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Screening of second messengers involved in photocyte bioluminescence control of three ophiuroid species (Ophiuroidea: Echinodermata)

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Summary

We investigated the effects of cyclic nucleotides (cGMP and cAMP) and inositol triphosphate/diacylglycerol pathways on the KCl-induced luminescence control of the ophiuroid species *Amphiura filiformis*, *Ophiopsila aranea* and *Ophiopsila californica*. Results show that dibutyryl-cGMP, the cGMP analogue, and sodium nitroprusside, the guanylyl cyclase activator, had no effect on the luminescence of *O. aranea* and *O. californica*. On the other hand, cGMP could be involved in an inhibitory control in *A. filiformis*. Dibutyryl-cAMP, the cAMP analogue, and forskolin, the adenylyl cyclase activator, had no effect on maximal light emission, but the adenylyl cyclase inhibitors MDL-12,330A and SQ22,536 affected the kinetics of light production in both *Ophiopsila* species and strongly reduced KCl-induced luminescence in *A. filiformis* and *O. aranea*, suggesting cAMP pathway involvement in photogenesis. The phospholipase C inhibitor U-73122 also strongly reduced KCl-induced luminescence in all three species but this effect seems to be unspecific since U-73343, the inactive analogue of U-73122, equally inhibited photogenesis. Therefore, the results suggest that luminescence control of *A. filiformis*, *O. aranea* and *O. californica* is mediated by cAMP in synergy with calcium.

Key words: invertebrate, echinoderm, ophiuroid, *Amphiura filiformis*, *Ophiopsila aranea*, *Ophiopsila californica*, bioluminescence, second messenger, cAMP, nervous system, photocyte.

Introduction

Bioluminescence presents a huge diversity in its phylogenetic distribution, function, biology, chemistry and control mechanisms (Hastings, 1983; Herring, 1987; Campbell, 1989). In echinoderms, for instance, several neuromediators [e.g. adrenaline, acetylcholine (ACh), 5-hydroxytryptamine (serotonin) and octopamine] are involved in the nervous control of light emission, according to species (for a review, see Mallefet, 1999). In ophiuroids, pharmacological studies have failed to reveal mediators involved in the luminous control of *Ophiopsila aranea* and *Ophiopsila californica*. By contrast, they have shown ACh to induce luminescence through muscarinic and nicotinic cholinergic receptors in *Amphiura filiformis* (Dewael and Mallefet, 2002a) and through muscarinic cholinergic receptors in *Amphipholis squamata* (De Bremaeker et al., 1996). Moreover, in *A. squamata*, ACh-induced photogenesis is modulated by amino acids, neuropeptides, purines and catecholamines (De Bremaeker et al., 1996, 1999b, 2000a).

While an increasing amount of information exists on the extrinsic control mechanisms of luminescence in ophiuroids, only few data are available concerning the intrinsic control mechanisms leading to photocyte photogenesis. The behaviour of all cells from one instant to another is governed by signalling systems that translate external information into a limited repertoire of internal signals, the second messengers (Cobb and Laverack, 1967). There are four main second messenger pathways discovered so far: cyclic AMP (cAMP), cyclic GMP (cGMP), inositol triphosphate/diacylglycerol (IP_3/DAG) and calcium (Ca^{2+}). Most studies concerning second messengers have been performed on vertebrates and they are found in all cell types studied so far. In echinoderms, it has been shown that Ca^{2+} (Mallefet et al., 1994, 1998), cyclic nucleotides and IP_3/DAG (De Bremaeker et al., 2000b) are involved in the luminescence control of the ophiuroid *A. squamata*. Dewael and Mallefet (2002b) have also documented the Ca^{2+} requirement for light emission in three other ophiuroid species.

The aim of this work is thus to identify second messengers triggering photogenesis in three ophiuroid species: *A. filiformis*, *O. aranea* and *O. californica*. This was achieved by testing agonists and antagonists of three second messenger pathways (cGMP, cAMP and IP_3/DAG) on light production. While most previous studies on bioluminescence control in ophiuroids have been carried out on whole arms or arm segments (De Bremaeker et al., 1996, 1999a,b; Dewael and Mallefet, 2002a,b), we analysed the luminous cells (photocytes) directly.

Materials and methods

Animals

Specimens of *Amphiura filiformis* (Müller 1776) were collected at the Kristineberg Marine Station (Fiskebäckskil, Sweden) by a Smith–McIntyre mud grab at a depth of 25–40 m. Animals were then kept in circulating natural seawater. Specimens of *Ophiopsila aranea* (Forbes 1843) were collected at the ARAGO Biological Station (C.N.R.S.; Banyuls-sur-Mer, France) by scuba diving at 20–25 m depth. Specimens of *Ophiopsila californica* (Clark 1921) were collected by the same technique at the Marine Sciences Institute of the University of California (Santa-Barbara). All these animals were transported to our laboratory in Belgium in aerated natural seawater and then kept in aquaria filled with a mixture of natural and artificial recirculating seawater at 12°C. Food (Marine Interpet, Liquifry & Co., Dorking, UK) was provided to ophiuroids once a week. Animals kept in captivity were used between one week and three months after collection. Control stimulations were carried out for each experiment to make sure that no difference in light capabilities appeared according to the captivity time.

Experiments on dissociated photocytes

The method used is based on that described by De Bremaeker et al. (2000b). First, the ophiuroids were anaesthetized by immersion in 3.5% $MgCl_2$ in artificial seawater (ASW: 400.4 mmol l^{-1} NaCl, 9.9 mmol l^{-1} $CaCl_2$, 9.6 mmol l^{-1} KCl, 52.3 mmol l^{-1} $MgCl_2$, 27.7 mmol l^{-1} Na_2SO_4, 20 mmol l^{-1} Tris, pH 8.3). Next, since the arms are the only luminescent body parts of the studied ophiuroid species, they were isolated from the disc and chopped into tiny pieces before enzymatic digestion and differential centrifugation. The enriched luminous-cell fraction was then divided into aliquot parts (200 µl), and light emission was measured with a FB12 Berthold luminometer (Pforzheim, Germany) linked to a personal computer. Injection of drugs was controlled to avoid mechanical stimulation of luminescence during the assays. Injections of corresponding volumes of ASW served as controls before the assays. These controls indicated that luminescence due to mechanical excitability was absent or negligible.

Stimulations

A stock solution of 400 mmol l^{-1} KCl was prepared in ASW without NaCl to keep the same osmolarity as normal ASW. Stimulations were performed by injection of potassium chloride (KCl) to a final dilution of 200 mmol l^{-1}; one experiment was carried out using 50 mmol l^{-1} KCl (see Results). For each experimental protocol, one aliquot part was stimulated in normal ASW, as a control, while the other preparations were first immersed in ASW containing the tested drug for 10 min before stimulation with KCl.

Drugs

The following drugs were used in this study: bisindolylmaleimide (GF 109203X; Sigma, Bornem, Belgium); N^6,2′-o-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP; Sigma); N^2,2′-o-dibutyrylguanosine 3′,5′-cyclic monophosphate (db-cGMP; Sigma); forskolin (FSK; ICN, Irvine, CA, USA); 3-isobutyl-1-methyl-xanthine (IBMX; Sigma); 1-[6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl]-1H-pyr-role-2,5-dione (U-73122; Sigma); 1-[6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl]-2,5-pyrrolidine-dione (U-73343; ICN); pentoxifylline (Sigma); *cis*-*N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine monohydrochloride (MDL-12,330A; Sigma); sodium nitroprusside (Na nitro; Sigma); 9-tetrahydro-2-furanyl-9H-purin-6-amine (SQ22,536; Sigma).

Solutions of db-cAMP, db-cGMP, IBMX, pentoxifylline, Na nitro and SQ22,536 were dissolved in ASW; solutions of FSK, GF 109203X, MDL-12,330A, U-73122 and U-73343 were dissolved in dimethylsulphoxide (DMSO) before dilution in ASW, with a maximum of 1% DMSO at final concentration. This final DMSO dilution alone had no effect. The concentrations used for the drugs were chosen from previous studies on echinoderm tissues (Soliman, 1984a,b; Gustafson, 1990; Karaseva and Khotimchenko, 1995; De Bremaeker et al., 2000b). Fresh solutions were prepared daily.

Different parameters were used in order to characterise the photogenesis: (1) L_{max}, the maximum level of light emission expressed as a percentage of the control; (2) L_{tot}, total amount of light emitted expressed as a percentage of the control; (3) *LT*, latency time, the time elapsed between stimulation and the beginning of the light emission; (4) TL_{max}, the time between onset of light production and maximum light emission.

Statistical analyses [analysis of variance (ANOVA), Dunnet and Tukey tests] were performed using SAS/STAT® software (SAS Institute Inc., 1990).

Results

The results of the various drugs used to characterise the three second messenger pathways are summarised in Table 1.

Effects of db-cGMP and guanylyl cyclase activator

The treatment of dissociated photocytes from *A. filiformis*, *O. aranea* and *O. californica* with 10^{-4} mol l^{-1} dibutyryl-cGMP (db-cGMP), a membrane-permeable analogue of guanosine 3′,5′-cyclic monophosphate (cGMP) and 10^{-3} mol l^{-1} sodium nitroprusside (Na nitro), an activator of guanylyl cyclase, did not induce luminescence without KCl application. After 10 min treatment with db-cGMP or Na nitro, 200 mmol l^{-1} KCl was applied. Controls were not treated with the drugs before KCl application. In both *Ophiopsila* species, no significant differences in the parameters of light emission were observed after KCl injection between the treated and the control photocytes (Fig. 1). On the other hand, in *A. filiformis*, 10^{-4} mol l^{-1} db-cGMP did inhibit light emission; only 5.89±1.68% of maximal light intensity (L_{max}) and 12.19±3.3% of the total amount of emitted light (L_{tot}) remained. Na nitro (10^{-3} mol l^{-1}) treatment was ineffective in all three species.

Table 1. *Summary of the effects of the various drugs used to characterise second messenger pathways on the light parameters of* A. filiformis, O. aranea *and* O. californica

Drug	Activity	Effect		
		A. filiformis	*O. aranea*	*O. californica*
db-cGMP	cGMP analogue (↑ cGMP)	–	0	0
Na nitro	Guanylyl cyclase activator (↑ cGMP)	0	0	0
db-cAMP	cAMP analogue (↑ cAMP)	0	0	0
Forskolin	Adenylyl cyclase activator (↑ cAMP)	0	0	0/+
MDL-12,330A	Adenylyl cyclase inhibitor (↓ cAMP)	–	–	–
SQ22,536	Adenylyl cyclase inhibitor (↓ cAMP)	–	–	–
IBMX	Phosphodiesterase inhibitor (↑ cGMP and cAMP)	–	0	0
Pentoxifylline	Phosphodiesterase inhibitor (↑ cGMP and cAMP)	0	0	0
U-73122	Phospholipase C inhibitor (↓ IP_3 and DAG)	–	–	–
U-73343	Inactive analogue of U-73122	–	–	–
GF 109203X	Protein kinase C inhibitor	0	0	0

–, inhibition; 0, no effect; 0/+, positive effect on light production (↓ of kinetic parameters).

Kinetic parameters (*LT* and TL_{max}) were not modified in any cases.

Effects of db-cAMP, adenylyl cyclase activator and inhibitors

The treatment of dissociated photocytes from *A. filiformis*, *O. aranea* and *O. californica* with 10^{-4} mol l^{-1} dibutyryl-cAMP (db-cAMP), a membrane-permeable analogue of adenosine 3′,5′-cyclic monophosphate (cAMP), 10^{-4} mol l^{-1} forskolin (FSK), an activator of adenylyl cyclase, as well as 10^{-5} mol l^{-1} MDL-12,330A and 10^{-5} mol l^{-1} SQ22,536, two inhibitors of adenylyl cyclase, did not induce luminescence prior to KCl application. Neither db-cAMP nor FSK showed a significant effect on 200 mmol l^{-1} KCl-induced luminescence (Fig. 2). Moreover, 10^{-4} mol l^{-1} FSK did not have any significant effect on 50 mmol l^{-1} KCl-induced luminescence (L_{max}) in all three species (Fig. 2). We tested FSK on 50 mmol l^{-1} induced luminescence to highlight a potential increase of light production since 50 mmol l^{-1} KCl does not trigger a maximal light response (~50% of 200 mmol l^{-1} KCl light response). Only in *O. californica* did 10^{-4} mol l^{-1} FSK decrease kinetic parameters of light production (*LT* and TL_{max}; Table 2).

On the other hand, MDL-12,330A and SQ22,536 strongly reduced 200 mmol l^{-1} KCl-induced luminescence in *A. filiformis* and *O. aranea* (L_{max}; $P<0.01$, $N=10$; Fig. 2). Total amount of emitted light was also significantly decreased in both species (Table 3), and TL_{max} was increased in *O. aranea* for both drugs (Table 2). In *O. californica*, MDL-12,330A strongly decreased light parameters (L_{max}, Fig. 2; L_{tot}, Table 3)

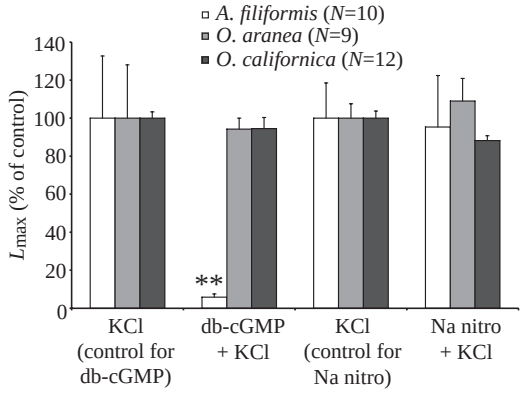

Fig. 1. Effects of 10^{-4} mol l^{-1} dibutyryl-cGMP (db-cGMP) and 10^{-3} mol l^{-1} sodium nitroprusside (Na nitro) on KCl-induced luminescence of *A. filiformis*, *O. aranea* and *O. californica* dissociated photocytes. Means ± S.E.M. are expressed as a percentage of photogenesis triggered by 200 mmol l^{-1} KCl in normal artificial seawater. $^{**}P<0.01$; *N*, number of stimulated aliquot parts.

Table 2. *Effect of forskolin (FSK), SQ22,536 and MDL-12,330A on the kinetic parameters (*LT *and* TL_{max}*) of KCl-induced luminescence on* O. californica *and* O. aranea *dissociated photocytes*

	LT (s)	TL_{max} (s)
O. californica (*N*=10)		
50 mmol l^{-1} KCl (control for FSK)	8.30±3.43	11.15±3.43
10^{-4} mol l^{-1} FSK	0.01*	1.25±0.33*
200 mmol l^{-1} KCl (control for SQ)	0.01	0.55±0.15
10^{-5} mol l^{-1} SQ22,536	0.01	3.20±0.86*
200 mmol l^{-1} KCl (control for MDL)	0.01	0.50±0.16
10^{-5} mol l^{-1} MDL-12,330A	0.01	3.72±0.73*
O. aranea (*N*=10)		
200 mmol l^{-1} KCl (control for MDL)	0.28±0.22	0.61±0.11
10^{-5} mol l^{-1} MDL-12,330A	0.20±0.08	2.00±0.37*
200 mmol l^{-1} KCl (control for SQ)	0.01	0.10±0.06
10^{-5} mol l^{-1} SQ22,536	0.05±0.04	2.60±0.71*

The results for *A. filiformis* are not shown since no significant effect of any of the drugs was observed on the kinetic parameters of light emission in this species. Means ± S.E.M. are expressed in seconds; values without S.E.M. are due to the fact that the same *LT* was observed for all repetitions. $^{*}P<0.01$; *N*, number of stimulated aliquot parts.

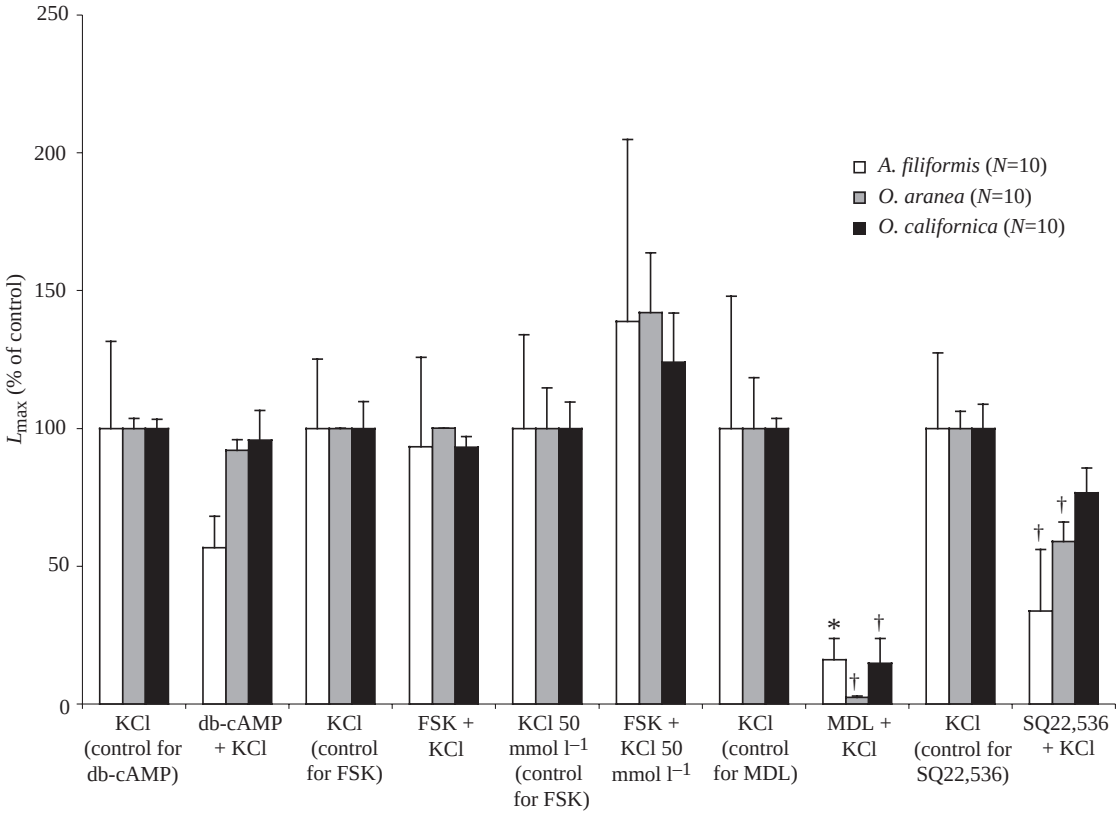

Fig. 2. Effects of 10^{-4} mol l^{-1} dibutyryl-cAMP (db-cAMP), 10^{-4} mol l^{-1} forskolin (FSK), 10^{-5} mol l^{-1} MDL-12,330A (MDL) and 10^{-5} mol l^{-1} SQ22,536 on KCl-induced luminescence of *A. filiformis*, *O. aranea* and *O. californica* dissociated photocytes. Means ± S.E.M. are expressed as a percentage of photogenesis triggered by 200 mmol l^{-1} KCl and 50 mmol l^{-1} KCl (for FSK) in normal artificial seawater. *P<0.05; †P<0.01; *N*, number of stimulated aliquot parts.

and also increased TL_{max} (Table 2). SQ22,536 did not significantly reduce L_{max} (Fig. 2) and L_{tot} (Table 3) but increased TL_{max} (Table 2) in this species.

Effects of phosphodiesterase inhibitors

In order to complete the study of a putative involvement of cyclic nucleotides on luminescence control in *A. filiformis*, *O. aranea* and *O. californica*, we tested two phosphodiesterase inhibitors, 10^{-4} mol l^{-1} IBMX and 10^{-4} mol l^{-1} pentoxifylline, which increase the intracellular level of both cAMP and cGMP. None of these drugs induced luminescence without KCl application. Fig. 3 shows that there is no effect of pentoxifylline on KCl-induced luminescence, while 10^{-4} mol l^{-1} IBMX largely reduced L_{max} (55.2±24.3% of control) solely in *A. filiformis*. Total amount of light was also decreased (L_{tot}=19.6±8.5% of control) in this species. Nevertheless, higher (5×10^{-4} mol l^{-1}) and lower (10^{-5} mol l^{-1}) IBMX concentrations did not affect the KCl-induced light emission in comparison with the 200 mmol l^{-1} KCl control (not shown). Kinetic parameters were not modified by these treatments in any of these species.

Effects of phospholipase C and protein kinase C inhibitors

In order to investigate the putative involvement of

Table 3. *Effect of MDL-12,330A, SQ22,536, U-73122 and U-73343 on the total amount of emitted light (L_{tot}) of KCl-induced luminescence on* A. filiformis, O. aranea *and* O. californica *dissociated photocytes*

	A. filiformis (*N*=10)	*O. aranea* (*N*=10)	*O. californica* (*N*=10)
10^{-5} mol l^{-1} MDL-12,330A	16.07±7.85†	7.02±1.20†	17.05±6.02†
10^{-5} mol l^{-1} SQ22,536	32.91±19.02†	79.75±6.15†	102.90±9.16
5×10^{-6} mol l^{-1} U-73122	40.74±14.43*	60.72±11.54†	89.22±8.33*
5×10^{-7} mol l^{-1} U-73122	38.82±16.25*	66.37±4.26*	121.60±8.72
5×10^{-6} mol l^{-1} U-73343	19.34±5.45†	25.11±3.03†	37.07±4.22†

Means ± S.E.M. are expressed as a percentage of photogenesis triggered by 200 mmol l^{-1} KCl in normal artificial seawater (controls=100%). *P<0.05, †P<0.01, *N*, number of stimulated aliquot parts.

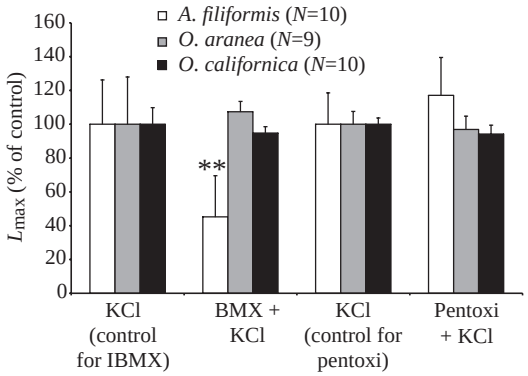

Fig. 3. Effects of 10^{-4} mol l^{-1} 3-isobutyl-1-methyl-xanthine (IBMX) and 10^{-4} mol l^{-1} pentoxifylline (pentoxi) on KCl-induced luminescence of *A. filiformis*, *O. aranea* and *O. californica* dissociated photocytes. Means ± S.E.M. are expressed as a percentage of photogenesis triggered by 200 mmol l^{-1} KCl in normal artificial seawater. $^{**}P<0.01$; *N*, number of stimulated aliquot parts.

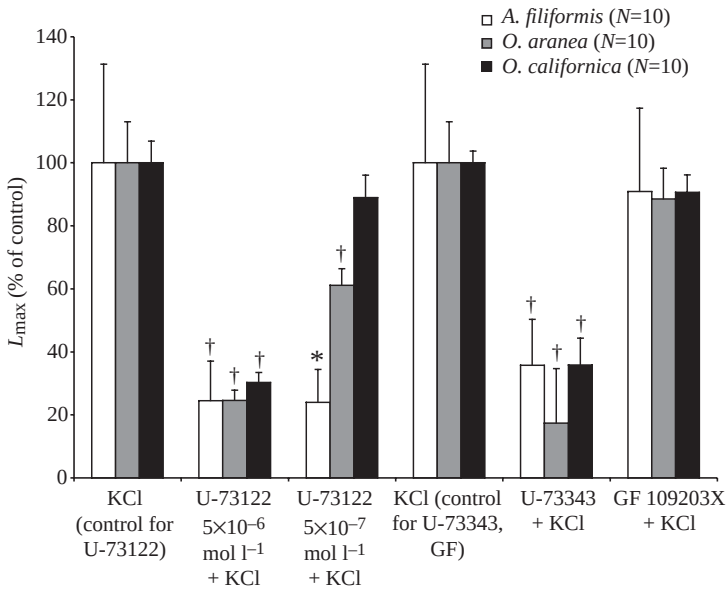

Fig. 4. Effects of 5×10^{-6} mol l^{-1} U-73122, 5×10^{-7} mol l^{-1} U-73122, 5×10^{-6} mol l^{-1} U-73343 and 5×10^{-7} mol l^{-1} GF 109203X on KCl-induced luminescence of *A. filiformis*, *O. aranea* and *O. californica* dissociated photocytes. Means ± S.E.M. are expressed as a percentage of photogenesis triggered by 200 mmol l^{-1} KCl in normal artificial seawater. $^{*}P<0.05$; $^{\dagger}P<0.01$; *N*, number of stimulated aliquot parts.

phosphoinositides in the triggering of photogenesis, we tested U-73122, a phospholipase C inhibitor. It inhibits the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) and therefore leads to a decrease in calcium mobilization from intracellular stores (Smallridge et al., 1992; Yule and Williams, 1992). The drug did not induce luminescence by itself but it strongly inhibited KCl-induced light emission in all three species at a concentration of 5×10^{-6} mol l^{-1} (L_{max}, Fig. 4; L_{tot}, Table 3). At 5×10^{-7} mol l^{-1}, U-73122 still inhibited light emission in *A. filiformis* and *O. aranea* (L_{max}, Fig. 4; L_{tot}, Table 3) but not in *O. californica*. Kinetic parameters were not modified in any conditions.

The inactive U-73122 analogue, U-73343, did not induce photogenesis by itself at a concentration of 5×10^{-6} mol l^{-1} but inhibited KCl-induced luminescence with the same efficiency as U-73122 in all three species (Fig. 4; Table 3). Kinetic parameters were not modified.

We also tested 5×10^{-7} mol l^{-1} GF 109203X, a protein kinase C inhibitor, in order to emphasise the putative activation of protein kinase C by DAG. Fig. 4 shows no effect of this drug on the KCl-induced luminescence in any of the species.

Discussion

One of the greatest methodological differences of this work, in comparison with most of the previous studies, is the use of isolated photocytes instead of arms or arm segments. This method is advantageous since it directly studies the effects of the drugs at the cell level and therefore rules out, for instance, indirect effects on nerve terminals. The problem of adsorption and penetration of the drugs to the photocytes because of the heavy calcification of the ophiuroid arms is also avoided by this technique.

In the present study, we used KCl (200 mmol l^{-1}) to trigger photogenesis since the neurotransmitters involved in the luminous control of both *Ophiopsila* species are still unknown. In *A. filiformis*, even if ACh seems to be the main transmitter in luminescence control (Dewael and Mallefet, 2002a), this drug has no effect on dissociated photocytes. This lack of response could be due to the dissociation process: the use of protease, a powerful enzyme that dissociates cells, may alter the cholinergic receptors on the photocyte membrane. Tests with papain, a milder enzyme (Gillis and Anctil, 2001; J. Mallefet, personal communication), failed to improve the results, suggesting that the dissociation process is not responsible for the lack of response. The absence of an ACh-induced response might reflect the absence of cholinergic receptors on the photocyte membrane. ACh receptors could be present on another cell membrane, closely associated to the photocytes. This has been observed by Dunlap et al. (1987), who showed that luminescence in the hydrozoan coelenterate *Obelia geniculata* was mediated by support cells through gap junctions. However, the presence of gap junctions has neither been demonstrated in ophiuroids (Y. Dewael, D. Sonny and J. Mallefet, unpublished) nor, for that matter, in any adult echinoderm species (Cobb, 1995).

Cyclic nucleotides

We used db-cGMP, the membrane-permeable cGMP analogue (Soliman, 1984a,b; Gustafson, 1990), and Na nitro, the guanylyl cyclase activator (Karaseva and Khotimchenko, 1995), to test the effect of an increase in intracellular cGMP on photogenesis. 10^{-4} mol l^{-1} db-cGMP had no effect on the

KCl-induced luminescence of dissociated photocytes from *O. aranea* and *O. californica*. By contrast, db-cGMP decreased the luminescence in *A. filiformis*. Although the effect is important (less than 6% of the control luminescence remains), the mechanism of an increase in intracellular cGMP remains unclear. Indeed, Na nitro, the activator of guanylyl cyclase, which is supposed to have the same final effect, i.e. an increase in intracellular cGMP level, did not affect light emission in any of the species. These contradicting results could come from the fact that guanylyl cyclase activation is not mediated by NO in ophiuroids, whereas Na nitro acts on NO production, which then stimulates guanylyl cyclase activation. Consequently, we suggest that the cGMP pathway is not involved in the luminescence of either *Ophiopsila* species but could be involved in an inhibitory luminescence modulation in *A. filiformis*. cGMP acting as a negative second messenger has already been observed in echinoderms: it blocks the reinitiation of meiosis in the oocytes of the holothurian *Stichopus japonicus* (Karaseva and Khotimchenko, 1995), it reduces the Ca^{2+} influx and counteracts the stimulatory action of Ca^{2+} in the muscular activity of larvae of the sea urchin *Psammechinus miliaris* (Gustafson, 1990). On the other hand, the cGMP pathway is not involved in the regulation of luminescence in the ophiuroid *A. squamata* (De Bremaeker et al., 2000b).

The cAMP analogue db-cAMP (Soliman, 1984a,b; Gustafson, 1990) and FSK, the adenylyl cyclase activator (Karaseva and Khotimchenko, 1995), had no effect on 200 mmol l^{-1} KCl-induced luminescence of dissociated photocytes from *O. aranea*, *O. californica* and *A. filiformis*. It is therefore assumed that an increase of both external and intracellular cAMP concentration in photocytes does not affect 200 mmol l^{-1} KCl-induced luminescence. On the other hand, a decrease of cAMP concentration, produced by the adenylyl cyclase inhibitors MDL-12,330A (Lippe and Ardizzone, 1991) and SQ22,536 (Fabbri et al., 1991; Lippe and Ardizzone, 1991; Goldsmith and Abrams, 1992; Shi and Bunney, 1992), strongly reduced L_{max} and L_{tot} in *A. filiformis* and *O. aranea*. It also increased TL_{max} in both *Ophiopsila* species.

These contradicting results may be explained by the nature of stimulation. Since KCl already triggers maximal intensity of light by photocyte depolarisation (Mallefet and Dubuisson, 1995; De Bremaeker et al., 2000b; Dewael and Mallefet, 2002a), it is not very likely to observe a potentiation of this maximal light emission. This is the reason why we tested the adenylyl cyclase activator FSK on non-maximal light emission triggered by 50 mmol l^{-1} KCl. This latter treatment did not significantly potentiate light production in all three species but decreased kinetic parameters of light production (*LT* and TL_{max}) in *O. californica*. This may result from the fact that cAMP is not the only pathway controlling luminescence, and increasing cAMP on its own does not subsequently increase light emission. Nevertheless, the effect of FSK on kinetic parameters in *O. californica* is important since it increases the rate of photocyte light response (shown by a decrease of *LT* and TL_{max}).

Given the results, we can suggest that the cAMP pathway is partly involved in the luminescence control of all three ophiuroid species. Similar results were observed in the ophiuroid *A. squamata*, where cAMP was involved in ACh-induced luminescence (De Bremaeker et al., 2000b). The second messenger cAMP is formed by the hydrolysis of ATP by adenylyl cyclase. When a neurotransmitter fixes G protein-coupled receptors, the heterotrimeric G protein binds to GTP and dissociates into G_{α}-GTP and $G_{\beta\gamma}$. The former product can stimulate adenylyl cyclase, leading to an increase of intracellular cAMP concentration (Hille, 2001). cAMP can activate protein kinase A, which is involved in the regulation of receptors and structural proteins and the opening and closing of ion channels. It will, among other effects, increase Ca^{2+} influx (Kennedy, 1994). Ca^{2+} and cAMP pathways may interact at several levels since cytosolic calcium can affect the cAMP cascade by binding to calmodulin. The calcium–calmodulin complex alters the activity of adenylyl cyclase and phosphodiesterase enzymes (Kebabian, 1992).

A previous study has shown that calcium movements are required in the luminescence of the three studied ophiuroid species (Dewael and Mallefet, 2002b). In the absence of extracellular calcium, photogenesis is strongly inhibited; pharmacological experiments indicate that calcium channels involved in the luminescence control appear to be of the voltage-dependent L-type in *A. filiformis* and *O. californica* but not in *O. aranea*. Eventually, the intracellular increase of Ca^{2+} concentration would lead to the triggering of the light reaction, through a mechanism not yet understood.

The phosphodiesterase inhibitors IBMX and pentoxifylline did not modify the KCl-induced luminescence in *O. aranea* and *O. californica*. In *A. filiformis*, only 10^{-4} mol l^{-1} IBMX decreased light emission; higher and lower concentrations had no effect. Knowing that IBMX is also an antagonist of adenosine receptors, the effect could be due to the blockade of these receptors. Although adenosine is not a transmitter for luminescence (Dewael and Mallefet, 2002a), one cannot exclude that IBMX inhibition might be due to blockade of adenosine neuromodulation of photogenesis. This neuromodulatory effect has been described in *A. squamata*, where adenosine potentiates ACh-induced luminescence (De Bremaeker et al., 2000a).

Phospholipase C and protein kinase C inhibitors

Treatment with U-73122, a phospholipase C inhibitor (Yule and Williams, 1992), strongly reduced KCl-induced luminescence in the three species at a concentration of 5×10^{-6} mol l^{-1}. At a lower concentration (5×10^{-7} mol l^{-1}), it still inhibited light emission in *A. filiformis* and *O. aranea* but not in *O. californica*. Since U-73122 has been reported to have some unspecific effects (Alter et al., 1994), we used its inactive analogue U-73343 (5×10^{-6} mol l^{-1}; Bleasdale et al., 1990; Smith et al., 1990) as a negative control. This drug did not induce photogenesis by itself but inhibited KCl-induced luminescence with the same efficiency as U-73122. It supports the evidence that the effects of U-73122 are unspecific. Moreover, quantitative assays of labelled IP_3 have shown the

absence of IP_3 production when photocytes are stimulated (results not shown), suggesting that bioluminescence control is not mediated by IP_3 production. Conversely, IP_3 involvement in luminescence control has previously been shown in *A. squamata* (De Bremaeker et al., 2000b), where it mediated ATP- and ACh-induced luminescence. These results highlight that luminous control mechanisms differ between ophiuroid species.

We tested the putative involvement of DAG in photogenesis control using GF 109203X, a protein kinase C inhibitor (Toullec et al., 1991; Martiny-Baron et al., 1993). This drug did not affect light emission, suggesting that DAG-activated protein kinase C is not used in photogenesis. Consequently, we suggest that the IP_3/DAG pathway is not involved in the luminescence control of all three ophiuroid species.

In conclusion, this study shows that IP_3 and DAG are not involved in the luminescence control of *Amphiura filiformis*, *Ophiopsila aranea* and *Ophiopsila californica*. cGMP could be involved in an inhibitory mechanism in *A. filiformis*. We suggest that the production of cAMP in photocytes of all three ophiuroid species is a step in the transduction signal leading to photogenesis. These rises in cAMP level and intracellular calcium concentration are therefore likely to be crucial factors responsible for light emission. The final steps of intrinsic luminescence control and the nature of luminescent systems remain to be discovered. We have presented evidence that luminescence control in ophiuroids is species-specific since the second messengers involved in bioluminescence control vary between species (*A. squamata*, *A. filiformis*, *O. aranea* and *O. californica*). This diversity had been observed at different levels previously: in extrinsic control mechanisms (Dewael and Mallefet, 2002a) and in calcium channels (Dewael and Mallefet, 2002b). It is also noteworthy that *A. filiformis* presents a higher intra-specific variability in luminous capabilities than *O. aranea* and *O. californica* (Dupont et al., 2001; Dewael and Mallefet, 2002a,b; present study).

Electrophysiological studies, using patch-clamp techniques and microspectrofluorimetric measurements of free intracellular calcium concentrations, are planned to bring some new clues to the signal transduction pathways of light emission in ophiuroids.

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