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Comparative Biochemistry and Physiology Part C 131 (2002) 153–160

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Calcium involvement in the luminescence control of three ophiuroid species (*Echinodermata*)

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Received 16 August 2001; received in revised form 2 November 2001; accepted 22 November 2001

Abstract

Although it has been shown that calcium is involved in the control of the luminous reaction of many invertebrate phyla, its role in Echinoderms is poorly documented. The aim of this work was to carry out a comparative study of calcium requirement of KCl-induced light emission by arm segments and dissociated luminous cells from three ophiuroid species, *Ophiopsila californica*, *O. aranea* and *Amphiura filiformis*. Results show a gradual inhibition of the luminescence when preparations are incubated in artificial sea water with lowered calcium concentration. The calcium substitutes Ba^{2+} and Sr^{2+} could act either as blockers or as substitutes, depending on the ophiuroid species; while calcium blockers Co^{2+}, Ni^{2+} and Cd^{2+} inhibit light emission in *A. filiformis* and in *O. californica*, but not in *O. aranea*. The nature of putative calcium voltage-gated channel has been studied pharmacologically using 1,4-dihydropyridine, benzodiazepine, phenylalkylamine and trifluoroperazine. From our results, it is proposed that calcium could act via an L-type voltage-gated calcium channel in *O. californica* and *A. filiformis* but not in *O. aranea*. The precise role of calcium in luminescence control still remains unknown; it could act as a second messenger or as a co-factor of the luminous reaction. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Invertebrate; Echinoderm; Ophiuroid; *Amphiura filiformis*; *Ophiopsila aranea*; *Ophiopsila californica*; Bioluminescence; Calcium; Nervous system

1. Introduction

Within the echinoderm phylum, luminescence has been mostly documented among the *Ophiuroidea* class, but little is known about the control mechanisms of the light emission. Recent studies on the small brittlestar *Amphipholis squamata* have shown that photogenesis is under nervous control. Acetylcholine is the main transmitter triggering light production (De Bremaeker et al., 1996, 1999a) though some other compounds (ATP, GABA, SALMFamides S1 and S2,...) modulate the cholinergic action (De Bremaeker et al., 1999b,c). Studying intrinsic control mechanisms, it has been shown that calcium was necessary for potassium chloride and acetylcholine-induced light emission by both isolated arms and dissociated photocytes of *A. squamata* (Mallefet et al., 1994, 1998). A calcium requirement for light emission has already been reported in other phyla like Polychaeta (Herrera, 1979), Cnidaria (Charbonneau and Cormier, 1979; Dunlap et al., 1987) and Ctenophora (Shimomura, 1985). In the hydrozoan coelenterate *Obelia geniculata*, the calcium ion is a co-factor for the luminous reaction since light emission is produced by photocytes containing a calcium-activated photoprotein (Dunlap et al., 1987) and in the polynoid polychaete worm *Hesperonoe complanata*, the light flashes are produced

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PII: S1532-0456(01)00288-5

when single calcium spikes occur (Herrera, 1979). In echinoderms, Brehm (1977) already showed that luminescence of entire arms of *Ophiopsila californica* was completely blocked in the absence of calcium.

Since it seems that the nervous control of bioluminescence in ophiuroids differs from species to species, the aim of this study was to investigate the involvement of calcium ion in the KCl-induced luminescence in three ophiuroid species: *Ophiopsila aranea* (Forbes, 1843), *O. californica* (Clarck, 1921) and *Amphiura filiformis* (O.F. Müller, 1776). Pharmacological experiments were performed in order to identify the type of calcium channel involved in the luminescence of these brittlestars.

2. Materials and methods

2.1. Animals

Specimens of *Ophiopsila aranea* were collected at the ARAGO biological station (C.N.R.S.) of Banyuls-sur-Mer (France) by SCUBA at 20–25 m depth whereas specimens of *Ophiopsila californica* were collected by the same techniques at the Marine Sciences Institute of the University of California (Santa-Barbara). Specimens of *Amphiura filiformis* were collected at the Kristineberg Marine Research Station (Fiskebäckskil, Sweden) by mechanical mud grab at 25–40 m depth. All these animals were transported to our laboratory in Belgium in aerated natural sea water and then kept in aquaria filled with a recirculating mixture of natural and artificial sea water at 12 °C. Food was provided once a week.

2.2. Experiments on arm segments

After anesthesia of the animals by immersion in 3.5% w/w $MgCl_2$ in artificial sea water (ASW), arms were isolated from the disc and divided into sections of eight articles (*Ophiopsila*) or 20 articles (*Amphiura*), which were then rinsed in ASW (NaCl 400.4 mM, $CaCl_2$ 9.9 mM, KCl 9.6 mM, $MgCl_2$ 52.3 mM, Na_2SO_4 27.7 mM, Tris 20 mM, pH 8.3).

2.3. Experiments on dissociated photocytes

The arms were isolated from the disc and chopped into tiny pieces before enzymatic digestion and differential centrifugation using the protocol described by Mallefet et al. (1998). The enriched luminous-cell fraction was then aliquoted (100 μl), and light emission was measured with a FB12 Berthold luminometer linked to a personal computer.

2.4. Stimulations

Stimulations were performed by injection of 200 mM potassium chloride (KCl). A stock solution of 400 mM KCl was prepared in ASW without NaCl, to keep roughly the same osmolarity than normal ASW. For each experimental protocol, one arm segment or one aliquot was stimulated in normal ASW, as a control, while the other preparations were first immersed in ASW containing the tested drug or the tested calcium concentration for at least 10 min before stimulation with KCl.

2.5. Drugs

The following drugs were used at these concentrations in this study: A-23187 (calcimycin 20 μM; ICN), strontium chloride hexahydrate ($SrCl_2$ 9.9 mM; Sigma), barium chloride dihydrate ($BaCl_2$ 9.9 mM; Janssen Chimica), cadmium chloride ($CdCl_2$ 1 mM; Sigma), cobalt(II) chloride anhydrous ($CoCl_2$ 5 mM; Janssen Chimica), nickel(II) chloride hexahydrate ($NiCl_2$ 5 mM; Janssen Chimica), diltiazem HCl (100 μM; RBI), nifedipine (100 μM; Sigma), (±)-verapamil (100 μM; Sigma), trifluoroperazine dihydrochloride (100 μM; Sigma). All solutions were diluted in ASW, except nifedipine and A-23187, which were dissolved in dimethylsulfoxide (DMSO), after which the solutions were diluted in ASW with respectively, 0.1% and 1% DMSO final concentration.

Statistical analyses (ANOVA) were performed using the SAS program (Statistic Analysis System).

Different parameters were used in order to characterize the photogenesis: (1) L_{max}: the maximum level of light emission expressed in Megaquanta per second (Mq s^{-1}) or as a percentage of the control; (2) *LT*: latency time, the time elapsed between the stimulation and the beginning of the light emission; and (3) TL_{max}: the time between onset of light production and maximum of light emission.

Table 1
Effect of calcium concentration on the maximal intensity of light emission induced by KCl 200 mM, from arm segments of *Ophiopsila californica*, *O. aranea* and *Amphiura filiformis*

	9.9 mM Ca^{2+} (control)	5 mM Ca^{2+}	0 mM Ca^{2+}
Ophiopsila californica ($n=15$)	100 ± 0	$45.45\pm6.04^{*}$	$6.22\pm1.62^{*}$
Ophiopsila aranea ($n=12$)	100 ± 0	95.8 ± 7.34	$10.66\pm4.06^{*}$
Amphiura filiformis ($n=20$)	100 ± 0	79.89 ± 11.22	$1.08\pm0.31^{*}$

Maximal intensities of light are expressed as a percentage of the light emitted in normal ASW (with 9.9 mM Ca^{2+}). Mean ± S.E.M. (standard error of mean).
* $P<0.01$; n = number of stimulated segments.

3. Results

3.1. Calcium concentration

Maximal intensities of light emitted by arm segments of the three ophiuroid species strongly decreased with the complete removal of Ca^{2+} in ASW (Table 1), compared to normal ASW with 9.9 mM of Ca^{2+} (*O. californica*: 59 562 ± 8863 Mq s^{-1}, *O. aranea*: 46 459 ± 10 734 Mq s^{-1}, *A. filiformis*: 12 213 ± 3492 Mq s^{-1}). Only *O. californica* showed a gradual decrease of luminescence when immersed in ASW with 5 mM of Ca^{2+}. Kinetic parameters of luminescence were also significantly altered by the absence of calcium (data not shown). Light productions were either slowed or progressively abolished (number of luminous responses decreased).

Fig. 1 shows original recordings from experiments on dissociated photocytes from *Ophiopsila aranea* (a), *O. californica* (b) and *Amphiura filiformis* (c). Control stimulation of the photocytes by 200 mM KCl in normal ASW produced a monophasic light emission (left graphs). The maximum of light emission (L_{max}) is reached nearly immediately in both *Ophiopsila* species, while in *Amphiura*, it took 5–10 s. In the middle graphs, when the photocytes were suspended in calcium-free ASW containing 1 mM EGTA before stimulation by Ca^{2+}-free KCl, no luminescence was detected. The subsequent addition of calcium in ASW partially restored light emission in *Ophiopsila* species (approx. 15% of the control intensity). In *A. filiformis*, the basal level of luminescence slightly increased. The kinetics of the light reaction was also slower. The right graphs on Fig. 1 show that the addition of ASW with 19.8 mM of Ca^{2+} on dissociated photocytes treated with calcium ionophore A-23187 (calcimycin 20 μM) for 10 min only triggers very weak light emission, less than 1% of the control in all the three species. Moreover, subsequent injection of KCl produces luminescence whose intensity is significantly lower than the control in *O. californica* and *O. aranea*; it represents 2.67 ± 0.72% ($n=9$) and 17.48 ± 14.05% ($n=10$), respectively. This was not the case in *Amphiura filiformis* where a mean value of 68.96 ± 47.53% ($n=12$) is observed. Here again, light emission was slower in all cases as revealed by the significant increase of latency time and time to reach the peak of luminescence, compared to the control. Latency time is significantly increased by 15%, 50% and 14% in *O. aranea*, *O. californica* and *A. filiformis*, respectively. TL_{max} is either not affected in *A. filiformis* response, or strongly increased: it is six-fold (*O. aranea*) and 11-fold (*O. californica*) larger than the control. It must be pointed out that spontaneous light emission was observed during incubation of the photocytes in solution containing the A-23187, even in calcium-free ASW.

3.2. Inorganic ions

Calcium substitutes, strontium (Sr^{2+} 9.9 mM) and barium (Ba^{2+} 9.9 mM) exhibited weak substitution of Ca^{2+} in the KCl-induced luminescence on *Ophiopsila aranea* arm segments, while in *Ophiopsila californica* and in *Amphiura filiformis*, more than 50% of control maximal intensity is reached (Fig. 2). Kinetic parameters were not modified in *A. filiformis* and in *O. aranea*, while latency time (*LT*) and time to reach maximal intensity (TL_{max}) were increased in *O. californica*. Calcium channel blockers cobalt (Co^{2+} 5 mM) and nickel (Ni^{2+} 5 mM) showed inhibitory effects on the luminescence of *A. filiformis* and *O. californica*, but not of *O. aranea*. Luminous reaction was slowed down since both *LT* and TL_{max} were increased. Cadmium (Cd^{2+} 1 mM) completely

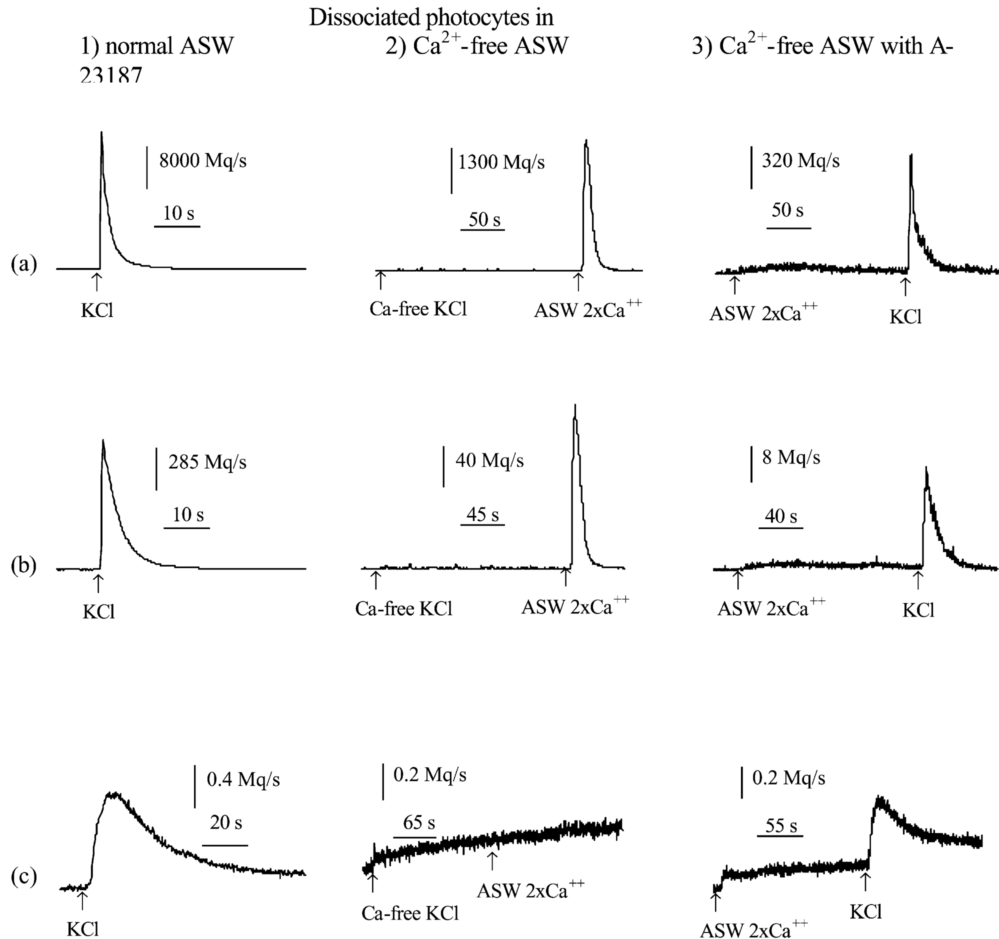

Fig. 1. Representative original recordings of light emissions of dissociated photocytes treated for 10 min with normal ASW (1); Ca-free ASW (2); and Ca-free ASW containing 20 μM A-23187 (3), from *Ophiopsila aranea* (a), *O. californica* (b) and *Amphiura filiformis* (c). Arrows show the stimulation of the photocytes with 200 mM KCl in normal ASW (KCl), with 200 mM KCl in Ca^{2+}-free ASW (Ca-free ASW), or with ASW containing double concentration of calcium (ASW $2\times Ca^{2+}$).

inhibited photogenesis in *A. filiformis* (remaining luminescence being not significantly different from zero) and in *O. californica*, only 18.7% ± 0.97% of the control luminescence was emitted. In *O. aranea*, 77.1% ± 8.15% was still produced. *LT* and TL_{max} increased in all three species (data not shown).

3.3. Pharmacology

Diltiazem (100 μM DILT), verapamil (100 μM VERA) and trifluoroperazine (100 μM TRIF) strongly reduced the light emission of both *O. californica* and *A. filiformis* isolated arms while nifedipine (100 μM NIF) only inhibited the light production of *O. californica* (Fig. 3). None of these drugs had any effect on the luminescence of *O. aranea*.

No statistical variations of kinetic parameters were detected for these treatments (data not shown).

4. Discussion

4.1. Isolated arms

Our experiments reveal that removing calcium from the external medium results in an inhibition of the KCl-triggered luminescence from isolated arm segments of *Ophiopsila aranea*, *Ophiopsila californica* and *Amphiura filiformis*. The low persistent level of light observed with isolated arms

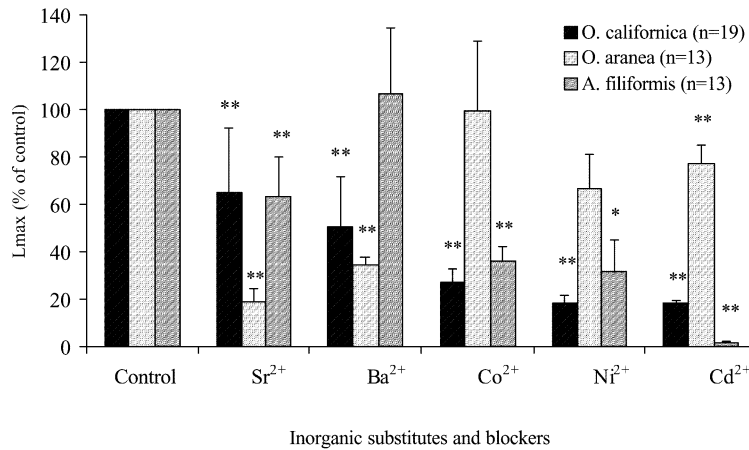

Fig. 2. Effect of inorganic calcium substitutes and blockers on maximal intensities (L_{max}) of arm segments. Means ± S.E.M. (standard error of mean) are expressed as a percentage of photogenesis triggered by 200 mM KCl in normal ASW. * = $P<0.05$, ** = $P<0.01$, n = number of stimulated segments.

incubated in a calcium-free solution might be due to small amounts of calcium released from crystalline skeleton, which contains approximately 90% calcium carbonate (Hernandez et al., 1987) and/or to the fact that most excitable cells always maintain some levels of intracellular calcium (Triggle, 1989). Calcium appears to be a necessary factor in the luminescence control in the three species of ophiuroids.

As shown in another luminous brittlestar, *Amphipholis squamata* (Mallefet et al., 1994), KCl-induced luminescence of isolated arms of the three species studied is slower when calcium concentration is decreased, indicating that the kinetic of the luminous reaction is calcium dependent.

It has been postulated that luminescent cells of ophiuroids might be of neural origin (Brehm and Morin, 1977) and that calcium was required for the compound action potential in the radial nerve cord of asteroids, echinoids and ophiuroids (Binyon and Hasler, 1970; Brehm, 1977; Smith et al., 1985). In *O. californica*, it has been shown that calcium-free ASW blocked all electrical activity on radial nerve cord (Brehm, 1977) as well as

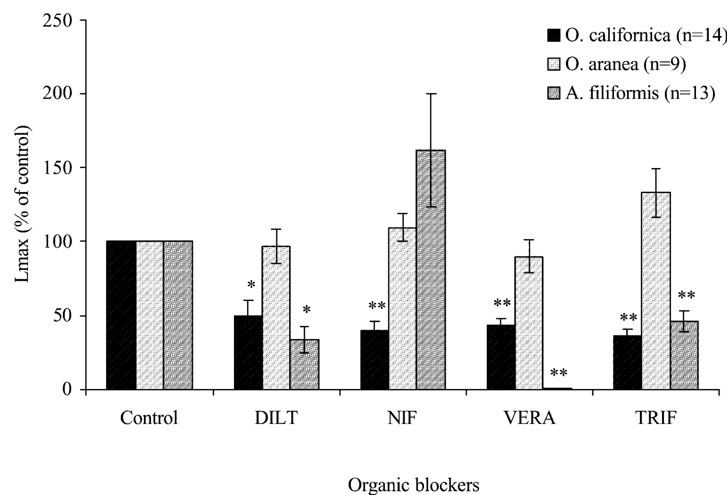

Fig. 3. Effect of calcium organic blockers on maximal intensities (L_{max}) of arm segments. Means ± S.E.M. (standard error of mean) are expressed as a percentage of photogenesis triggered by 200 mM KCl in normal ASW. * = $P<0.05$, ** = $P<0.01$, n = number of stimulated segments, DILT: diltiazem, NIF: nifedipine, VERA: verapamil, TRIF: trifluoroperazine.

luminescence (present study). Consequently, we suggest that light production by isolated arms of *O. aranea*, *O. californica* and *A. filiformis* share some common properties with those attributed to nervous elements of echinoderms.

Most calcium channels are also known to be permeable to barium and strontium; Ba^{2+} is even more permeant than Ca^{2+} through calcium channels (Hille, 2001). These two divalent cations substitute for calcium in maintaining regenerative responses, and this permeability to Ba^{2+} and Sr^{2+} is one of the criteria used to identify calcium spikes (Hagiwara and Byerly, 1981a,b). Therefore, Ba^{2+} and Sr^{2+} were chosen to replace Ca^{2+} in ASW and to observe their effect on photogenesis. Our results show that these two calcium substitutes only weakly replace Ca^{2+} in triggering the light emission of *O. aranea*, while they induce a nearly normal photogenesis in *O. californica* and in *A. filiformis*. Brehm (1977) noted that Sr^{2+} substitutes for Ca^{2+}, but Ba^{2+} only stimulates a very weak light production in *Ophiopsila californica* whole arm. Although these results partially contradict ours, it must be pointed out that the preparations used are different in the two studies: whole arms in Brehm's study vs. arm segments in this work. Nevertheless, conflicting reports on the effects of Ca^{2+} substitutes are found in the literature. Mallefet et al. (1994) reported an inhibitory effect of Sr^{2+} and Ba^{2+} on light emission of *Amphipholis squamata*. Berrios et al. (1985) showed that Ba^{2+} (11 mM) was unable to replace Ca^{2+} and furthermore, that it irreversibly blocked nervous electrical activity in spines of the sea urchin *Diadema antillarum*. On the contrary, Sr^{2+} not only replaced Ca^{2+} in electrical conduction, but also doubled the amplitude of action potentials. Cobb and Moore (1988) indicated that Sr^{2+} seemed to be a good Ca^{2+} substitute for action potentials in neurons of the brittlestar *Ophiura ophiura* while Ba^{2+} had variable effects: either it substituted for Ca^{2+} or blocked electrical activity. According to all these studies, Sr^{2+} and Ba^{2+} do not seem to be reliable substitutes for calcium in echinoderms. In luminous ophiuroids, they may enter the cell through calcium channels, but do not have the same efficiency as calcium in triggering light emission once in the cytoplasm.

Three inorganic Ca^{2+}-channels blockers were also used in our study: cobalt, nickel and cadmium (Co^{2+} 5 mM, Ni^{2+} 5 mM and Cd^{2+} 1 mM). Cobalt and nickel were found to strongly inhibit photogenesis in *A. filiformis* and in *O. californica*, but not in *O. aranea*. The inhibitory effect of inorganic calcium channels blockers was confirmed by the use of cadmium since luminous reaction was completely abolished in *A. filiformis*, strongly reduced in *O. californica*, but not decreased in *O. aranea*. Stockbridge and Ross (1986) reported that nerve cells from the giant barnacle *Balanus nubilus* did not produce action potentials when either Ni^{2+} or Co^{2+} blocked Ca^{2+}-channels. In echinoderms, cobalt (5 mM) and cadmium (2 mM) cause an irreversible blockade of action potentials in nerve cells of the brittlestar *Ophiura ophiura* (Cobb and Moore, 1988) and in spines of the sea urchin *Diadema antillarum* (Berrios et al., 1985).

Our results suggest that calcium is necessary for light emission of *Ophiopsila aranea*, *Ophiopsila californica* and *Amphiura filiformis*. Assuming that, further steps were realized in order to identify the subtype of calcium channels involved, based on their specific pharmacological properties (Brehm and Morin, 1977; Triggle, 1999). In our pharmacological experiments, we have shown that KCl-induced luminescence of isolated arms of *O. californica* was reduced by L-type voltage-gated calcium channel blockers diltiazem (benzodiazepine), nifedipine (1,4-dihydropyridine), verapamil (phenylalkylamine) (Triggle, 1990, 1992; Rampe and Triggle, 1993) and by trifluoroperazine, a calmodulin inhibitor. The same results were observed in *A. filiformis*, except for nifedipine, which had no effect. On the contrary, none of these blockers had an inhibitory effect on *O. aranea*.

Our results support the hypothesis that Ca^{2+} channels involved in the luminescence of *O. californica* are of the L-type. According to the literature, we propose that voltage-gated calcium channels involved in the light emission of *A. filiformis* might also be of the L-type, but with no affinity for nifedipine since only diltiazem, verapamil and trifluoroperazine inhibit light emission. Channels involved in *O. aranea* luminescence seem to be of another type since the light response is not modified when preparations are treated with L-type voltage-gated calcium channel blockers.

It is noteworthy that all classes of calcium channels has been characterized in vertebrates and may differ in some invertebrates. Further experiments will be done in order to identify precisely

the type of Ca^{2+}-channels involved in luminescence control.

On isolated arm segments, we have seen that inorganic substances had some effect on the kinetics of ophiuroid luminescence (LT and TL_{max} longer), while organic blockers did not. We can then assume that these two different classes of drugs do not share the same mode of action. The use of patch-clamp techniques should confirm this hypothesis thanks to the direct measure of ion currents through the channels.

4.2. Dissociated photocytes

On the arm segment, we have seen that the removal of Ca^{2+} from the external medium nearly abolished luminescence in all three species, external calcium being necessary to induce light emission by KCl stimulation. This hypothesis was confirmed by experiments on dissociated photocytes, since the light production of photocytes in calcium-free ASW was not statistically different from the light produced by mechanical stimulation of dissociated photocytes triggered by normal ASW injection (data not shown). The addition of ASW with 19.8 mM Ca^{2+} after depolarization of the photocytes with Ca^{2+}-free KCl only triggered a weak light emission in *Ophiopsila* aliquots, and no light at all in *Amphiura* **preparation**. This absence of response after these treatments might reflect the importance of membrane potential in the control mechanisms; photocytes being unable to produce light as a consequence of disturbance of the control mechanisms by membrane depolarization without calcium.

The addition of ASW after treatment of the dissociated photocytes with calcium ionophore A-23187 does not induce light emission. When KCl is injected onto the photocytes treated with A-23187, light emission is significantly decreased, in the *Ophiopsila* preparation, but not in the *Amphiura* ones. This decrease might be the consequence of light emission by the photocytes during pretreatment with A-23187 in calcium-free ASW in *Ophiopsila aranea* and in *O. californica*. We suggest that this light emission is produced by the depletion of intracellular stores of calcium by A-23187.

In conclusion, this study shows from this study that calcium movements are necessary to activate the luminous reaction. Nevertheless, calcium does not seem to be directly involved in the primary light-emitting reaction of the *Ophiopsila californica* luminescence (Shimomura, 1986). Although the precise role of calcium in ophiuroid light emission remains unknown, strict requirement of this ion is observed. We suggest that the control mechanisms of light emission appear to show differences from species to species: L-type voltage-gated calcium channels are involved in the photogenesis of *Amphiura filiformis* and *Ophiopsila californica*, but not in *O. aranea*. These differences, observed in the same *Ophiopsila* **genus** are supported by Ward's clustering method on Euclidian matrix of distance computed on luminescence parameters. Using this technique, it has been suggested that *O. aranea* and *O. californica* are not closely related (Dupont et al., 2001).

It must be pointed out that drugs used on the arm segment preparations could act either directly on the photocytes or on the nerves controlling photocytes. Further studies, using patch-clamp techniques are being developed in order to characterize precisely the location and the types of calcium channels involved in the control of the luminescence in these three ophiuroid species.

Acknowledgments

We thank Drs P. Gailly, J. Lebacq and R. Hill for their comments on this manuscript. We also thank I. Cogneau for technical assistance. We acknowledge financial supports to Y. Dewael and J. Mallefet from an EEC Large Scale Facility and ARI at Kristineberg Marine Station, Fiskebäckskil, Sweden; Fonds Léopold III at Arago Laboratory, Banyuls-sur-Mer, France and F.N.R.S. short stay to J. Mallefet at Marine Sciences Institute, U.C.S.B., California, United States of America. J. Mallefet is a Research Associate of the National Fund for Scientific Research (F.N.R.S., Belgium). Research supported by a FRIA grant for Y. Dewael.

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