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Review

Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems

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Summary

Prostaglandins (PG) are oxygenated derivatives of C20 polyunsaturated fatty acids including arachidonic and eicosapentaenoic acids. In mammals, these compounds have been shown to play key roles in haemostasis, sleep–wake regulation, smooth muscle tone, and vaso-, temperature and immune regulation. In invertebrates, PGs have been reported to perform similar roles and are involved in the control of oogenesis and spermatogenesis, ion transport and defence. Although there is often a detailed understanding of the actions of these compounds in invertebrates such as insects, knowledge of their mechanism of biosynthesis is often lacking. This account provides a critical review of our current knowledge on the structure and modes of biosynthesis of PGs in invertebrates, with particular reference to aquatic invertebrates. It emphasises some of the most recent findings, which suggest that some PGs have been misidentified.

Prostaglandins in invertebrates can be categorised into two main types; the classical forms, such as PGE_2 and PGD_2 that are found in mammals, and novel forms including clavulones, bromo- and iodo-vulones and various PGA_2 and PGE_2 esters. A significant number of reports of PG identification in invertebrates have relied upon methods such as enzyme immunoassay that do not have the necessary specificity to ensure the validity of the identification. For example, in the barnacle *Balanus amphitrite*, although there are PG-like compounds that bind to antibodies raised against PGE_2, mass spectrometric analysis failed to confirm the presence of this and other classical PGs. Therefore, care should be taken in drawing conclusions about what PGs are formed in invertebrates without employing appropriate analytical methods. Finally, the recent publication of the *Ciona* genome should facilitate studies on the nature and mode of biosynthesis of PGs in this advanced deuterostomate invertebrate.

Key words: barnacle, coral, cyclooxygenase, eicosanoid, leukotriene, prostaglandin, prostaglandin D synthase, tunicate, *Ciona intestinalis*, *Balanus amphitrite*.

Introduction

Prostaglandins, together with thromboxanes (collectively termed prostanoids), are fatty acid derivatives of significant importance in many physiological processes. These compounds are formed following the action of cyclooxygenases (COX) and associated enzymes on C20 polyunsaturated fatty acid precursors released from phospholipids in membranes. Nearly all mammalian cell types have the biosynthetic machinery to produce at least one type of prostanoid. The same C20 fatty acid substrates can also be acted upon by lipoxygenases to produce mono- di- and tri-hydroxy derivatives such as leukotrienes, lipoxins and resolvins. The final route is the cytochrome P_{450} pathway that can convert C20 fatty acids to hydroxylated derivatives. Collectively these compounds are called eicosanoids; the term derived from the Greek *eikosi* that refers to the C20 backbone in the parent fatty acid.

Prostaglandins (PGs) were first discovered in the 1930s by von Euler and colleagues, who found a substance produced by the prostate gland that caused smooth muscle contraction. They christened the active substance ‘prostaglandin’, but it was over 30 years until the structure and mode of biosynthesis of these fatty acid derivatives became fully understood. PGs have many basic physiological functions where they act as ‘local’ hormones. For example, thromboxane (Tx) A_2 and prostacyclin (PGI_2) generated by platelets and endothelial cells, respectively, regulate the aggregatory behaviour of

platelets during haemostatic episodes (Moncada and Vane, 1979). Other PGs, including PGD_2 and PGE_2, are regulators of sleep–wake activity in mammals (Hayaishi 2000). For instance, in rat models, infusion of PGD_2 specifically increases the duration of sleep in a dose-dependent way (Hayaishi et al., 1990). PGE_2 also influences the central nervous system (CNS) in terms of temperature regulation, in which it acts as an endogenous pyrogen (see review by DuBois et al., 1998). Several PGs target smooth muscle cells, causing their contraction or relaxation. This is of particular importance in parturition where $PGF_{2\alpha}$ is an activator of myometrial contraction and cervical ripening (Johnson and Everitt, 2000). In the kidney PGs, including PGE_2 and PGI_2, modulate haemodynamics as a result of their vasodilatory activity and also have an effect on both salt and water balance (DuBois et al., 1998; Frolich and Stichtenoth, 1998). Finally, PGs play a complex role in inflammation, not only in the early stages as pro-inflammatory mediators but also at a later stage in eliciting resolution (Colville-Nash and Gilroy, 2000).

Aquatic invertebrates have played significant roles in our understanding of the biological activities of PGs. In 1969, Weinheimer and Spraggins discovered that one species of coral (*Plexaura homomalla*) contains up to 8% of its dry mass as PG esters. For a short time, in the absence of other available routes to synthesize PGs, this coral provided a ready source of precursors for the synthesis of such compounds for use in studies with humans and other mammalian models. From the many studies that have followed over the last 30 years, it is apparent that PGs play important roles in reproduction, ion transport and defence across a wide range of invertebrates (reviewed in Stanley, 2000). For instance, in insects detailed research has revealed that PGs function in egg laying, immune defence mechanisms and chloride transport (see reviews by Stanley-Samuelson, 1990; Stanley and Miller, 1998; Stanley, 2000). Despite a growing understanding of the roles of PGs in invertebrates (reviewed by Stanley, 2000), the nature of the products formed and their mode of biosynthesis are still largely unknown, particularly in non-insectan forms. This account therefore focuses on these aspects of PG biology and reviews some recent findings from aquatic invertebrates including corals, barnacles and tunicates. It questions whether all of the reports of PG identification and presence in invertebrates are valid in light of these recent findings.

Prostanoid biosynthetic pathways in mammals

The great majority of our knowledge of PG and Tx generation comes from studies using mammals. Hence this section briefly reviews the mechanism of prostanoid biosynthesis in these animals with particular emphasis on enzymatic activities. The principal substrate for prostanoid synthesis in mammals is the C20 polyunsaturated fatty acid, arachidonic acid (20:4n-6) although other fatty acids can act as substrates, including eicosapentaenoic acid (20:5n-3) and eicosatrienoic acid (20:3n-6). PGs derived from arachidonic acid are termed 2-series PGs, while eicosatrienoic and eicosapentaenoic acids result in the formation of 1- and 3-series PGs, respectively. The enzyme at the heart of prostanoid biosynthesis is COX, also termed PGH synthase. This enzyme is responsible for the generation of PGH_2 from arachidonate *via* the highly unstable endoperoxide, PGG_2 (Fig. 1). There are several forms of COX. The first, termed COX-1, is usually constitutively expressed in nearly all cell types within mammals, while the second, COX-2, is mainly inducible and only expressed by a more limited range of cell types. COX-1 is often described as the ‘housekeeping’ form of the enzyme because it is responsible for the generation of PGs of importance in physiological and haemostatic events. COX-2, on the other hand, has been found to be rapidly expressed in inflammatory conditions and is the target for a new group of non-steroidal anti-inflammatory drugs such as celecoxib and rofecoxib that have negligible effects on the constitutive COX-1 (Hawkey, 1999). The recent controversial finding of a third type of COX, COX-3, derived from the *COX-1* gene that is expressed in the cerebral cortex and heart, and is sensitive to analgesic/antipyretic drugs such as acetaminophen (Chandrasekharan et al., 2002), has given new insights into the mechanism of action of such agents (Chandrasekharan et al., 2002; Warner and Mitchell, 2002). It remains to be established if variants of COX-2 will be discovered (Chandrasekharan et al., 2002).

As can be seen from Fig. 1, the ultimate product of COX activity, PGH_2, is subject to further conversion to give rise to the generation of ‘classical’ PGs including PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (prostacyclin) as well as TxA_2. For such generation to occur, further enzyme activity is usually required. For example, PGD synthases, responsible for the generation of PGD_2 from arachidonate, consist of at least two evolutionarily distinct enzymes: a haemopoietic form expressed in mast cells, Th2 lymphocytes and platelet precursors, and a lipocalin-type PGD synthase found in the brain, testes and heart (Urade and Eguchi, 2002). The haemopoietic form of PGD synthase is a member of the sigma-class glutathione *S*-transferase family that has widespread distribution in multicellular organisms (Thomson et al., 1998). PGE synthases also consist of both membrane-associated and cytosolic forms (Murakami et al., 2002). The dramatic increase in PGE_2 generation in some inflammatory states appears to result from the induction of one of the membrane-associated PGE synthases (termed mPGES-1), and the stimuli responsible for the induction of COX-2 expression also induce the expression of this type of PGE synthase (Reddy and Herschman, 1997; Mancini et al., 2001; Umatsu et al., 2002). The recent addition of a second membrane-associated form of PGE synthase (mPGES-2) that is linked to both COX-1 and COX-2 (Murakami et al., 2003) emphasises the potential complexity of the relationship between PGE synthases and COX-1 and COX-2. Various cytosolic glutathione *S*-transferases also have the ability to convert PGH_2 to PGE_2 and other PGs (Ujihara et al., 1988). Finally, TxA and PGI synthases are distinct members of the diverse cytochrome P_{450} superfamily (Hara et al., 1994; Ullrich et al., 2001; Wang and Kulmacz, 2002).

As well as the ‘classical’ PGs, mention should be made of

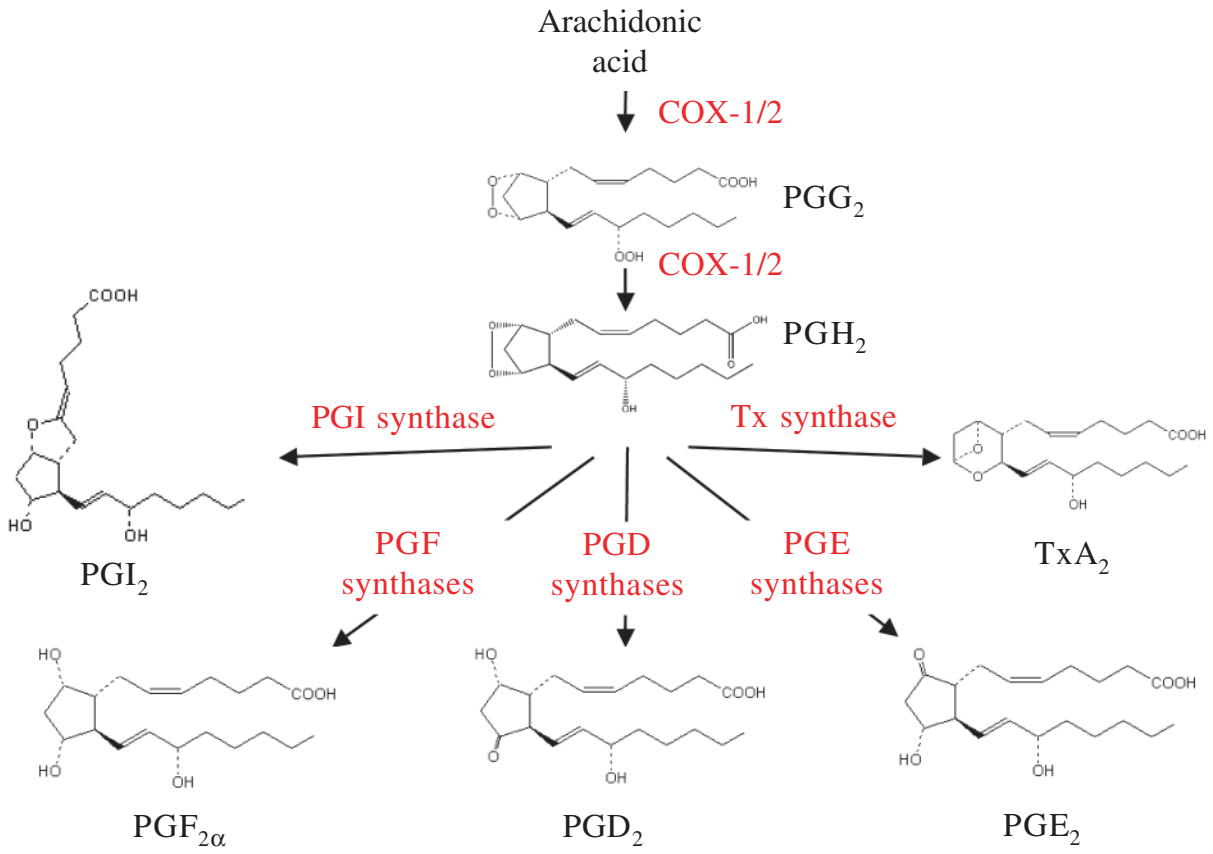

Fig. 1. Biosynthetic routes for the generation of 'classical' prostaglandins (PG) as found in mammals.

several additional forms including PGA_2, PGB_2 and PGJ_2. The J-type PGs are unusual in that they contain a cyclopentenone ring. PGD_2 is the precursor for the non-enzymatic generation of PGJ_2 and related forms such as Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Hirata et al., 1988). PGA_2 (also called medullin) is a non-enzymatic dehydration product of PGE_2, although the extent of its generation and biological activity in mammals remains unclear.

Following their biosynthesis, PGs are exported from cells across the cell membrane and bind to specific receptors on target cells. They can also be carried across membranes by a PG transporter (PGT; Kanai et al., 1995; Pucci et al., 1999). The finding that PGT is expressed in cell types that synthesize and release PGs may suggest that the transporter is involved in the re-uptake of PGs, either as a way of negating their leakage and/or facilitating the transport of such molecules to target nuclear receptors (Bao et al., 2002).

Our understanding of the nature and diversity of prostanoid receptors has increased dramatically in the last two decades. Each of the main type of prostanoid has its own specific G protein-coupled receptor. These are classified into five types termed EP, DP, FP, IP and TP, corresponding to the main prostanoids, PGE, PGD, PGF, PGI and TxA, respectively (Tsuboi et al., 2002). According to Tsuboi et al. (2002) with the exception of the EP receptors, all the others consist of a single type. The EP receptors for PGE consist of four main sub-types, EP_1–EP_4, in which each has a distinctive structure, signalling pathway and tissue distribution (Wright et al., 2001). The terminal product of PGD_2 breakdown, namely 15-deoxy-$\Delta^{12,14}$-PGJ_2, has its own specific nuclear receptor, the γ form of the peroxisome proliferator-activated receptor (PPARγ). This receptor is an important regulator of adipocyte differentiation (Negishi and Katoh, 2002).

Evidence for prostanoid generation in non-insectan invertebrates

One of the earliest descriptions of PG generation in invertebrates comes from the work of Christ and van Dorp (1972) who studied the ability of a wide range of invertebrates and vertebrates to synthesize PGs from radiolabelled eicosatrienoic acid. They found that conversion of this substrate to PGE_1 occurs in tissue homogenates from *Mytilus* (mussel), *Homarus* (lobster), *Lumbricus* (earthworm) and *Cyanea* (jellyfish) but not *Anthoplexaura* (coral), although the levels of conversion were reported to be rather small. Since these initial findings, there have been many reports of PG biosynthesis in a wide range of invertebrates. The nature of the PGs generated in insects has received particular attention and, as the results of these studies have been recently reviewed elsewhere (Stanley and Miller, 1998; Stanley, 2000), this current account focuses on non-insectan invertebrates only (Table 1).

Table 1. *Prostaglandin (PG) generation in non-insectan invertebrates*

Genus/species	Tissue(s)	PG generated	Analytical method	Functional significance	Reference
Sponges					
Reniera mucosa	All	Mucosin	NMR, HPLC*	–	Casapullo et al. (1997)
Cnidarians					
Gersemia fruticosa	Soft tissues	PGD_2, PGE_2, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$	HPLC, GC-MS	–	Varvas et al. (1993, 1999)
Clavularia viridis	Whole organism	Chlorovulones I–IV	NMR	–	Iguchi et al. (1985)
Clavularia viridis	Whole organism	Bromovulones and iodovulones	NMR	–	Iguchi et al. (1986); Watanabe et al. (2001)
Clavularia viridis	Whole organism	Clavulones, clavirins	NMR	–	Kikuchi et al. (1982); Iwashima et al. (1999)
Dendronephthya sp., *Dendrophyllia* sp., *Tubipora musica*	Whole organism	Bromovulones, bromopunaglandins	HPLC, NMR	Antibacterial, defence against predation	Řezanka and Dembitsky (2003)
Plexaura homomalla (coral)	Soft tissues	Various PGA_2 and PGE_2 esters	NMR	Defence against predation?	Weinheimer and Spraggins (1969); Gerhart (1986); Groweiss and Fenical (1990)
Telesto riisei (octocoral)	Whole organism	Punaglandins	NMR, MS	–	Baker et al. (1985); Baker and Scheuer (1994)
Nematodes					
Brugia malayi	Whole microfilariae	6-keto-$PGF_{1\alpha}$, PGE_2, PGD_2 but no $PGF_{2\alpha}$ or TxB_2	Radio TLC and HPLC; EIA	Assistance with invasive properties of parasite in host	Liu et al. (1990, 1992)
Wuchereria bancrofti	Whole microfilariae	PGE_2	EIA		Liu et al. (1992)
Platyhelminthes					
Schistosoma mansoni	Cercariae	$PGE_{1/2}$, PGD_2, PGA_2	HPLC, RIA	Parasite penetration of host	Fusco et al. (1985, 1986, 1993)
Molluscs					
Argopecten purpuratus (scallop)	Gonad	PGE_2, $PGF_{2\alpha}$	RIA	Gonadal development	Martínez et al. (1999)
Mytilus edulis (mussel)	Muscle, gill, mantle	Various classical PGs and PG-like compounds	TLC	–	Srivastava and Mustafa (1985)
Ligumia subrostrata	Gill homogenates	PGE_2, $PGF_{2\alpha}$	RIA	Ion balance	Saintsing et al. (1983); Hagar et al. (1989)
Lymnaea stagnalis	Accessory sex glands	PG-like compounds	HPLC	–	Clare et al. (1986)
Octopus vulgaris	Heart	PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2	Radio TLC	Control of cardiac function	Agnisola et al. (1994)
Patinopecten yessoensis (scallop)	Various	$PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$, TxB_2	HPLC, GC-MS	Spawning behaviour	Osada et al. (1989)
Tethys fimbria	Mantle, cerata and reproductive gland	PG 1,15-lactones of $PGE_{2/3}$ and $PGF_{2/3\alpha}$	GC-MS, EIMS, NMR	Chemical defence, control of oocyte fertilisation/production, smooth muscle contraction	Cimino et al. (1989, 1991a,b); Di Marzo et al. (1991)
Annelids					
Hirudo medicinalis (medicinal leech)	Head region	6-keto- $PGF_{1\alpha}$-like'	RIA	Inhibition of host platelet aggregation?	Nikonov et al. (1999)

Table 1. *Continued*

Genus/species	Tissue(s)	PG generated	Analytical method	Functional significance	Reference
Crustaceans					
Balanus amphitrite (barnacle)	Whole cyprid larvae	PGE	EIA	Inhibition of larval settlement	Knight et al. (2000)
Carcinus maenas (shore crab)	Blood cells	PGE, TxB, 6-keto-$PGF_{1\alpha}$	RIA	–	Hampson et al. (1992)
Penaeus japonicus (kurama prawn)	Whole haemolymph and ovary	$PGF_{2\alpha}$ and PGE_2	HPLC/RIA	Control of ovarian development	Tahara and Yano (2003)
Procambarus paeninsulanus (Florida crayfish)	Ovary	$PGF_{2\alpha}$, PGE_2		Ovulation ($PGF_{2\alpha}$)	Spaziani et al. (1993, 1995)
Acari					
Amblyomma americanum (lone star tick)	Whole haemolymph, salivary glands and saliva	PGE_2, $PGF_{2\alpha}PGD_2$, PGA_2/PGB_2	RIA/GC-MS and bioassay; radio-TLC	Assistance in tick feeding	Bowman et al. (1996); Pedibhotla et al. (1997); Aljamali et al. (2002)
Urochordates					
Ciona intestinalis (sea squirt)	Tunic, basket, ovary, intestine and heart	PGE, PGF	EIA	–	Knight et al. (1999); Pope and Rowley (2002)

*EIA, enzyme immunoassay; EIMS, electron impact mass spectrometry; GC-MS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; RIA, radioimmunoassay; TLC, thin layer chromatography.

As can be seen from Table 1, the PGs formed in invertebrates appear to fall into two categories, namely novel PGs only found in invertebrates, and the classical PGs (e.g. PGE_2, PGD_2 etc.) found in both invertebrates and vertebrates. The early studies of Weinheimer and Spraggins (1969) with the coral *P. homomalla* not only noted the unusual stereochemistry of the PGs formed (*R* rather than the *S* forms found in vertebrates) but also made the important finding that rather than the classical PGs, the main products synthesized were esters of PGA_2 and PGE_2 (Fig. 2). Subsequently, a number of other novel PGs have been reported from a diverse range of cnidarians and sponges including chloro-, bromo- and iodo-vulones, clavulones, punaglandins and mucosin (Table 1; Fig. 2). Several of these products have received much attention due to their potential antitumour activity (e.g. Iguchi et al., 1985, 1986; Honda et al., 1988; Iwashima et al., 1999). Their functional significance in the animals producing such compounds is unclear, but they may provide defence against predation by fish (Gerhart, 1991) as well as protecting against microbial attack (Řezanka and Dembitsky, 2003). Their potential as anti-predatory factors has, however, been questioned (Pawlik and Fenical, 1989) and further experimental work is required to confirm the original observations.

One of the most impressive series of studies on PG biosynthesis in invertebrates comes from the work on the opistobranch mollusc, *Tethrys fimbria* (Cimino et al., 1989, 1991a,b; Di Marzo et al., 1991). These authors showed conclusively that this mollusc generates novel PG derivatives, the PG 1,15-lactones, apparently derived from PGE_2 and $PGF_{2\alpha}$. The product profile in *T. fimbriae* also differs between the mantle, cerata and reproductive glands (Cimino et al., 1991b; Di Marzo et al., 1991), with PGs formed in the mantle exported to the cerata and reproductive glands where further structural modification occurs. This regional-specific generation of PGs may imply that these products perform different functions such as defence in the cerata, and control of the reproductive processes in the ovary/testis (Di Marzo et al., 1991). Because these studies have fully characterised the products formed and their mode of biosynthesis, *T. fimbria* would make a good model for detailed investigations aimed to determine the functional significance and mechanism of action of the PGs formed.

As can be seen from Table 1, there are many reports of the generation of classical PGs, particularly PGE_2, PGD_2 and $PGF_{2\alpha}$, in invertebrates. A significant number of these have employed techniques such as enzyme immunoassay (EIA), radioimmunoassay (RIA) and thin layer chromatography (TLC) that alone do not provide the specificity to confidently report on the presence of absence of various PGs. For instance, Knight et al. (1999) used commercially available EIA kits to determine if PGE and PGF immunoreactivity was formed in ionophore-challenged tissues from the tunicate, *Ciona intestinalis*. Because this approach without HPLC or some other form of

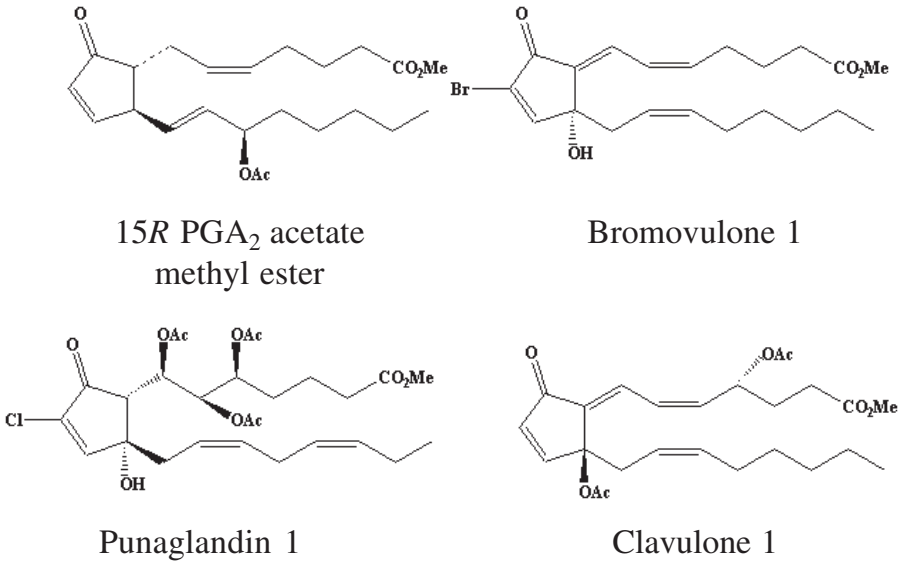

Fig. 2. Structure of some of the novel prostaglandin-like compounds formed in invertebrates. Structures from LipidBank (http://lipidbank.jp/index00.shtml).

high-resolution purification cannot differentiate between 2- and 3-series PGs (and other non-PG components), they expressed their results as 'ng immunoreactive PGE' rather than ng PGE_2. Others, however, have taken it for granted that the product identified and quantified by EIA or RIA is only that defined by the assay (e.g. Hagar et al., 1989; Martínez et al., 1999; Tahara and Yano, 2003), despite the possibility of the presence of alternative fatty acid substrates in these animals. It must be remembered that the specificity of these assays totally depends on the antibodies used as well as the degree to which samples have been extracted prior to the assay. Most of the antibodies employed show low reactivity with other classical PGs so that in defined cell types/tissues in mammals this approach presents few problems. However, in invertebrates with the potential for novel PGs that have not been screened for cross-reactivity with the antibodies, such an approach has clear limitations. An additional problem arises because many aquatic invertebrates, unlike terrestrial mammals, have significant amounts of arachidonic and eicosapentaenoic acids in their phospholipids (Stanley, 2000). Both of these can act as substrates for PG generation and EIA and RIA do not differentiate between the products formed. For instance, both PGE_2, formed from arachidonate, and PGE_3, derived from eicosapentaenoate, react equally with the antibodies in some commercial PGE_2 EIA kits. As discussed by Taylor and Wellings (1994), unless full structural analysis is achieved, there is little point in blindly using quantitative approaches, such as EIA, that lack specificity. Essentially, confidence in the accuracy of the identification and the quantification can only be achieved by a combination of approaches such as solid phase extraction prior to separation of analytes by high performance liquid chromatography (HPLC) or TLC, followed by mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy to provide full structural identification and an appreciation of the stereochemistry of the products. In very few cases has such an approach been employed in the studies reported in Table 1 and therefore the results reported are equivocal.

Several authors have reported the presence of 'PG-like' compounds in some invertebrates. For example, in the blue mussel *Mytilus edulis* the products of incubating gill, mantle or muscle with [^{14}C]arachidonic acid included one or more PG-like compounds that had an *rf* value on TLC similar to authentic PGE_2, $PGF_{2\alpha}$ and PGD_2 (Srivastava and Mustafa 1985). Aspirin and indomethacin inhibited the generation of radiolabelled material, suggesting that they are products of COX activity. Overall, however, there was no convincing evidence that the compounds formed were identical to classical PGs. Similarly, leeches (*Hirudo medicinalis*) are said to produce a PGI_2-like substance (Nikonov et al., 1999). Although the active substance inhibits human platelet aggregation and reacts with antiserum to 6-keto-$PGF_{1\alpha}$, the stable breakdown product of PGI_2, no structural data were provided. It is entirely possible that the active factor is a PG, but not necessarily PGI_2. In the snail *Lymnaea stagnalis*, the principal prostanoid synthesized following incubation of various tissues with radiolabelled arachidonate, did not correspond chromatographically to any authentic classical PG and was hence termed 'PG-like' (Clare et al., 1986). The COX inhibitor, aspirin, at 10 mmol l^{-1} reduced but did not completely eliminate the generation of this putative PG.

Finally, there are examples of studies with some invertebrates where authors have used known classical PGs and other eicosanoids in bioassays without first screening by any analytical method to see if the compound of interest is synthesised in the animal studied. Such an approach often results in the finding of biological activity without any indication that the animal or tissue under study can synthesize the appropriate substance. An example of this comes from work with sand dollars (*Echinaracnius parma*) where leukotriene B_4 (LTB_4), a 5-lipoxygenase product, has been found to regulate intracellular Ca^{2+} levels in eggs (Silver et al., 1994) when studies with eggs from other echinoderms (sea urchins, *Stronglyocentrotus purpuratus*) have shown categorically that the lipoxygenase products generated did not

include LTB_4 (Hawkins and Brash, 1987). Hence the natural eicosanoid that regulates calcium changes in echinoderm eggs is highly unlikely to be LTB_4.

Recent insights from studies on barnacles

Barnacles have a complex life cycle involving free swimming larval stages that give rise to a sessile adult (Fig. 3). There are two main stages of this life cycle during which eicosanoids are thought to play key roles in the signalling pathways that may control barnacle development. The first is following fertilisation, when the fertilised eggs (embryos) are brooded in the mantle cavity of the adult. Upon hatching, the larvae are liberated into the surrounding water. This hatching process appears to be triggered by 'barnacle hatching factor' that is thought to consist of a 'cocktail' of different eicosanoids including the lipoxygenase products, hepoxilin A_3 (Vogan et al., 2003) and various mono-, di- and tri-hydroxy fatty acid derivatives (Hill et al., 1993). Whether PGs have hatching activity is unclear, although Clare et al. (1982) found that crude barnacle hatching factor from *Balanus balanoides* prepared in the presence of the COX inhibitor, aspirin, lacked hatching activity, perhaps indicating COX involvement in its generation. They also noted that dried coral extract from *P. homomalla* has barnacle hatching factor activity, suggesting that a range of eicosanoids are likely to be involved and that the ligand specificity of the triggering process may be limited.

The second stage that may be influenced by eicosanoids is settlement when the cyprid larvae attach to the substratum, prior to a radical metamorphosis that ultimately gives rise to sessile adults (Fig. 3). Knight et al. (2000) demonstrated in *Balanus amphitrite* that PGE_2, PGE_3 and the stable synthetic analogue of PGE_2, 15,15-dimethyl-PGE_2, caused a dose-dependent inhibition of larval settlement, while indomethacin, a COX inhibitor, stimulated this process. They concluded from these preliminary findings that PGs might play key roles in controlling larval settlement. Studies using EIA alone found that the soft tissues of *B. amphitrite* generate significant amounts of PGE immunoreactive material (Knight et al., 2000), but taking into account the problems of using EIA alone for PG identification already discussed, such preliminary results required confirmation. Therefore, the potential biosynthesis of PGs by adult and larval barnacles was studied using a combination of solid phase extraction of analytes, separation by reverse phase-HPLC, followed by mass spectrometry (MS) of fractions found to have immunoreactivity in EIA. Such an approach was chosen to categorically identify all potential PGs generated. HPLC-negative ion electrospray MS of calcium ionophore-challenged barnacle tissues revealed two major peaks with PG-like masses and elution times. Firstly, a component with a retention time of ~14.23 min eluting ~0.7 min earlier than the authentic $PGF_{3\alpha}$, which generated a deprotonated ($M\text{-}H^-$) ion at an *m/z* 353, and secondly, a component that eluted at ~16.43 min between authentic standards $PGF_{2\alpha}$ and PGE_2 (equivalent to peaks I and II, respectively, in Fig. 4), generating an $M\text{-}H^-$ at *m/z* 351. In order to boost product generation and overcome the problems of low sensitivity (ng levels) on HPLC-MS, *B. amphitrite* tissue samples were pre-incubated with the exogenous fatty acids EPA and AA. This generated two additional peaks with PG-like masses, an *m/z* 353 species with a retention time of ~18.54 min and an *m/z* 351 species, which eluted at ~20.66 min (peaks III and IV, respectively, in Fig. 4). However, when samples were pre-incubated with the COX inhibitor indomethacin (25 μmol l^{-1}), all four peaks remained, suggesting that the peak identities were either non-prostanoid

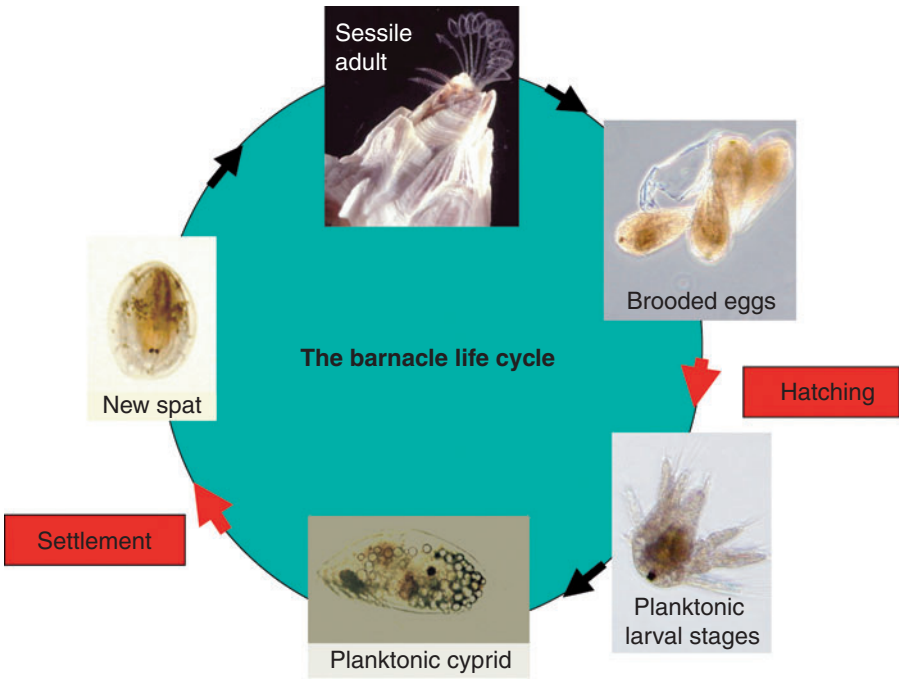

Fig. 3. Life cycle of barnacles and the times when eicosanoids are thought to play a role in development (red boxes). Following fertilisation the eggs are brooded in the mantle cavity where hatching is under the control of hatching factors. The resulting planktonic larval stages undergo several moults until giving rise to the cyprid stage that uses its antennules to probe for suitable settlement sites. At settlement, these moult to give rise to juveniles (spat) that also grow and moult to give rise to a filter-feeding sedentary adult.

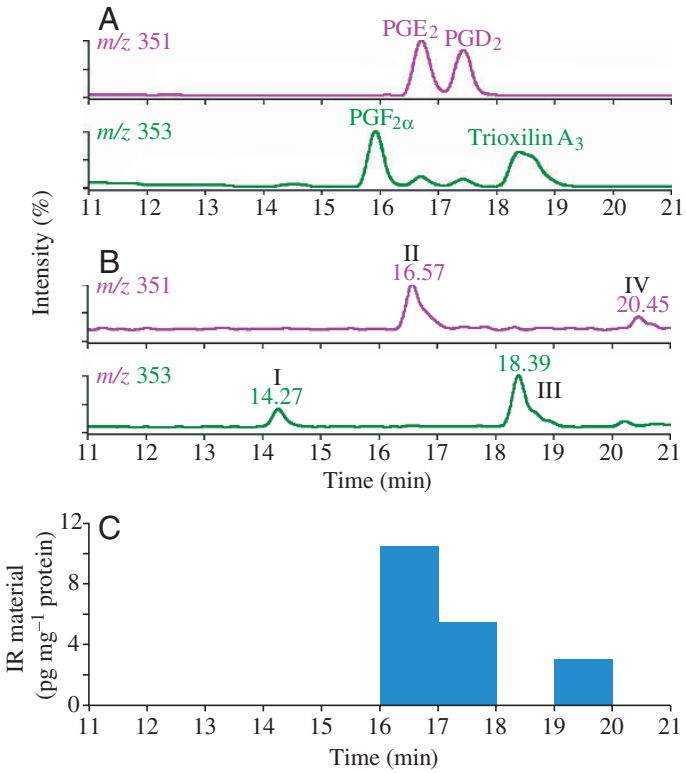

Fig. 4. Ionophore-challenged *B. amphitrite* larval sample showing generation of prostaglandin (PG)-like material prepared in the absence of the COX inhibitor, indomethacin. (A) HPLC-MS trace showing characteristic peak generation in the *m/z* 351 and 353 ion channels for the eicosanoid standards, PGE_2, PGD_2, $PGF_{2\alpha}$ and trioxilin A_3. (B) Larval sample showing peaks with prostaglandin-like masses (I–IV) on HPLC-MS prepared in parallel and run under identical HPLC-MS conditions to the standards in A. (C) HPLC fractions collected from material shown in B that was subsequently lyophilised, resuspended in buffer and assayed on a total PG Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) designed to react with all classical PGs. This immunoreactivity was significantly reduced when barnacle samples were pre-incubated with indomethacin (20 µmol l^{-1}) prior to ionophore challenge (not shown).

or that they were prostanoids derived *via* a non-COX route. HPLC fractions containing PG-like material were derivatised for electron impact GC-MS. This revealed the peak identities to be the lipoxygenase products, trioxilin A_4 (peak II, Fig. 4) and trioxilin A_3 (peak III, Fig. 4). The two remaining PG-like peaks (I and IV, Fig. 4) could not be identified on electron impact GC-MS. Thus, the presence of any classical prostanoids in *B. amphitrite* including $PGE_{2/3}$, $PGF_{2/3\alpha}$, $PGD_{2/3}$, $TxA_{2/3}$, $PGI_{2/3}$ as well as $PGA_{2/3}$ and $PGJ_{2/3}$ could not be confirmed on either HPLC-MS or GC-MS. Hence it was concluded that they are either not produced or they are present in levels below the detection limit (ng) on HPLC/GC-MS. The latter hypothesis was further supported by the repeated detection of >100 pg mg^{-1} protein of PG immunoreactivity on total PG, PGE and PGF EIA kits predominantly in HPLC fractions between 14–18 min, but particularly in the 16–17 min time fraction e.g. (Fig. 4). When samples were prepared in the presence of indomethacin (a COX inhibitor), immunoreactivity was completely suppressed, suggesting that this material was probably derived through a COX route (i.e. PG-like) and was not the result of antibody cross reactivity with trioxilin A_4 or other lipoxygenase-derived products.

Overall these barnacle studies highlight the fact that it is extremely easy to mis-identify other compounds (e.g. trioxilins) as PG-like compounds if no electron impact GC-MS work is conducted. It also indicates the problems encountered in gaining structural elucidation when material is generated in extremely low levels (i.e. sub-ng), as appears to be the case in *B. amphitrite*.

Prostanoid biosynthetic pathways in invertebrates

Until recently there was a dearth of information about how PGs are generated in invertebrates. Initially, PG generation in corals, at least, was thought to proceed *via* a collaborative mechanism involving 8(*R*) lipoxygenase and allene oxide synthase activity (e.g. Corey et al., 1987; Song and Brash, 1991). However, it is clear from a number of reports that PG generation in a wide range of invertebrates is subject to inhibition by the presence of COX inhibitors such as indomethacin, aspirin and ibuprofen (e.g. Knight et al., 1999) suggesting the existence of a COX-derived mechanism for PG biosynthesis. More recent biochemical studies using the Arctic coral *Gersemia fruticosa* have demonstrated that radiolabelled arachidonate can be converted to the unstable intermediate PGG_2 (Varvas et al., 1999). The cDNA that codes for a COX isozyme was subsequently cloned from this coral (Koljak et al., 2001) and the deduced amino acid sequence of the *G. fruticosa* COX revealed the presence of Ile^{523} that mainly confers the specificity of this enzyme towards COX inhibitors. In COX-2 the amino acid at this position is valine (Val^{523}), while in all known COX-1 isozymes it is isoleucine (Gierse et al., 1996; Garavito and DeWitt, 1999). As the coral COX contains isoleucine at this position, it is insensitive to COX-2 selective inhibitors such as nimesulide but subject to inhibition by the broad-spectrum COX inhibitor, indomethacin (Koljak et al., 2001). These and other key findings on the structure of coral COX (Valmsen et al., 2001) show that COX activity probably occurs widely within all multicellular invertebrates and is therefore likely to be central in PG generation in all protostomate and deuterostomate animals. Presumably the coral COX is the forerunner of the typical vertebrate COX-1 and COX-2 isozymes. What remains unanswered, however, is at what stage in metazoan evolution did the different forms of COX appear? As bony fish have been shown to have both a constitutive COX-1 as well as an inducible COX-2 with strong sequence homology to their mammalian counterparts (Zou et al., 1999; Roberts et al., 2000) this key event in the evolution of these two isozymes probably predates the emergence of bony fish some 350 Myr ago. Whether the more primitive jawed cartilaginous fish, such as sharks and rays, and the jawless lampreys and hagfishes, express one or two isoforms of COX is currently unknown but a recent report on the cloning

of COX cDNA from shark *Squalus acanthias* rectal glands has revealed the existence of a single, constitutively expressed, isoform of COX sharing some features of both COX-1 and COX-2 (Yang et al., 2002). These findings could arguably support the hypothesis that sharks may only have a single COX isoform, but this tentative conclusion remains to be investigated.

Not only has the existence of COX been shown in some corals but also potential mechanisms for the biosynthesis of the unusual PG esters have been proposed (Valmsen et al., 2001). In this, the action of COX on arachidonate leads to the generation of an unstable PG endoperoxide similar to PGH_2 found in mammals but with the *R* rather than the *S* configuration at C15 (Fig. 5). Following this COX-mediated stage, the 15(*R*)-PGE_2 formed is converted to its methyl ester and acetylated to give rise ultimately to the large amounts of stable 15*R*-PGA_2-methyl ester and 15*R*-acetate-PGA_2-methyl ester stored in these animals.

Recent findings by Brash and colleagues on lipoxygenases in *P. homomalla* may explain how clavulones and related cyclopentenone eicosanoids are formed (Boutaud and Brash, 1999; Tijet and Brash, 2002). This coral contains an unusual allene oxide synthase – lipoxygenase fusion protein. Tijet and Brash (2002) have suggested that clavulones are formed by a pathway that commences with the action of 8(*R*)lipoxygenase on arachidonic acid to give rise to 8*R* hydroperoxyeicosatetraenoic acid that is subsequently converted to allene oxide by the allene oxide synthase activity. Subsequently, this gives rise to clavulones by a method analogous to that employed in plants in the formation of jasmonic acid from linolenic acid (Tijet and Brash, 2002). This provides a much needed explanation of how clavulones and related forms may be synthesized in marine invertebrates.

Little is known of the presence of any of the other enzymes involved in the generation of classical PGs in invertebrates with the exception of PGD synthase in parasites. Since the discovery of Fusco et al. (1985) that the penetration of the human host by cercariae of *Schistosoma mansoni* is apparently

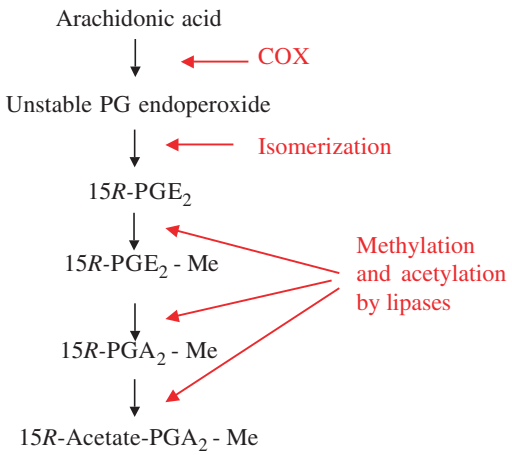

Fig. 5. The proposed mechanism of biosynthesis of prostaglandins in the coral, *Plexaura homomalla* (modified from Valmsen et al., 2001).

influenced by PGs, there has been heightened interest in the possibility that both protozoan and metazoan parasites may improve their success of survival either by generation of PGs themselves or by modifying the host's ability to generate PGs. Haemopoietic PGD synthase is a member of the sigma-class glutathione *S*-transferase (GST) family (Kanaoka et al., 2000). GSTs in general are multifunctional enzymes found in both invertebrates and vertebrates, and it is unlikely that all of the sigma-class forms will have PG synthase activity because some lack the amino acid residues involved in substrate (PGH_2) binding (Thomson et al., 1998). Recently, however, both the sigma class GSTs from the filarial parasite *Onchocerca volvulus* (Sommer et al., 2003) and *Schistosoma* (Johnson et al., 2003) have been shown to convert PGH_2 to PGD_2, while in *Ascaridia galli* a purified GST has PGE synthase activity (Meyer et al., 1996). In the case of the *O. volvulus* GST (*Ov*-GST-1), this enzyme is located at the margins of the parasite, in the cuticle and hence in a prime location to influence the host responses. Similarly, in *Brugia malayi* and *Wuchereria bancrofti*, the parasite microfilariae become coated in PGE_2 following *in vitro* culture as a result of its generation in the parasites (Liu et al., 1992). As PGs have been shown to be involved in immune regulation in mammals and some other vertebrates (e.g. Garrone et al., 1994; Knight and Rowley, 1995) as well as in inflammation (Colville-Nash and Gilroy, 2000), the synthesis of these compounds by parasites could affect the host immune response favouring parasite survival and host penetration (Daugschies and Joachim, 2000; Noverr et al., 2003).

Insights from the *Ciona* genome

Ciona intestinalis is a member of the Phylum Chordata that includes the vertebrates, urochordates (sea squirts, salps) and cephalochordates (amphioxus). This deuterostomate invertebrate has probably retained many of the features of the ancient chordates prior to the emergence of the ancestors of the early vertebrates. Hence, it has been a popular model animal for comparative immunologists interested in tracing the roots of the vertebrate immune system (Cooper and Parrinello, 2001) and developmental biologists who have employed ascidians, including *C. intestinalis,* to study gene expression during development (Jeffery, 2002). Towards the end of 2002 the draft genome of *C. intestinalis* was published (Dehal et al., 2002) and the sequence database established (http://genome.jgi-psf.org/ciona4/ciona4.home.html). A recent cDNA/expressed sequence tag study has also identified a number of genes expressed in the haemocytes (blood cells) of *C. intestinalis* (Shida et al., 2003). Previous studies on eicosanoid generation in *C. intestinalis* mainly focussed on the putative lipoxygenase products rather than PGs (Knight et al., 1999; Pope and Rowley, 2002). Knight et al. (1999) did, however, report that PG generation as measured by EIA was selectively inhibited by COX-2 rather than COX-1 inhibitors. They deduced from this that the constitutive form of COX expressed in *C. intestinalis* is COX-2 like in terms of those amino acids that

confer such selectivity (Gierse et al., 1996). This conclusion is borne out by the *Ciona* genome project where ~95% sequence homology with various piscine COX-2 was found. No gene encoding for a further COX-isoform is apparent in the current annotated database, possibly implying that only a single form of COX exists in *C. intestinalis*. Other interesting findings include a gene coding for PGD synthase that shows >95% homology with other glutathione-dependent PGD synthases (haemopoietic PGD synthases) and known invertebrate and vertebrate glutathione *S*-transferases. Homologues of the mammalian PGT transporter and the EP_4 receptor for PGE_2 are also annotated in the *Ciona* database. The current annotations fail to identify any further PG/Tx synthase genes or any of the other receptor family for prostanoids. As the database is subject to further probing such genes may be still be found, but it has been concluded by Dehal et al. (2002) that the genes missing from the current assemblage are probably absent from the genome itself. The study also noted the paucity of genes coding for rhodopsin-like heterotrimeric GTP-binding protein coupled receptor family of which the PG receptors are constitutive members. While it may be premature to speculate further, it appears as if *Ciona* may only have one receptor type for PGs with high homology to the EP_4 receptor subtype. In the apparent absence of the enzymes required for TxA_2 and PGI_2 (prostacyclin) generation and their respective receptors, it is tempting to suggest that such molecules are absent from *Ciona* and perhaps all invertebrates. Their evolutionary origins may be linked to the emergence of haemostatic mechanisms based on fibrin generation and its interaction with platelets/thrombocytes that happened with the appearance of the first vertebrates (Rowley et al., 1997).

Concluding remarks

To our knowledge, no prostanoid receptors have been cloned from any invertebrate and only in a few selected cases (e.g. corals) outside the Insecta do we have even a basic understanding of the nature of the prostanoids formed and their modes of biosynthesis. This is clearly an unsatisfactory situation if an understanding of how such molecules influence physiological events in these animals is to be achieved. The recent publication of the *Ciona* genome, coupled with an extensive knowledge of this organism's developmental biology, physiology and immunobiology, makes this a key model animal for future eicosanoid research in a deuterostomate invertebrate that will dissect both the pathways for eicosanoid biosynthesis and how such molecules are involved in signalling events at the molecular level.

List of abbreviations

GST	glutathione *S*-transferase
CNS	central nervous system
COX	cyclooxygenase
EIA	enzyme immunoassay
HPLC	high performance liquid chromatography
mPGES	membrane associated PGE synthase
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
PG	prostaglandin
PGI_2	prostacyclin
PGT	prostaglandin transporter
RIA	radioimmunoassay
TLC	thin layer chromatography
Tx	thromboxane

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