx. 1:1 w/w). The aqueous residues from the acetone extracts of mantle or cerata were then extracted with diethyl ether and purified on a $SiO_2$ column (benzene/diethyl ether), and by HPLC (μ-porasil, 4.5 × 30 mm [Waters, Ass.], n-hexane/ethyl acetate 85/15 v/v, flow rate 1 ml/min). Yields were consistent and higher than 70%. After HPLC, aliquots of the fractions corresponding to the lactone peaks were counted or acetylated. Acetylation was performed in 500 μl of anhydrous pyridine with 100 μl of acetic anhydride. After 3 h the reaction was stopped by adding 2 ml of methanol and the reaction mixture was dried under a stream of nitrogen, and then submitted to HPLC purification of the

Table 1. Quantitative data for PG free acids and 1,15-lactones in the mantle (M) and cerata (C) of *Tethys fimbria* in μg/g dry weight of tissue ± SE.

| | $PGE_3$ | $PGE_2$ | $PGE_3$ lact. | $PGE_2$ lact. | $PGF_{3\alpha}$ 11-ac. lact. | $PGF_{2\alpha}$ 11-ac. lact. | $PGA_3$ lact. | $PGA_2$ lact. |
|---|---|---|---|---|---|---|---|---|
| $C_{n=3}$ | 98 ± 11 | 37 ± 8 | 245 ± 46 | 221 ± 43 | 139 ± 56 | 30 ± 2 | 124 ± 30 | 10 ± 2 |
| $M_{n=3}$ | 75 ± 20 | 20 ± 8 | 89 ± 16 | 99 ± 7 | 199 ± 10 | 60 ± 2 | 97 ± 7 | 2 ± 1 |

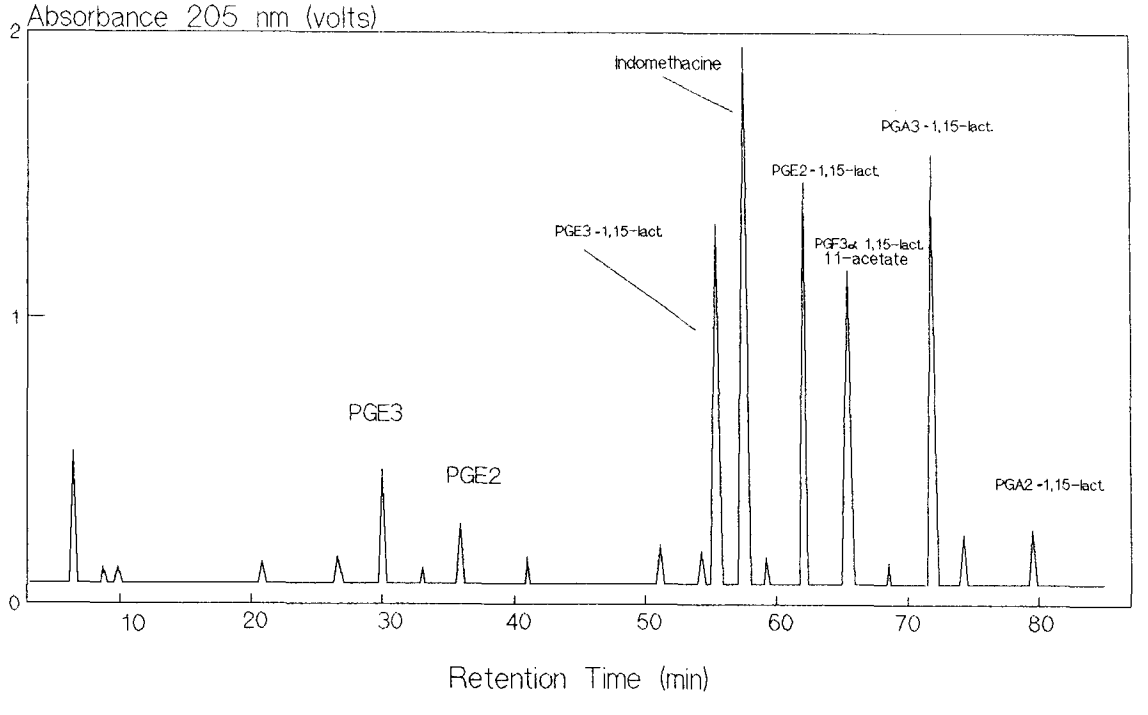


Figure 2. UV Absorbance (205 nm) elution profile of a sample from *T. fimbria* cerata. Conditions were: Spherisorb 5 μm column (ODS-2, 4.5 × 25 mm) eluted with a 90-min gradient from 30% to 70% acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid, flow rate = 1 ml/min.

mono- and di-acetyl derivatives. Radioactivity counts were determined with 35% efficiency in a TriCarb liquid scintillation counter using Insta-Fluor II scintillation liquid, both from Packard. Recovery of radioactivity in purified lactones ranged between 0.5 and 2.5% of the injected counts.

Extraction of the tissue with 5% acetic acid in water + 15 μM indomethacin, and purification with Sep-pak cartridges (Waters, Ass.) washed with 10% methanol/85% water/5% acetic acid and eluted with 100% methanol, were used when both PG-acids and lactones had to be purified and/or quantitated. After Sep-pak, the extracts were purified on a Spherisorb 5-μm column (ODS-2, 4.5 × 25 mm) eluted with a 90-min gradient from 30 to 70% acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid, flow rate = 1 ml/min. UV absorbance was monitored at 205 nm. PG amounts were determined using this HPLC system and PG standards, commercially available or previously purified from *T. fimbria*, to calibrate the column. The detection limit was 1 μg. Acquisition, integration and calibration were performed using a Maxima 820 chromatography workstation (Waters, Ass.).

Experiments in which [$^3$H]-$PGE_2$-1,15-lactone, obtained pure from the biosynthesis experiments, was converted back into tritiated $PGE_2$, were carried out in vivo in *T. fimbria* cerata. Labelled lactone (50 000 dpm) was injected into three different points of several cerata of comparable size immediately after their detachment from molested molluscs. The appendages, in duplicate, were either frozen (Time 0) or kept contracting for different periods of time. Extraction of the tissue and HPLC purification were then conducted in order to isolate labelled $PGE_2$ and $PGE_2$-1,15-lactone. The recovery of radioactivity in acid + lactone was consistent and was about 50%.

*Results and discussion*

The first series of experiments was aimed at determining whether PG-1,15-lactones could be synthesized in vivo from the corresponding acids and, hence, whether the former could be considered as an end-product of the latter. [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ were injected into the mantle of several specimens of *T. fimbria*. After different times of incubation the molluscs were frozen and the mantle and the cerata extracted separately. The extracts were purified by means of $SiO_2$ chromatography and straight phase HPLC. HPLC fractions containing PG-1,15-lactones were collected. Aliquots were used for counting and for acetylation which, after a second purification, confirmed that the incorporated radioactivity, retained in the mono- or diacetylated lactones, was not due to impurities. High incorporation of label was found in $PGF_{2\alpha}$-11-acetate and $PGE_2$-1,15-lactones, respectively, when [$^3$H]-$PGF_{2\alpha}$ or [$^3$H]-$PGE_2$ were injected. Labelled $PGE_2$-1,15-lactone and $PGE_2$ were also found upon injection of [$^3$H]-$PGF_{2\alpha}$, which indicated an unusual oxidative step from $PGF_{2\alpha}$ to $PGE_2$ free acids and/or lactones. As expected, no radioactivity was incorporated in $PGF_{3\alpha}$-11-acetate-, $PGE_3$- and $PGA_3$-1,15-lactones.

In all experiments the specific radioactivity of $PGE_2$-1,15-lactone decreased in the mantle and increased in the cerata with increasing incubation times (Fig. 3). This finding suggested that $PGE_2$-1,15-lactone is synthesized from $PGE_2$ mainly in the mantle, where the labelled precursors were injected, and then quickly transported into the cerata where it accumulates (an analogous observation could not be made for $PGF_{2\alpha}$ 1,15-lactone 11-acetate, which is more abundant in the mantle [table 1]). This hypothesis was confirmed by the quantitative data reported in table 1 and by four in vivo experiments summarized in table 2. 1) One day's incubation of [$^3$H]-$PGE_2$ in a specimen without cerata produced tritiated $PGE_2$-lactone. 2) Injection of labelled $PGE_2$-1,15-lactone, from the previous biosynthetic experiments, into the mantle of an intact specimen resulted in the finding of radiolabelled lactone also in the cerata. 3) Incubations of intact isolated cerata with [$^3$H]-$PGE_2$ for 6 h never produced a significant incorporation into the lactone, whereas 4) after a 6-h incubation in the intact mollusc the labelled lactone could be isolated from the cerata.

The next experiments were carried out in the cerata with the aim of investigating whether $PGE_2$- (and $PGE_3$-) 1,15-lactone, once transported to this tissue, could be converted in vivo back to the corresponding acids.

Spontaneous release of cerata from a few specimens of *T. fimbria* was induced and the appendages, in triplicate, were either kept contracting for different periods of time or immediately frozen at − 80 °C. The tissue was then extracted and analyzed by HPLC in order to quantitate PG free acids and lactones. As shown in fig. 4A, the amounts of $PGE_2$- and $PGE_3$-1,15-lactones were already considerably reduced after 15 min. Conversely, $PGE_2$ and $PGE_3$ levels were much higher than those observed in cerata at time 0. In order to confirm that these newly formed PGs originated from the lactones and not from de novo synthesis, radiolabelled $PGE_2$-1,15-lactone was injected into cerata which were treated as described above. Figure 4B shows that tritiated $PGE_2$, purified by HPLC, was formed during these experiments and that radioactivity incorporated into $PGE_2$-1,15-lactone decreased correspondingly. It is worthwhile mentioning that, when left for 4 h in a heat-inactivated homogenate of cerata, aliquots of [$^3$H]-$PGE_2$-1,15-lactone were not converted into $PGE_2$, thus ruling out the possibility that the conversion observed in vivo was merely a chemical degradation.

From the experiments described so far we can conclude that $PGE_2$- and, conceivably, $PGE_3$-1,15-lactones are: 1) synthesized from PGs mainly in the mantle; 2) mostly transferred into the cerata; 3) converted back into PGs mainly upon detachment of the cerata and during their spontaneous contractions. This is, to the best of our knowledge, the first example of a mechanism by which

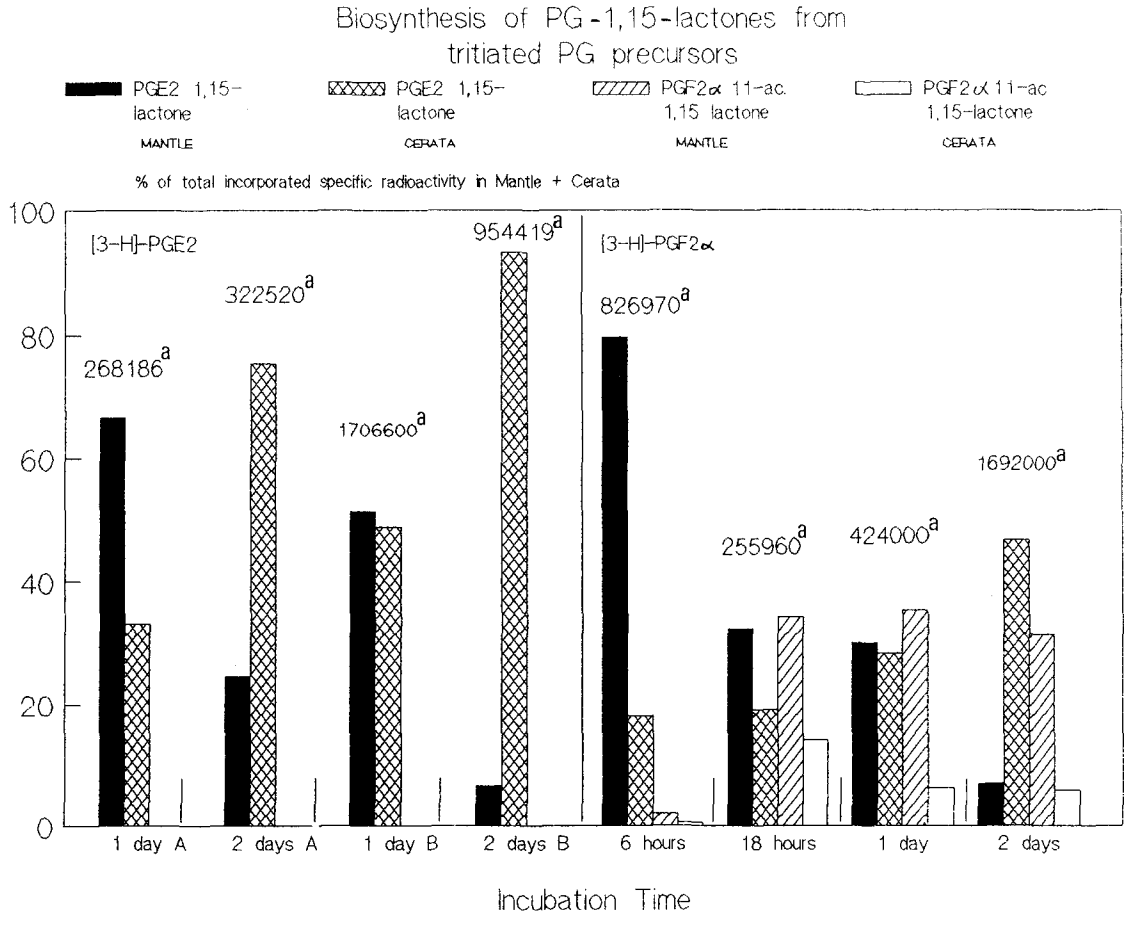


Figure 3. Biosynthesis of PG-1,15-lactones from tritiated PG precursors. Results are expressed as % of total incorporation because of inter-animal variations. [a] = Total specific incorporation for each experiment obtained adding together the specific incorporations (cpm/mg) observed separately in mantle and cerata for each radioactive lactone.

Table 2. Summary of the experiments conducted to show the transport of $PGE_2$ 1,15-lactone from the mantle to the cerata.

| | Total cpm found in $PGE_2$-1,15-lactone | |
|---|---|---|
| | Mantle | Cerata |
| Injecting 5 μCi tritiated $PGE_2$ into a specimen with no cerata (1 day) | 10200 | – |
| Injecting 25,000 cpm (1 mg) of tritiated $PGE_2$-1,15-lactone into an intact specimen (1 day) | 6500 | 3500 |
| Injecting 5 μCi tritiated $PGE_2$ into isolated cerata (6 h) (repeated three times) | – | N.D. |
| Injecting 5 μCi tritiated $PGE_2$ into an intact specimen (6 h) | 6120 | 5880 |

PGs, in the form of structurally-related compounds, are stored in vivo ready to be released in response to a mechanical stimulus to effect their biological action.

However, this action should not be considered simply as a mechanism of chemical deterrence since: 1) HPLC analysis of the supposedly defensive secretion of the mollusc revealed the absence of PGs and the presence of $PGE_2$-, $PGE_3$- and $PGA_3$-1,15-lactones, which did not produce the free acids even after the mucus was left at room temperature for many hours; 2) assays for ichthyotoxic activity against the mosquito fish (*Gambusia affinis*)[7] showed that, while PGs did not exhibit any toxicity, the lactones were toxic at concentrations ranging between 1 and 10 μg/ml (the most toxic compound was $PGE_3$-1,15-lactone whereas $PGF_{2\alpha}$- and $PGF_{3\alpha}$-1,15-lactone 11-acetates, which are only minor metabolites in the mucus, were non-toxic to mosquito fish). It must be said that the lack of ichthyotoxic activity described here for PG-acids is in contrast with previous observations suggesting that $PGA_2$ induces either an emetic response or an anti-feedant effect[8] in fish. However, the first effect was described for the 15(R) isomer of $PGA_2$, and the anti-feedant effect of this PG could only be observed at extremely high concentrations.

Therefore, PGs are not the defense allomones of *T. fimbria* although they are mainly synthesized during its typical defensive behavior, the detachment of the cerata. This mollusc seems to have developed a very economical way of exploiting PG-1,15-lactones for more than one purpose: a) as bioactive lactones, in the defensive secretion or, as might be the case for PGF-lactones[6], for other

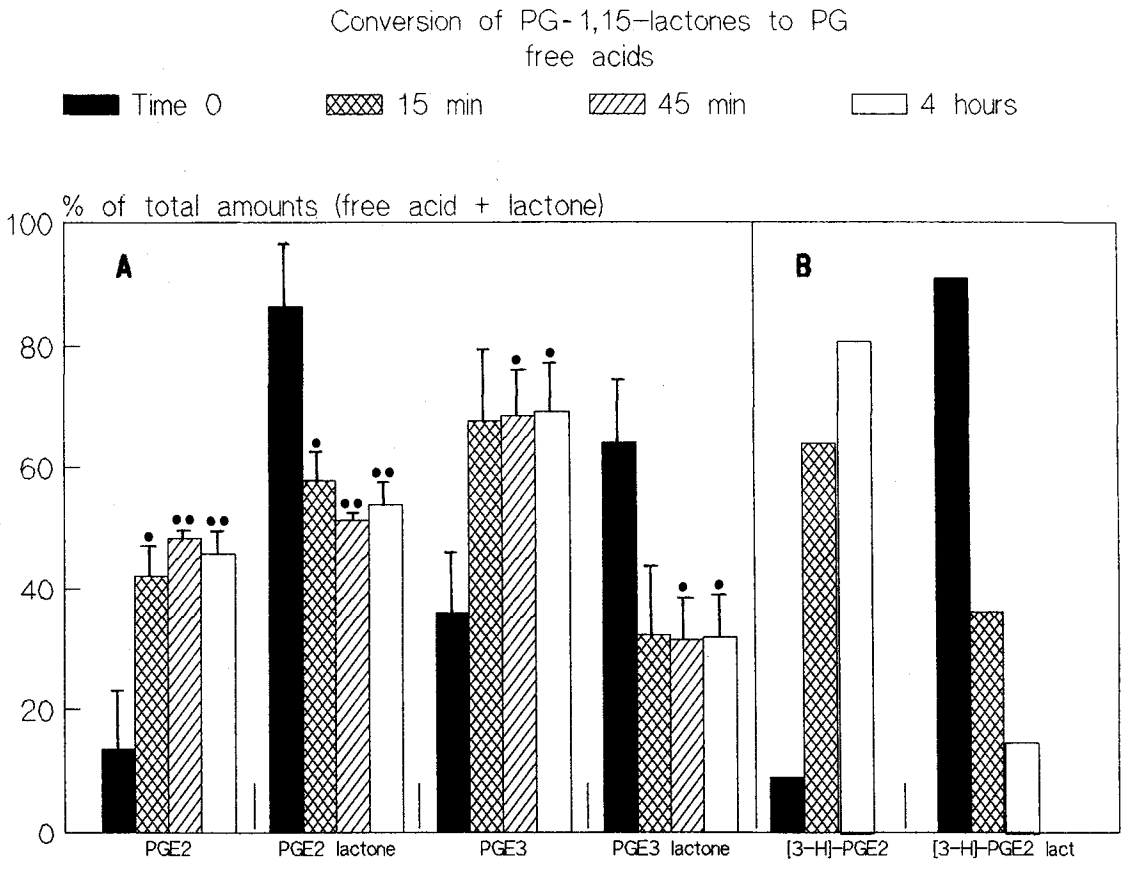


Figure 4. Conversion of PG lactones to PG's determined by: *A* quantitative measurement, as described in fig. 2, for each of the cerata at different times after detachment (n = 3, ± SE); *B* experiments with radiolabelled $PGE_2$-1,15-lactone. In *A*, average total amounts (acid + corresponding lactone) were 76 ± 7 and 92 ± 14 µg (n = 12, ± SE) respectively for $PGE_2$ and $PGE_3$. In *B*, statistical analysis was conducted using Student's unpaired t-test. ● = $p < 0.05$, ●● = $p < 0.025$.

uses in tissues different from the cerata; b) as inactive precursors of bioactive PGs within the cerata (or the mantle, considering that this tissue also contains PGs, [table 1]). Indeed, a pharmacological study conducted on PG-1,15-lactones before their discovery in natural sources showed that, apart from a certain antifertility activity, these compounds do not possess the wide range of pharmacological properties exerted by PGs[9]. For example, $PGE_2$-1,15-lactone did not cause contraction or release in smooth muscle preparations where $PGE_2$ is usually very active.

One hypothesis is that free PGs derived from PG lactones might intervene in the spontaneous and prolonged contractions typical of *T. fimbria* cerata. Histological analyses of this tissue showed the presence of contractile fibers similar to those of vertebrate smooth muscle, which is usually either relaxed or contracted by $PGE_2$[1]. Contractions of the cerata are probably needed to facilitate mucus secretion, and it may be in this context that PGs are involved in *T. fimbria* defensive mechanisms. The findings described here may be a starting point for future biochemical, pharmacological and electrophysiological studies aimed at fully understanding the role played by PG-1,15-lactones and free acids in this and other opisthobranch molluscs.

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