The Journal of Experimental Biology 211, 599-605
Published by The Company of Biologists 2008
doi:10.1242/jeb.009225

Reconstitution of a chemical defense signaling pathway in a heterologous system

Staci A. Padove Cohen[1], Hanns Hatt[2], Julia Kubanek[3] and Nael A. McCarty[1,*]

[1]Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA, [2]Cell Physiology, Ruhr-Universitaet Bochum, Bochum 44780, Germany and [3]Chemistry and Biochemistry, Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA

*Author for correspondence (e-mail: nael_mccarty@oz.ped.emory.edu)

Accepted 3 December 2007

SUMMARY

Chemical signaling plays an important role in ecological interactions, such as communication and predator–prey dynamics. Since sessile species cannot physically escape predators, many contain compounds that deter predation; however, it is largely unknown how predators physiologically detect deterrent chemicals. Few studies have investigated ecologically relevant aversive taste responses in any predator. Our objective was to determine if a signaling pathway for detecting marine sponge-derived deterrent compounds could be reconstituted in a heterologous expression system to ultimately facilitate investigation of the molecular mechanism of such an aversive behavioral response. Zebrafish (*Danio rerio*) rejected artificial diets laced with sponge chemical defense compounds that were previously shown to deter a generalist marine predator, *Thalassoma bifasciatum*, suggesting that zebrafish can recognize deterrent compounds relevant to coral reef systems. Transcripts made from a zebrafish cDNA library were expressed in a heterologous system, *Xenopus laevis* oocytes, and tested for chemoreceptor activation *via* electrophysiology, using the cystic fibrosis transmembrane conductance regulator (CFTR) as a reporter. Oocytes expressing gene sequences from the library and CFTR exhibited a CFTR-like electrophysiological response to formoside and ectyoplasides A and B, sponge defense compounds. Therefore, the chemical defense-activated signaling pathway can be reconstituted in *Xenopus* oocytes. Kinetics of the responses suggested that the responses to formoside and ectyoplasides A and B were receptor-mediated and capable of using the $G_{\alpha s}$ signaling pathway in this system. This bioassay has the potential to lead to the identification of genes that encode receptors capable of interacting with deterrent chemicals, which would enable understanding of predator detection of chemical defenses.

Key words: chemical defense, oocyte expression system, electrophysiology, chemoreceptor.

INTRODUCTION

Organisms detect and disseminate chemical stimuli to perceive their environments and communicate with other organisms (Dusenbery, 1992). However, the molecular basis for chemoreception is not well understood; although some genes encoding putative receptors are known (Buck and Axel, 1991), their specific ligands remain largely unidentified. Conversely, some ligands have been identified, but their responsive chemoreceptors have not (Mombaerts, 2004). Many sessile or slow-moving terrestrial and marine organisms utilize defensive chemicals to protect them from predation, colonization by bacteria, and overgrowth by neighboring organisms (Paul et al., 2006). For example, chili peppers contain capsaicin, a pain-inducing compound that reduces predation by select vertebrates (Caterina et al., 1997). Triterpene glycosides produced by *Erylus formosus* and *Ectyoplasia ferox* protect these Caribbean sponges from predation, microbial attachment, and overgrowth by competing sponges (Kubanek et al., 2002). The decorator crab *Libinia dubia* reduces its predation by decorating its carapace with *Dictyota menstrualis* (Stachowicz and Hay, 1999), a chemically defended brown alga which contains isoprenoid compounds that deter predation and prevent larval settlement on the surface of these plants (Schmitt et al., 1995). Chemical defense compounds, like those utilized by this wide variety of organisms, are secondary metabolites produced either by the organism, a bacterial symbiont, or are sequestered from another species (Moore, 2006).

Defensive compounds could act on predators in a variety of ways. For example, the mediator of the noxious response to chili peppers is the capsaicin receptor, TRPV1, a member of the TRP family of ion channels, which causes the activation of a pain pathway in mammals (Caterina et al., 1997) but not birds (Jordt and Julius, 2002). Some defensive compounds have been shown to be phagomimics that distract predators, who attempt to eat the emitted defensive compounds while the prey escapes (Kicklighter et al., 2005). Several species of ascidians produce inorganic acids that cause them to be unpalatable to potential predators and damage cells of the organisms that ingest them (Stoecker, 1980; Lindquist et al., 1992; Pisut and Pawlik, 2002). Alternatively, phlorotannins, found in marine algae, and tannins, found in terrestrial plants, form indigestible complexes with plant nutrients or inactivate digestive enzymes by binding to them (Mole and Waterman, 1987; Boettcher and Targett, 1993; Targett and Arnold, 2001). Some deterrent compounds are hypothesized to be toxic (Lindquist and Hay, 1995); and potential predators have unknown molecular detection methods to prevent them from ingesting prey bearing these and other unpalatable compounds.

Marine sponges contain a variety of secondary metabolites that are known to be unpalatable to reef predators (Chanas et al., 1997; Assmann et al., 2000; Waddell and Pawlik, 2000; Duque et al., 2001; Kubanek et al., 2001; Pawlik et al., 2002) yet we know very little about how these compounds are perceived by potential predators, other than the fact that predators rapidly reject foods containing these compounds. A study of the cellular effects of chemical deterrents from marine sponges (Bickmeyer et al., 2004) suggested that 4,5-dibromopyrrole-2-carboxylic acid, a deterrent

compound found in *Agelas* sponges, may alter calcium homeostasis of chemoreceptive cells. However, this study investigated calcium responses in rat adrenal cells and *Aplysia* (sea hare) neurons, which are only distantly related to natural predators of sponges; therefore, this physiological response may not occur in fish chemoreceptive cells.

It is likely that most cases of deterrence are mediated by a chemosensory response based upon odor or taste; that is, a predator's chemoreceptors most probably respond to deterrent compounds from prey, as they have the ability to respond to numerous chemicals (Mombaerts, 2004). Chemoreceptors for known odorants or tastants are often G protein-coupled receptors (GPCRs), which may couple to ion channels, such as bitter receptors; in some cases, receptors form ion channels themselves, as in the case of sour receptors (Lindemann, 2001; Mombaerts, 1999). Both bitter and sour taste receptors cause aversive responses in many organisms and help organisms detect unripe fruits, spoiled food and potentially harmful compounds, and to avoid tissue damage by acids (Lindemann, 2001; Oike et al., 2007). Because predatory fish have been observed to reject foods containing chemical defense compounds within 1 s of ingestion (Chanas et al., 1997; Kubanek et al., 2000; Assmann et al., 2000; Pawlik et al., 2002), we hypothesized that ion channels (known to cause immediate cellular responses involved in sour and bitter taste) may be involved, either directly as receptors for these deterrent compounds or *via* coupling to chemosensory receptors. The ligands that interact with chemoreceptors have been identified in very few cases, and relatively little is known about chemoreceptors that respond to chemical deterrents (Caterina et al., 1997). Identifying a gene encoding such a chemoreceptor and investigating its signaling response could be very useful in studying predator–prey interactions on a molecular, behavioral and evolutionary level.

The long-term goal of this study was to identify a gene encoding a receptor whose ligand acts as a chemical defense in a marine organism, by functionally screening a fish cDNA library, in order to investigate the molecular mechanism of an aversive behavioral response. A logical species choice for the library was bluehead wrasse (*Thalassoma bifasciatum*); a common predator on coral reefs (Lindquist et al., 1992) that is one of several reef fishes shown to respond to a variety of chemical defense compounds (Chanas et al., 1997; Assmann et al., 2000; Kubanek et al., 2000; O'Neal and Pawlik, 2002; Pawlik et al., 2002; Pisut and Pawlik, 2002; Kicklighter et al., 2003; Jones et al., 2005). Since cDNA libraries from this species, and other generalist reef fishes, are not publicly available, we utilized a library constructed from a different, model fish species, the zebrafish *Danio rerio*. The *D. rerio* genome is highly characterized, and high quality libraries are publicly available. Since chemical defense compounds are noxious, and many organisms have protective mechanisms to detect these types of chemicals in order to avoid them, we hypothesized that zebrafish may also be able to detect them. First, we used a behavioral assay to confirm that zebrafish are able to detect sponge chemical defense compounds that also induce aversive responses in reef fishes. We then determined that a deterrent signaling pathway responsive to one of these compounds could be reconstituted by expressing a zebrafish cDNA library in *Xenopus* oocytes.

MATERIALS AND METHODS

Chemicals

Isoproterenol (Iso), forskolin, 3-isobutyl-1-methylxanthine (IBMX), octanal, sodium alginate, calcium chloride and ethanol were obtained from Sigma-Aldrich (St Louis, MO, USA). Sceptrin (a defensive sponge compound) was obtained from A.G. Scientific (San Diego, CA, USA). All other sponge-derived compounds were isolated from sponge tissues by following previously described methods (Chanas et al., 1997; Kubanek et al., 2000; Kubanek et al., 2002).

Animals

Female *Danio rerio* Hamilton were obtained from Carolina Biological Supply (Burlington, NC, USA). All fish were housed singly in partitioned 40 l aquaria and maintained at 23–27°C in a 12 h:12 h L:D cycle. *Xenopus laevis* (Daudin 1802) were obtained from Xenopus Express (Dexter, MI, USA) and housed in an aquatic habitat (Aquaneering, Inc., San Diego, CA, USA). Methods of animal handling were in accordance with the NIH guidelines and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology.

Fish feeding assays

Palatability assays using the zebrafish *D. rerio* were performed as previously reported for marine fishes (Pawlik and Fenical, 1992; Pawlik et al., 1995). Briefly, isolated sponge compounds, triterpene glycosides or brominated alkaloids, were dissolved in a minimal amount (<0.01%) of methanol and incorporated into a matrix of aqueous sodium alginate (0.06 g ml^{-1}) and freeze-dried squid (0.03 g ml^{-1}). Concentrations of sponge compounds incorporated into the mixture were chosen based on concentrations known to be deterrent to bluehead wrasse and the amount of compound available to assay. The mixture was packed into a 1 ml syringe, which had an attached 200 μl pipette tip with a slightly enlarged opening, and ejected into a 0.25 mol l^{-1} $CaCl_2$ solution to solidify the artificial food. The resulting noodle was rinsed with deionized water, to remove excess $CaCl_2$, and sliced into 3 mm pellets. Control pellets were identical to experimental pellets except that they contained methanol without sponge compound. A minimal amount of food coloring (<1%) was added to both mixtures to ensure experimental pellets were similar in appearance to control. Using a Pasteur pipette, these pellets were offered to individual zebrafish in a randomized order (N=7–10 fish), and rejection or acceptance was assessed for each fish. Rejection of a pellet was defined as up to three or more unsuccessful attempts by a single fish to ingest the pellet; if the fish swallowed the pellet within three attempts it was considered accepted. If a pellet treated with sponge compound was rejected, this was always followed with a control pellet to ensure that rejection was not due to satiation. Statistical analysis was performed using a Fisher's exact test (one-tailed; P<0.05) to determine whether fish responded differently to treated *vs* control food pellets.

Molecular biology manipulations

A whole zebrafish (*D. rerio*) cDNA plasmid library constructed in the pExpress-1 vector and size selected for larger inserts (average size is 2 kb) was obtained from the I.M.A.G.E. Consortium (distributed by Open Biosystems, Huntsville, AL, USA). Dr David Gadsby (Rockefeller University, NY, USA) kindly provided the construct encoding the human cystic fibrosis transmembrane conductance regulator (CFTR) in the pGEMHE vector, and Dr Brian Kobilka (Stanford University, CA, USA) kindly provided the construct encoding the human beta 2 adrenergic receptor (β_2AR) in the pSP65 vector. A construct encoding rat aldehyde olfactory receptor OR-I7 was constructed in the pSMYC vector (Wetzel et al., 1999). All cDNA plasmids were isolated from DH5α or DH10B cells with Qiaprep spin kits (Qiagen, Valencia, CA, USA),

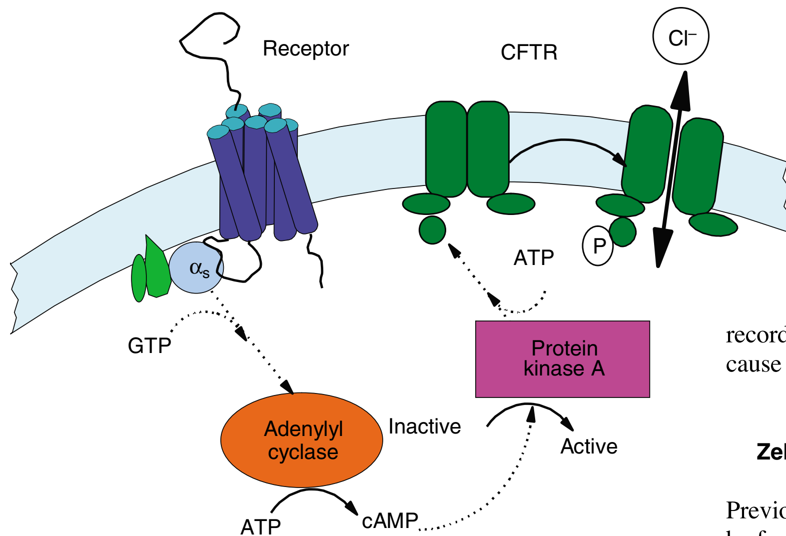

Fig. 1. $G_{\alpha s}$ signaling pathway utilized in bioassay. The cyclic AMP-dependent response in oocytes co-expressing the zebrafish cDNA library and CFTR is activated by the binding of a ligand to a membrane receptor, causing the receptor to interact with a G protein ($G_{\alpha s}$). Upon dissociation from the heterotrimeric G-protein–receptor complex, the α subunit activates adenylyl cyclase. This action leads to a cAMP signaling cascade, ending in the activation of CFTR. The output of this cascade is measured by electrophysiology and is the basis of the functional assay.

linearized, and *in vitro* transcribed into cRNA (mMessage mMachine, Ambion, Austin, TX, USA).

Electrophysiology

We hypothesized that a known chemoreceptor that endogenously couples to the G protein G_{olf} could activate a G_s-coupled signaling cascade, which would result in the opening of heterologously expressed CFTR ion channels in the plasma membrane of oocytes expressing this channel, thus changing current. Both $G_{\alpha olf}$ and $G_{\alpha s}$ lead to activation of adenylyl cyclase and, subsequently, protein kinase A (PKA). CFTR is a PKA-activated chloride channel, and its activation, *via* the adenylyl cyclase signaling cascade, can be measured using the two-electrode voltage clamp (TEVC) technique (McCarty et al., 1993). *Xenopus laevis* oocytes are a convenient tool for electrophysiological investigations of GPCRs and ion channels. These relatively large cells impale easily with two electrodes so that TEVC can be employed to measure whole cell currents. Furthermore, most of the proteins that constitute the $G_{\alpha S}$ protein signaling machinery are endogenously expressed within oocytes (Fig. 1), and these cells have been utilized in many other instances to reconstitute GPCR signaling cascades (Lubbert et al., 1987; Abaffy et al., 2006). The TEVC technique was used to detect electrophysiological responses in the oocytes through a change in current brought about by the activation of the receptor in question that in turn triggered a signaling cascade.

X. laevis oocytes were isolated from adult females and prepared as previously described (Fuller et al., 2004; McDonough et al., 1994). Various combinations of library transcript (2.5–10 ng), CFTR transcript (1.25–5 ng), and β_2AR transcript (0.5–2 ng) were microinjected into stage V oocytes. After incubation for 48–96 h in L-15 medium (Invitrogen, Carlsbad, CA, USA) at 17°C, oocytes were tested by TEVC, using a GeneClamp 500 amplifier (Axon Instruments, Sunnyvale, CA, USA). Recording solution was ND96 (96 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} KCl, 5 mmol l^{-1} Hepes; pH 7.50) with 1.8 mmol l^{-1} $CaCl_2$. Oocytes were treated with deterrent compounds dissolved in ND96 buffer and a minimal amount of solvent (ethanol or DMSO), usually ~0.01% final concentration, *via* a gravity perfusion system that exchanged the entire recording chamber in approximately 1 min. If CFTR was activated by a chemoreceptor-mediated signaling cascade, the electrophysiological response would be a slow, broad change in current that slowly returns to baseline. Whole oocyte currents were recorded at V_M=–60 mV. Application of vehicle in ND96 did not cause a change in current.

RESULTS

Zebrafish reject marine sponge compounds known to be aversive to reef fishes

Previous studies have shown that blueheaded wrasse were deterred by formoside (natural concentration: 7.9 mg ml^{-1}), sceptrin (natural concentration: 5.3 mg ml^{-1}), ectyoplasides A and B (natural concentration: 5.3 mg ml^{-1}) and oroidin (natural concentration: 1.4 mg ml^{-1}) at and/or below their naturally occurring concentrations (Chanas et al., 1997; Assmann et al., 2000; Kubanek et al., 2000; Kubanek et al., 2002). Zebrafish rejected foods laced with formoside, sceptrin and ectyoplasides A and B at the same or slightly higher concentrations than those known to deter a common predator on coral reefs, the bluehead wrasse (Table 1). These results suggest that zebrafish possess chemoreceptors that are able to detect at least some marine chemical defenses. However, zebrafish did not have an aversive response to oroidin, even at more than seven times the concentration that was previously found to be aversive to bluehead wrasse (Chanas et al., 1997); therefore, as previously observed, chemosensing can be species specific (Lindquist and Hay, 1995; Kaissling, 1996), and aversive patterns vary based upon chemical structure (Lindel et al., 2000; Lane and Kubanek, 2006).

Receptor-mediated responses can be reconstituted in *Xenopus* oocytes

In order to characterize chemoreceptors and identify potential signaling pathways, we sought to functionally express them in a heterologous cell expression system, *Xenopus* oocytes, which endogenously contain G protein signaling machinery. Fig. 2A shows direct stimulation of CFTR in oocytes by exposure to IBMX, a membrane-permeant inhibitor of phosphodiesterase which leads to sustained activation of PKA and a characteristically slow, broad response that slowly returned to baseline when IBMX was removed from the bathing solution. CFTR can also be activated by exposure to forskolin, a membrane-permeant activator of adenylyl cyclase (Fig. 2B). When the rat aldehyde olfactory receptor, OR-I7, was heterologously expressed in oocytes along with CFTR, CFTR activity increased in response to octanal, an OR-I7 ligand (Fig. 2B), suggesting that this GPCR-mediated signaling pathway can be reconstituted in oocytes.

After determining that signaling by a known chemoreceptor can be reconstituted in oocytes, we sought to determine whether we could reconstitute other receptor-mediated signaling pathways in oocytes expressing a zebrafish cDNA library. When the odorant octanal and the β-adrenergic receptor agonist isoproterenol were applied separately to the oocytes expressing both the library and CFTR, a substantial increase in CFTR activity was observed (Fig. 2C) that did not occur in cells without the cDNA library (data

Table 1. Zebrafish (*Danio rerio*) are deterred by some sponge chemical defenses

Test compound	Concentration (mg ml^{-1})*	Test pellet	Pellets eaten	Pellets rejected	Result
Formoside	15.8 [15.0 mmol l^{-1}]	Treatment	0	10	Rejected (P<0.01)
		Control	10	0	
Sceptrin	1.0 [1.6 mmol l^{-1}]	Treatment	7	2	Accepted (P=0.24)
		Control	9	0	
	5.0 [8.1 mmol l^{-1}]	Treatment	0	7	Rejected (P<0.01)
		Control	7	0	
Oroidin	5.0 [28.0 mmol l^{-1}]	Treatment	10	0	Accepted (P=1.00)
		Control	10	0	
	10.0 [56.0 mmol l^{-1}]	Treatment	9	0	Accepted (P=1.00)
		Control	9	0	
Mix of ectyoplasides A and B	5.3 [2.8 mmol l^{-1}]	Treatment	0	9	Rejected (P<0.01)
		Control	9	0	

*Naturally occurring concentrations of test compounds: formoside, 7.9 mg ml^{-1}; sceptrin, 5.3 mg ml^{-1}; ectyoplasides A and B, 5.3 mg ml^{-1}; oroidin, 1.4 mg ml^{-1}.

not shown). These data suggest that the zebrafish library included clones encoding a receptor that may be homologous to OR-I7, which is activated by octanal, and a receptor homologous to the β-adrenergic receptor family, which is activated by isoproterenol. CFTR served as a read-out in this assay, since the response to isoproterenol was not observed in cells expressing the library alone (Fig. 2D). Fig. 2D shows a very slight change in current in response to formoside (note the change in scale) that occurred in oocytes only expressing the library, suggesting that when CFTR is not overexpressed a G_s pathway is still activated by formoside; expression of CFTR allows enhanced detection of the stimulation of the pathway.

Responses to chemical defense compounds can be reconstituted in *Xenopus* oocytes

Since receptor-mediated responses to a known odorant could be obtained from cDNA library-expressing oocytes, we hypothesized that chemical defense signaling pathways could be reconstituted in these cells as well, allowing the measurement of electrophysiological response to a chemical defense compound. Oocytes co-expressing the library, CFTR and β_2AR were treated with the marine sponge-derived compounds, which we showed (Table 1) lead to a behavioral response in zebrafish. β_2AR was included to potentially increase functional expression of chemoreceptors (Hague et al., 2004). Library-expressing oocytes did not have a detectable response to either oroidin (Fig. 3A) or sceptrin (Fig. 3B) when these compounds were applied in the bathing solution. Exposure to a concentration of 10 μmol l^{-1} ectyoplasides A and B (Fig. 3C) and 5 μmol l^{-1} formoside (Fig. 4) led to a 0.03±0.01 μA (± s.e.m.; range 0–0.1 μA; N=11) and 0.2±0.07 μA (± s.e.m.; range 0.1–0.8 μA; N=15) response, respectively. These concentrations were considerably lower than those utilized in the behavioral assays because higher concentrations of these compounds (at least tenfold) were toxic to oocytes. The application of formoside or ectyoplasides A and B to oocytes expressing the library led to an electrophysiological response that reflected activation of CFTR (Fig. 3C and Fig. 4A), which was not seen in control (Fig. 3D and Fig. 4B). The response

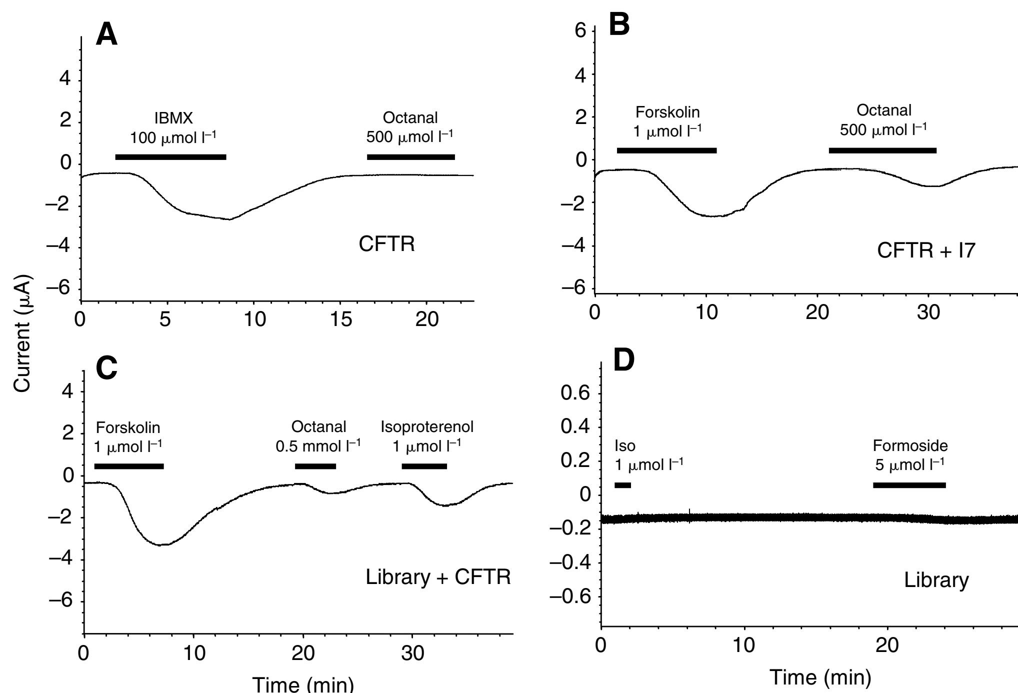

Fig. 2. Receptor-mediated responses in oocytes expressing zebrafish cDNA library or OR-I7. (A) Current changed in response to IBMX, an activator of CFTR, but did not change in response to octanal, an agonist of the OR-I7 chemoreceptor, in cells heterologously expressing CFTR alone. (B) Both forskolin, an activator of CFTR, and octanal caused increased CFTR activity when applied to oocytes expressing OR-I7 and CFTR. (C) Both isoproterenol (Iso), an agonist of β_2AR, and octanal caused increased CFTR activity when applied to oocytes expressing the library and CFTR but caused no change in oocytes expressing CFTR alone (data not shown). (D) Oocytes expressing only the library had a very slight change in current in response to formoside. Note the difference in time scale from A to D.

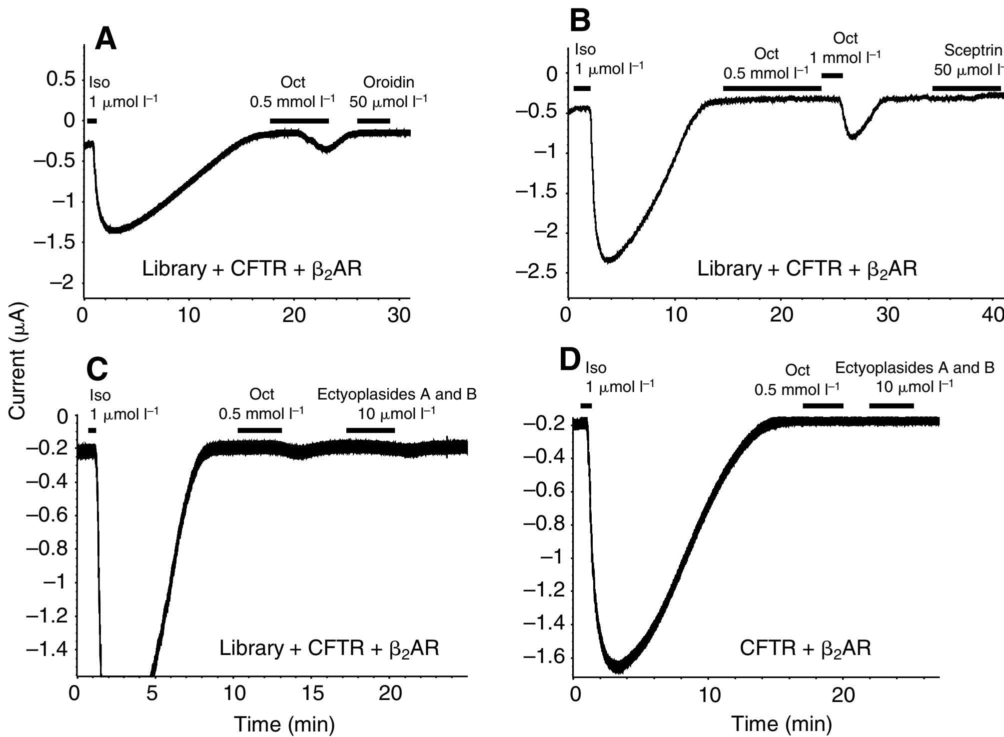

Fig. 3. Electrophysiological responses to chemical deterrents. No electrophysiological change was seen in response to application of oroidin (A) or sceptrin (B). A mixture of ectyoplasides A and B caused a slight change in current (C), indicating activation of CFTR *via* the G_s signaling pathway, which was not observed in control cells (D). Iso, isoproterenol; Oct, octanal.

to formoside was more robust than the response to ectyoplasides A and B, since all cells expressing library, β_2AR and CFTR responded to formoside but not all cells responded to ectyoplasides A and B. Interestingly, this change in current in response to formoside usually occurred only when the compound was applied after the activation of β_2AR with isoproterenol (Fig. 4A), suggesting that the activation of the $G_{\alpha s}$-mediated pathway may enhance the response to formoside to a detectable level. The response to formoside, unlike ectyoplasides A and B, was very repeatable (*N*>15) and not seen in oocytes not expressing the library (Fig. 4B). Furthermore, multiple presentations of formoside to a library-expressing oocyte did not cause repeatable responses within the same experiment, but with considerable time between presentations (e.g. 3 h), a second presentation of formoside could lead to a second response of similar magnitude (data not shown). These results suggest that the formoside and ectyoplasides A and B signaling pathways were successfully reconstituted in cells expressing the zebrafish library.

DISCUSSION

Reconstitution of chemical defense signaling pathways

The molecular detection of chemical defense compounds has rarely been investigated (Bickmeyer et al., 2004) and, therefore, it is generally unproven whether chemical defense compounds are detected in a receptor-mediated manner. We reconstituted the chemical defense signaling pathways for formoside and ectyoplasides A and B, marine sponge compounds, in *Xenopus* oocytes and showed an electrophysiological response to these compounds (Fig. 3C and Fig. 4). Interestingly, ectyoplasides A and B and formoside are from the same class of molecules, triterpene glycosides, and the electrophysiological responses to these compounds are also similar. The response to these compounds was observed only in cells expressing the zebrafish cDNA library, indicating that the electrophysiological change occurred because of a receptor–ligand interaction. These putative receptors appear to function as GPCRs that may activate an ion channel in fish endogenously expressing these genes because the response to

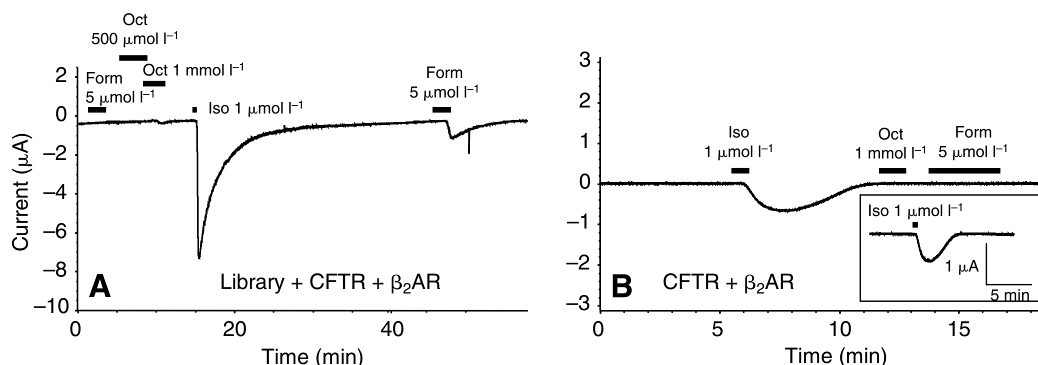

Fig. 4. Formoside induces an electrophysiological response in zebrafish cDNA library-expressing oocytes. Formoside (Form) caused a response in library-expressing cells after activation of the G_s pathway with isoproterenol (Iso; A). No responses to formoside or octanal (Oct) were seen in oocytes without library (B). Note the difference in time scale between A and B. Inset shows the response to isoproterenol in B on the same time scale as A.

formoside and ectyoplasides A and B only occurred in oocytes that were expressing the zebrafish cDNA library and was amplified when the ion channel CFTR was co-expressed (Fig. 2D and Fig. 4). Activation of an endogenous ion channel *via* these putative receptors may lead to depolarization of the receptor-encoding cell, sending the signal for higher order processing.

Unlike the receptor-mediated response to formoside, it is possible that other sponge compounds cause tissue or cellular damage or a general cellular response, as may be the case with sceptrin, one of the *Agelas*-derived defense compounds utilized in the palatability assays (Table 1). The mechanism of action of sceptrin has been investigated in rat adrenal cells, where it appeared to have an effect on calcium homeostasis (Bickmeyer et al., 2004). Sceptrin may not cause a receptor-mediated response in zebrafish, as no electrophysiological change occurred in response to this compound in our experiments (Fig. 3B). Alternatively, zebrafish sensory cells may exhibit an electrophysiological response to sceptrin that was not measurable in our heterologous expression assay. Oroidin, which zebrafish accepted in the palatability assay (Table 1) but was rejected by coral reef fish (Chanas et al., 1997), also does not appear to cause a receptor-mediated response in this assay (Fig. 3A). These data, combined with the behavioral data, suggest that zebrafish either: (1) do not possess a chemoreceptor capable of detecting oroidin, or (2) do possess a chemoreceptor capable of detecting oroidin, but its activation causes an acceptance rather than a rejection response. If the second scenario is true, then zebrafish chemoreceptor cells that express this receptor may be wired differently than the same cells in a species that rejects this compound, such as bluehead wrasse.

Interaction between β_2AR and receptors that detect deterrent compounds

Some cDNA library-expressing oocytes did not respond to formoside until after isoproterenol-induced stimulation of the $G_{\alpha s}$ signaling pathway through β_2AR (Fig. 4). There are several possible mechanisms that may explain why activation of β_2AR is sometimes required in oocytes prior to a response to formoside. β_2AR expression leads to cell surface expression of the mouse M71 olfactory receptor (Hague et al., 2004) and may similarly facilitate cell surface expression of the formoside receptor. G proteins are known to be redistributed in response to the activation of GPCRs (Milligan, 1993; AbdAlla et al., 2000; Cordeaux and Hill, 2002). Therefore, it is possible that $G_{\alpha s}$ proteins are redistributed upon activation of β_2AR, and this action increases the formoside receptor response by providing the receptor with additional G proteins. β_2AR is also known to sequester $G_{\alpha s}$ proteins such that other receptors cannot utilize them for signaling (Vasquez and Lewis, 2003), and these G proteins may be made available to the formoside receptor by activating β_2AR with isoproterenol. Alternatively, β_2AR could present G proteins to the formoside receptors, perhaps *via* receptor heterodimerization. Other receptors have been thought to do this, such as the bradykinin receptors, which are hypothesized to present G proteins to the angiotensin receptor, thus increasing their signaling ability (AbdAlla et al., 2000; Cordeaux and Hill, 2002). Activation or expression of β_2AR could also recruit other GPCRs, such as formoside receptors, to the plasma membrane, where they become functional. It could also lead to phosphorylation or dephosphorylation of G protein binding sites, ultimately affecting signaling output. Interestingly, stimulation of the G_S signaling pathway with isoproterenol in the olfactory bulb is known to enhance conditioned olfactory learning in rat pups (Sullivan et al., 1989). Since β-adrenergic receptors are co-expressed along with olfactory receptors in some olfactory sensory cells (Kawai et al., 1999), activation of this pathway in fish peripheral cells may increase formoside signaling in the periphery, to ultimately enhance the rejection process by potential predators of marine sponges.

Protective mechanisms and evolutionary implications

Although all of the compounds tested in our palatability assays are found in marine sponges, our experiments used the freshwater zebrafish *Danio rerio*. Although not ecologically relevant because of the geographic separation of these two organisms, this finding may have evolutionary implications as the behavioral rejection response of the zebrafish to some marine sponge compounds (Table 1) indicates that this aversion may be evolutionarily conserved in fish, while other chemically mediated interactions are more species-specific (Lindquist and Hay, 1995; Kaissling, 1996). Furthermore, because the response to formoside appears to be receptor-mediated, the receptor(s) involved in the detection of this compound also may be conserved. Conservation of receptors that detect potentially harmful compounds would not be surprising given that these receptors would afford an evolutionary advantage to organisms that would be predisposed to avoid noxious prey, and a variety of marine and terrestrial organisms produce triterpene glycosides (Zhang et al., 2006; Ukiya et al., 2007), which are known in some organisms to act as defenses (Kubanek et al., 2000). Many organisms exploit such a predisposition, such as the directed-deterrence of chili plants. Chilies contain capsaicin, a compound that deters predation by mammals possessing a nociceptor capable of activating a pain pathway in response this compound (Caterina et al., 1997). However, the equivalent avian receptor contains a mutation that renders birds insensitive to capsaicin (Jordt and Julius, 2002); birds readily consume chilies and effectively disperse their seeds (Tewksbury and Nabhan, 2001). Therefore, these plants benefit by containing a chemical defense, as do marine sponges.

The potentially widespread occurrence of an aversive response in a predator also would be advantageous for prey species that possess these chemical deterrents, making it more likely that a variety of potential predators would be inclined to avoid these prey as food. For example, as shown by field experiments, formoside (Kubanek et al., 2002) and some other marine chemical defense compounds (Chanas et al., 1997; Vervoort et al., 1998; Wilson et al., 1999) are deterrent to a variety of generalists (i.e., predators that utilize multiple resources). However, some specialists (i.e. predators that specialize on particular prey) have a higher tolerance to defensive compounds and are typically not deterred by defensive compounds of their preferred prey (Hay et al., 1990; Pennings et al., 1996). Our results suggest that marine sponges are broadly defended by deterrent compounds, since several sponge compounds deter feeding by a fish not present in the sponges' natural environment (Table 1). Because our data demonstrate that consumers from two very different habitats have the ability to detect some of the same deterrent compounds, suggesting that neither species has evolved resistance to these chemical defenses, sponge geographic distribution patterns may not be predominantly limited by predation pressure by generalist fishes.

Implications of the reconstitution of a defense pathway in frog oocytes

This work demonstrates that a chemical deterrent signaling pathway can be reconstituted in *Xenopus* oocytes and strongly suggests that encoded within this zebrafish cDNA library is a receptor that responds to the chemical defense compound, formoside. A receptor for ectyoplasides A and B also may exist in

this library. Using this expression system and electrophysiological assays that direct subdivision of the library clones into smaller and smaller groups, it is possible that the clones encoding these receptors may be isolated from the library and used to study predator detection of chemical defenses. This approach is expected to lead to identification of chemoreceptors used for detection of chemical defense compounds such as formoside.

LIST OF ABBREVIATIONS

CFTR	cystic fibrosis transmembrane conductance regulator
DMSO	dimethylsulfoxide
FORM	formoside
GPCR	G protein-coupled receptor
IBMX	3-isobutyl-1-methylxanthine
Iso	isoproterenol
Oct	octanol
PKA	protein kinase A
TEVC	two-electrode voltage clamp
β_2AR	beta-2 adrenergic receptor

The authors acknowledge a NSF-IGERT fellowship and a NSF-IGERT mini-grant to S.A.P.C. and a Blanchard Assistant Professorship to J.K. The authors thank Melissa H. Hutchins for marine sponge collections, Jennifer L. Jackson for insightful comments on the manuscript, and the members of the McCarty and Kubanek labs for helpful discussions regarding this work.

REFERENCES

Abaffy, T., Matsunami, H. and Luetje, C. W. (2006). Functional analysis of a mammalian odorant receptor subfamily. *J. Neurochem.* **97**, 1506-1518.
AbdAlla, S., Lother, H. and Quitterer, U. (2000). AT_1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* **407**, 94-98.
Assmann, M., Lichte, E., Pawlik, J. R. and Kock, M. (2000). Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera. Mar. Ecol. Prog. Ser.* **207**, 255-262.
Bickmeyer, U., Drechsler, C., Kock, M. and Assmann, M. (2004). Brominated pyrrole alkaloids from marine *Agelas* sponges reduce depolarization-induced cellular calcium elevation. *Toxicon* **44**, 45-51.
Boettcher, A. A. and Targett, N. M. (1993). Role of polyphenolic molecular-size in reduction of assimilation efficiency in *Xiphister mucosus. Ecology* **74**, 891-903.
Buck, L. and Axel, R. (1991). A novel multigene family may encode odorant receptors – a molecular-basis for odor recognition. *Cell* **65**, 175-187.
Caterina, M. J., Schumacher, M. A., Tomoinaga, M., Rosen, T. A., Levine, J. D. and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816-823.
Chanas, B., Pawlik, J. R., Lindel, T. and Fenical, W. (1997). Chemical defense of the Caribbean sponge *Agelas clathrodes* (Schmidt). *J. Exp. Mar. Biol. Ecol.* **208**, 185-196.
Cordeaux, Y. and Hill, S. J. (2002). Mechanisms of cross-talk between G protein-coupled receptors. *Neurosignals* **11**, 45-57.
Duque, C., Bonilla, A., Bautista, E. and Zea, S. (2001). Exudation of low molecular weight compounds (thiobismethane, methyl isocyanide, and methyl isothiocyanate) as a possible chemical defense mechanism in the marine sponge *Ircinia felix. Biochem. Syst. Ecol.* **29**, 459-467.
Dusenbery, D. B. (1992). *Sensory Ecology: How Organisms Acquire and Respond to Information.* New York: W. H. Freeman.
Fuller, M. D., Zhang, Z. R., Cui, G., Kubanek, J. and McCarty, N. A. (2004). Inhibition of CFTR channels by a peptide toxin of scorpion venom. *Am. J. Physiol.* **287**, C1328-C1341.
Hague, C., Uberti, M. A., Chen, Z. J., Bush, C. F., Jones, S. V., Ressler, K. J., Hall, R. A. and Minneman, K. P. (2004). Olfactory receptor surface expression is driven by association with the β_2-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **101**, 13672-13676.
Hay, M. E., Duffy, J. E., Paul, V. J., Renaud, P. E. and Fenical, W. (1990). Specialist herbivores reduce their susceptibility to predation by feeding on the chemically defended seaweed *Avrainvillea longicaulis. Limnol. Oceanogr.* **35**, 1734-1743.
Jones, A. C., Blum, J. E. and Pawlik, J. R. (2005). Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *J. Mar. Biol. Ecol.* **322**, 67-81.
Jordt, S. E. and Julius, D. (2002). Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **108**, 421-430.
Kaissling, K. E. (1996). Peripheral mechanisms of pheromone reception in moths. *Chem. Senses* **21**, 257-268.
Kawai, F., Kurahashi, T. and Kaneko, A. (1999). Adrenaline enhances odorant contrast by modulating signal encoding in olfactory receptor cells. *Nat. Neurosci.* **2**, 133-138.
Kicklighter, C. E., Kubanek, J., Barsby, T. and Hay, M. E. (2003). Palatability and defense of some tropical infaunal worms: alkylpyrrole sulfamates as deterrents to fish feeding. *Mar. Ecol. Prog. Ser.* **263**, 299-306.
Kicklighter, C. E., Shabani, S., Johnson, P. M. and Derby, C. D. (2005). Sea hares use novel antipredatory chemical defenses. *Curr. Biol.* **15**, 549-554.
Kubanek, J., Pawlik, J. R., Eve, T. M. and Fenical, W. (2000). Triterpene glycosides defend the Caribbean reef sponge *Erylus formosus* from predatory fishes. *Mar. Ecol. Prog. Ser.* **207**, 69-77.
Kubanek, J., Fenical, W. and Pawlik, J. R. (2001). New antifeedant triterpene glycosides from the Caribbean sponge *Erylus formosus. Nat. Prod. Lett.* **15**, 275-285.
Kubanek, J., Whalen, K. E., Engel, S., Kelly, S. R., Henkel, T. P., Fenical, W. and Pawlik, J. R. (2002). Multiple defensive roles for triterpene glycosides from two Caribbean sponges. *Oecologia* **131**, 125-136.
Lane, A. L. and Kubanek, J. (2006). Structure-activity relationship of chemical defenses from the freshwater plant *Microanthemum umbrosum. Phytochemistry* **67**, 1224-1231.
Lindel, T., Hoffmann, H., Hochgurtel M. and Pawlik, J. R. (2000). Structure-activity relationship of inhibition of fish feeding by sponge-derived and synthetic pyrrole-imidazole alkaloids. *J. Chem. Ecol.* **26,** 1477-1496.
Lindemann, B. (2001). Receptors and transduction in taste. *Nature* **413**, 219-225.
Lindquist, N. and Hay, M. E. (1995). Can small rare prey be chemically defended: the case for marine larvae. *Ecology* **76**, 1347-1358.
Lindquist, N., Hay, M. E. and Fenical, W. (1992). Defense of ascidians and their conspicuous larvae: adult vs. larval chemical defenses. *Ecol. Monogr.* **62**, 547-568.
Lubbert, H., Hoffman, B. J., Snutch, T. P., Vandyke, T., Levine, A. J., Hartig, P. R., Lester, H. A. and Davidson, N. (1987). cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of messenger RNA-injected *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **84**, 4332-4336.
McCarty, N. A., McDonough, S., Cohen, B. N., Riordan, J. R., Davidson, N. and Lester, H. A. (1993). Voltage-dependent block of the cystic fibrosis transmembrane conductance regulator Cl^- channel by two closely related arylaminobenzoates. *J. Gen. Physiol.* **102**, 1-23.
McDonough, S., Davidson, N., Lester, H. A. and McCarty, N. A. (1994). Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron* **13**, 623-634.
Milligan, G. (1993). Agonist regulation of cellular G-protein levels and distribution – mechanisms and functional implications. *Trends Pharmacol. Sci.* **14**, 413-418.
Mole, S. and Waterman, P. G. (1987). Tannic acid and proteolytic enzymes: enzyme inhibition or substrate deprivation? *Phytochemistry* **26**, 1.
Mombaerts, P. (1999). Seven-transmembrane proteins as odorant and chemosensory receptors. *Science* **286**, 707-711.
Mombaerts, P. (2004). Genes and ligands for odorant, vomeronasal and taste receptors. *Nat. Rev. Neurosci.* **5**, 263-278.
Moore, B. S. (2006). Biosynthesis of marine natural products: macroorganisms (Part B). *Nat. Prod. Rep.* **23**, 615-629.
Oike, H., Nagai, T., Furuyama, A., Okada, S., Aihara, Y., Ishimaru, Y., Marui, T., Matsumoto, I., Misaka, T. and Abe, K. (2007). Characterization of ligands for fish taste receptors. *J. Neurosci.* **27**, 5584-5592.
O'Neal, W. and Pawlik, J. R. (2002). A reappraisal of the chemical and physical defenses of Caribbean gorgonian corals against predatory fishes. *Mar. Ecol. Prog. Ser.* **240**, 117-126.
Paul, V. J., Puglisi, M. P. and Ritson-Williams, R. (2006). Marine chemical ecology. *Nat. Prod. Rep.* **23**, 153-180.
Pawlik, J. R. and Fenical, W. (1992). Chemical defense of *Pterogorgia anceps*, a Caribbean gorgonian coral. *Mar. Ecol. Prog. Ser.* **87**, 183-188.
Pawlik, J. R., Chanas, B., Toonen, R. and Fenical, W. (1995). Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* **127**, 183-194.
Pawlik, J. R., McFall, G. and Zea, S. (2002). Does the odor from sponges of the genus *Ircinia* protect them from fish predators? *J. Chem. Ecol.* **28**, 1103-1115.
Pennings, S. C., Weiss, A. M. and Paul, V. J. (1996). Secondary metabolites of the cyanobacterium *Microcoleus lyngbyaceus* and the sea hare *Stylocheilus longicauda*: palatability and toxicity. *Mar. Biol.* **126**, 735-743.
Pisut, D. P. and Pawlik, J. R. (2002). Anti-predatory chemical defenses of ascidians: secondary metabolites or inorganic acids? *J. Exp. Mar. Biol. Ecol.* **270**, 203-214.
Schmitt, T. M., Hay, M. E. and Lindquist, N. (1995). Constraints on chemically mediated coevolution – multiple functions for seaweed secondary metabolites. *Ecology* **76**, 107-123.
Stachowicz, J. J. and Hay, M. E. (1999). Reducing predation through chemically mediated camouflage: indirect effects of plant defenses on herbivores. *Ecology* **80**, 495-509.
Stoecker, D. (1980). Chemical defenses of ascidians against predators. *Ecology* **61**, 1327-1334.
Sullivan, R. M., Wilson, D. A. and Leon, M. (1989). Norepinephrine and learning-induced plasticity in infant rat olfactory system. *J. Neurosci.* **9**, 3998-4006.
Targett, N. and Arnold, T. M. (2001). Effects of secondary metabolites on digestion in marine herbivores. In *Marine Chemical Ecology* (ed. J. B. McClintock and B. J. Baker), pp. 391-411. Boca Raton: CRC Press.
Tewksbury, J. J. and Nabhan, G. P. (2001). Seed dispersal – directed deterrence by capsaicin in chilies. *Nature* **412**, 403-404.
Ukiya, M., Akihisa, T., Yasukawa, K., Koike, K., Takahashi, A., Suzuki, T. and Kimura, Y. (2007). Triterpene glycosides from the flower petals of sunflower (*Helianthus annuus*) and their anti-inflammatory activity. *J. Nat. Prod.* **70**, 813-816.
Vasquez, C. and Lewis, D. L. (2003). The β_2-adrenergic receptor specifically sequesters G_s but signals through both G_s and $G_{i/o}$ in rat sympathetic neurons. *Neuroscience* **118**, 603-610.
Vervoort, H. C., Pawlik, J. R. and Fenical, W. (1998). Chemical defense of the Caribbean ascidian *Didemnum conchyliatum. Mar. Ecol. Prog. Ser.* **164**, 221-228.
Waddell, B. and Pawlik, J. R. (2000). Defenses of Caribbean sponges against invertebrate predators. I. Assays with hermit crabs. *Mar. Ecol. Prog. Ser.* **195**, 125-132.
Wetzel, C. H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G. and Hatt, H. (1999). Specificity and sensitivity of a human olfactory receptor functionally expressed in Human Embryonic Kidney 293 cells and *Xenopus laevis* oocytes. *J. Neurosci.* **19**, 426-7433.
Wilson, D. M., Puyana, M., Fenical, W. and Pawlik, J. R. (1999). Chemical defense of the Caribbean reef sponge *Axinella corrugata* against predatory fishes. *J. Chem. Ecol.* **25**, 2811-2823.
Zhang, S. Y., Yi, Y. H. and Tang, H. F. (2006). Bioactive triterpene glycosides from the sea cucumber *Holothuria fuscocinerea. J. Nat. Prod.* **69**, 1492-1495.