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Effect of β-adrenergic antagonists on bioluminescence control in three species of brittlestars (Echinodermata: Ophiuroidea)

S. Dupont*, J. Mallefet, C. Vanderlinden

Laboratory of Marine Biology and Biodiversity Research Centre, Université catholique de Louvain, Bâtiment Kellner, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

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Abstract

The role of adrenaline in the nervous control of bioluminescence in three brittlestar species, *Amphiura filiformis*, *Amphipholis squamata*, and *Ophiopsila aranea*, was assessed by testing two different β-adrenergic antagonists (propranolol and labetalol) over a wide concentration range (10^{-10}–10^{-3}M). We compared the effects of analogues (active vs. inactive) of the same substance (L- and D-enantiomers of propranolol). Propranolol presented both specific and nonspecific effects: (i) nonspecific effects were observed at the higher concentrations tested (10^{-4} and 10^{-3}M) in all three species; (ii) specific effects were detected only at the lower concentrations tested (10^{-6}–10^{-5}M). In *A. squamata*, the involvement of adrenaline in the nervous control of luminescence is supported by propranolol and labetolol specific inhibition. The neuropharmacological implications of nonspecific effects, the involvement of adrenaline and the interspecific differences in the brittlestar nervous control of bioluminescence are discussed.

Keywords: *Amphiura filiformis*; *Amphipholis squamata*; Bioluminescence; Echinoderm; Labetalol; Nonspecific effects; *Ophiopsila aranea*; Propranolol

1. Introduction

In view of its importance to human health, pharmacology is much better understood in vertebrates and especially in mammals than in invertebrates. The use of these specific drugs developed originally for medical applications is often extended to invertebrate pharmacological preparations with the assumption that invertebrates have similar receptors (Lacoste et al., 2001a,b; Pierobon et al., 2001; Dewael and Mallefet, 2002a,b). Nevertheless, numerous unexpected effects are sometimes observed. It is then important to be cautious in the interpretation of the results because (i) differences might exist between invertebrate and vertebrate receptor sensitivity; that is, a drug developed for vertebrates does not necessarily have similar effects when used in invertebrates; (ii) in some cases, nonspecific effects have been observed in vertebrates; and (iii) high concentrations (up to 10^{-3} M) are sometimes required in invertebrates (e.g., Anctil et al., 1982; De Bremaeker, 1999; Dewael, 2002).

Physiological control of bioluminescence (the emission of visible light by living organisms) in echinoderms has been studied in our laboratory for 15 years. Since light emission in brittlestars is a phenomenon controlled by the nervous system, the obtained information from its investigation has provided information on echinoderm pharmacology: neuromediators, neuromodulators, second messengers, etc. (Mallefet, 1999; De Bremaeker et al., 2000b; Dewael, 2002; Vanderlinden et al., 2003). Although some of the drugs used are specific for echinoderms (e.g., neuropeptide S1; Elphick et al., 1991), most come from vertebrate studies.

It was shown that the nervous control of brittlestar bioluminescence is a very complex phenomenon which can show different implications in closely related species (De Bremaeker et al., 1999, 2000a,b; Dewael and Mallefet, 2002a,b; Dewael, 2002). In particular, pharmacological studies failed to reveal mediators involved in the luminous control of *Ophiopsila aranea*. On the other hand, previous results showed that acetylcholine is the main neurotransmitter inducing luminescence in *Amphipholis squamata* (De Bremaeker et al., 1996) and *Amphiura filiformis*

* Corresponding author. Tel.: +32-1047-3475; fax: +32-1047-3476.
E-mail address: dupont@bani.ucl.ac.be (S. Dupont).

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(Dewael and Mallefet, 2002b), and different substances appear to modulate the luminous response. This was particularly well described in *A. squamata* where amino acids, catecholamines, neuropeptides and purines appear to positively or negatively modulate light emission triggered by acetylcholine (De Bremaeker et al., 1999, 2000a).

A new attractive hypothesis suggests that adrenaline may play a key role in bioluminescence control in *A. squamata*. Preliminary results revealed a strong inhibition of light emission after treatment with β-adrenergic antagonists (Dupont et al., 1999; Dupont, 2002). The present work confirms the involvement of adrenaline in the control of luminescence in *A. squamata* and explores the possible occurrence of this control mechanism also in other luminous brittlestars. Two β-adrenergic antagonists (propranolol and labetalol) were chosen for this study. Treatments with two stereoisomeric forms of propranolol were tested in order to discriminate specific and nonspecific effects on the bioluminescence of three selected brittlestar species (*A. squamata*, *A. filiformis* and *O. aranea*). The origin of effects, the involvement of adrenaline and the interspecific differences in the nervous control are discussed.

2. Materials and methods

2.1. Animals

Three species of luminous brittlestars obtained from different locations were employed as follows. (i) Specimens of *A. squamata* Delle Chiaje 1828 were collected in Langrune-sur-Mer (France) from the rocks in the tidal zone; (ii) specimens of *A. filiformis* Müller 1776 were collected from mud at 25–40 m depth in Fiskebäckskil (Sweden); and (iii) specimens of *O. aranea* Forbes 1843 were collected in Banyuls-sur-Mer (France) from the encrusting calcareous seaweed zone (coralligene) at 20–25 m depth. All animals were transported in aerated seawater to our laboratory (Belgium) and then kept in aquaria filled with recirculating natural and artificial seawater (ASW) at 12 °C. Liquid food was provided to the brittlestars once a week (Liquifry Marine Interpet).

2.2. Dissection

Animals were anaesthetized by immersion in 3.5% w/w $MgCl_2$ in ASW. Arms, which are the only luminous body part in the three selected species, were removed from the disc. Since the three species differ remarkably in size, experiments were carried out on whole arms in *A. squamata*, on arm pieces of eight segments and 20 segments in *O. aranea* and in *A. filiformis*, respectively. Twelve preparations were used for each treatment. The arms or arm pieces were 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) before stimulation.

2.3. Stimulation

Maximal light emission was triggered by KCl application (200 mM). This KCl-induced luminescence mimics the natural phenomenon triggered by mechanical stimulations during interactions with a predator (Mallefet et al., 1992; Fauville et al., 2003). A stock solution of 400 mM KCl was prepared in ASW without NaCl, to maintain the same osmolarity as in normal ASW. In one specimen, the five arms or the five arm pieces cut at the same distance from the disc were considered to produce light of the same intensity, provided that they were of equal length (Mallefet et al., 1992; Dewael and Mallefet, 2002b). For each experimental protocol, one preparation (arm or arm piece) was immersed in normal ASW containing 10^{-3} M adrenaline and immediately after stimulated with 200 mM KCl, as a control; in contrast, the other four preparations were first immersed in ASW containing the tested drug for 10 min before addition of adrenaline and KCl stimulation. Measurement of light emission was carried out using a FB12 Berthold luminometer linked to a laptop. Each light response was characterized by its maximal intensity (L_{max} in Megaquanta per second) and expressed as a percentage of the control.

2.4. Drugs

Propranolol is a well-known β-adrenergic antagonist which has been used frequently to investigate the adrenaline pathway in marine animals (Baguet and Marechal, 1978; Christophe and Baguet, 1981, 1985; Anctil et al., 1982; Rees and Baguet, 1990; Fong et al., 1994). Two asymmetric forms (enantiomers) are available, the L(−) form being 100 times more effective in blocking β-receptors than the D(+) form (Tripathi, 1993). Both forms and the racemic mixture (DL) of propranolol (Sigma) were tested in this study. Labetalol (Sigma), another β-adrenergic receptor-blocking agents was also tested (Jaboureck-Bouttier et al., 1999). Adrenaline racemic mixture was obtained from Fluka. All solutions were diluted in ASW. A consistent preselected concentration (10^{-3} M) of adrenaline and a wide concentration range (10^{-10}–10^{-3} M) of propranolol and labetalol were used. Rather high concentrations of drugs, commonly used also in other brittlestars are employed in this work because of the heavy calcification of their arms, which impairs absorption and penetration into the luminous cells (photocytes; Dewael and Mallefet, 2002b).

2.5. Statistical analyses

Each mean value is expressed with its standard error of mean (mean ± S.E.M.); analysis of variance (ANOVA), *t*-test and Dunnet test were used to determine the significant differences between the groups. All methods are designed under the assumption that the data are normally distributed. Shapiro and Wilk's (1965) analysis of variance

was used to check that the data were a random sample from a normal distribution. When data were not normally distributed or when heteroscedasticity occurred, a square-root transformation of data was performed as indicated by Sokal and Rohlf (1995). The shape of the dose–response curve was obtained by the least-square method from the following equation of a growth curve:

$$y = \frac{C}{1 + e^{\left[\frac{x-A}{-B}\right]}}$$

Analyses were performed using SAS/STAT® software's capabilities (SAS Institute, 1990).

3. Results

3.1. Adrenaline-induced luminescence

Adrenaline treatment at a final concentration of 10^{-3} M triggered light emission in 56% and 37% of the tested preparations in *A. squamata* and *A. filiformis*, respectively. In both species, the maximum intensity of light emitted (L_{max}) was significantly lower than the subsequent luminescence due to 200 mM KCl. The average adrenaline induced luminescence represented only 1.21 ± 0.42% and 11.45 ± 4.14% of the responses to KCl in *A. squamata* and *A. filiformis*, respectively. No luminescence was observed in *O. aranea* after adrenaline treatment.

3.2. Propranolol-induced luminescence

None of the three species preparations was affected by low concentrations (10^{-10}–10^{-5} M) of either form of propranolol (Table 1). Treatment with high propranolol concentration (10^{-3} M) of *A. squamata* preparations resulted in light responses whose maximum intensities (L_{max}) varied between 11.1% and 15.4% of the control depending on the propranolol forms used (Table 1). On average, 67% to 83% of the treated preparations produced light. At 10^{-4} M, propranolol resulted in a weaker L_{max} (0.17% to 1.7% of the control) less often, since only 16–42% of the tested preparations produced light. Similar results were obtained for *A. filiformis*: a significant difference in the L_{max} (>36% of the control) occurred in most of the treated preparations (>90%) with all the tested propranolol forms at 10^{-3} M (Table 1). A weak luminescence (L_{max} < 3.5%) was also observed after treatment with propranolol at 10^{-4} M in less than 64% of the tested preparations. In *O. aranea*, although very weak light emissions were detected only with DL-propranolol (Table 1), observed values were not significantly different from zero (p>0.05 with Student's t-test).

3.3. Effect of propranolol on KCl-induced luminescence

At high concentrations (10^{-4} and 10^{-3} M), a strong inhibition of KCl-induced luminescence was observed for all the tested species and all the propranolol forms (D-, L- and DL-; Figs. 1–3), the only exception being observed in *O. aranea* where no significant inhibitory effect was revealed after treatment by DL-propranolol at 10^{-4} M. For the lower concentrations (< 10^{-4} M), differences between species and propranolol forms were observed as follows. (i) In *A. filiformis* and *O. aranea* (Figs. 2 and 3), the treated preparations did not show any significant difference from the controls for the concentration range 10^{-10} M up to 10^{-5} M in the three propranolol forms; moreover, no significant difference was observed between propranolol forms for each concentration (p>0.05). (ii) The situation was more complex in *A. squamata* (Fig. 1) where differences were observed between propranolol forms. The less active propranolol form (D-) did not have any effect since no significant difference was observed between the treated arms and the control for the concentration range 10^{-10} M up to 10^{-5} M. For the two other treatments (L- and DL-propranolol), a significant inhibition appeared at 10^{-5} and 10^{-6} M. Even if dose–response-like relations were obtained after curve fitting in some cases for the three species, the only real dose–response curves (i.e., showing significant effects at various contiguous concentrations 10^{-6} M up to 10^{-3} M)

Table 1
Luminous responses of the brittlestars *Amphipholis squamata*, *Amphiura filiformis* and *Ophiopsila aranea* to D-, L-and DL-propranolol at 10^{-3} and 10^{-4} M (% of responses: % of tested material producing light; L_{max}: maximal light intensity, expressed as % of the control; n = 12 for each treatment and each species)

	D-Propranolol		L-Propranolol		DL-Propranolol	
	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M
Amphipholis squamata						
% of responses	41.7	75	41.7	66.7	16.7	83.3
L_{max} (% of control)	0.24 ± 0.16	15.43 ± 6.86	1.70 ± 0.19	11.13 ± 7.47	0.17 ± 0.12	14.36 ± 5.27
Amphiura filiformis						
% of responses	25	100	25	100	63.6	90.9
L_{max} (% of control)	0.49 ± 0.02	59.83 ± 26.61	0.18 ± 0.14	36.24 ± 13.38	3.54 ± 1.40	78.47 ± 45.47
Ophiopsila aranea						
% of responses	0	0	0	0	8.3	16.7
L_{max} (% of control)	/	/	/	/	0.006	0.15 ± 0.0001

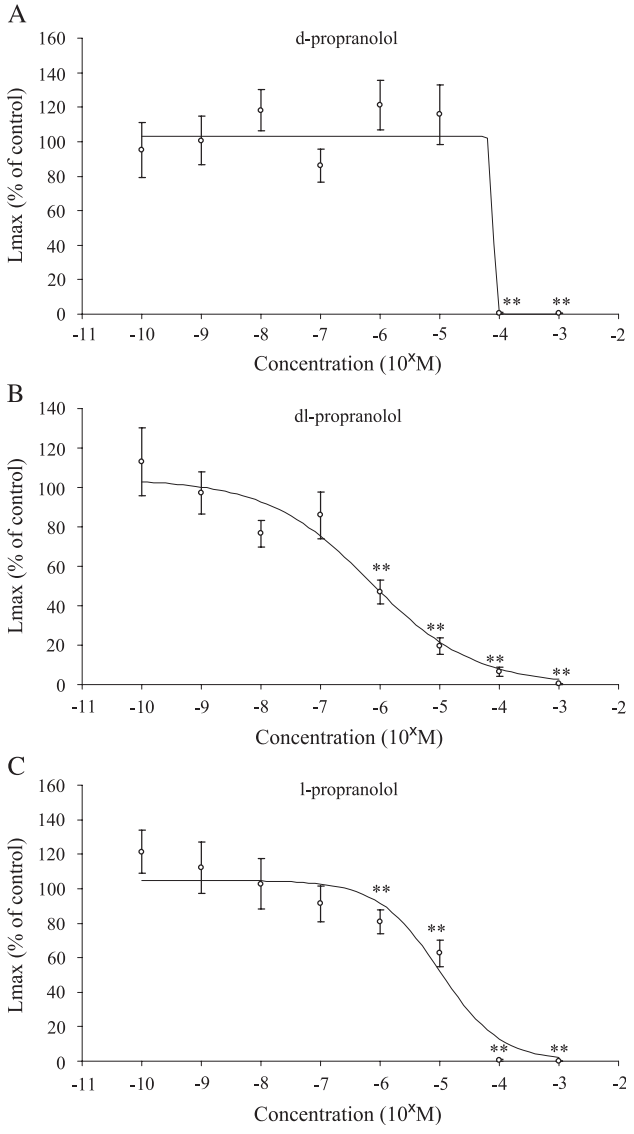

Fig. 1. Relation between different concentrations (10^{-10}–10^{-3} M) of propranolol forms [(A) D-propranolol, (B) DL-propranolol, and (C) L-propranolol] and maximal light intensity (L_{max} in % of control; mean ± S.E.M.) of KCl-induced luminescence in *A. squamata*. **Significant difference between control and treated arms ($p < 0.01$, Dunnet test); $n = 12$ for each treatment.

were obtained in *A. squamata* treated with L- and DL-propranolol.

3.4. Effect of labetalol on KCl-induced luminescence

Labetalol treatment never induced luminescence in all three species at any tested concentrations (10^{-10}–10^{-3} M). An inhibitory effect was observed only at high concentrations (10^{-4} and 10^{-3} M) in *A. squamata*, where a dose–response curve was derived (Fig. 4A). Treatment with labetalol did not exert any inhibitory effect on KCl-induced luminescence of *A. filiformis* and *O. aranea* preparations (Fig. 4B and C).

4. Discussion

Our results obtained with propranolol show two different trends. (i) At high concentrations (10^{-3} and 10^{-4} M) a homogenous inhibitory effect on KCl induced luminescence can be observed for all stereoisomeric forms in all species. (ii) At lower concentrations ($< 10^{-4}$ M), the inhibition of KCl-induced luminescence shows a sigmoidal dose–response curve only for the active (L- and DL-) forms of propranolol in *A. squamata*. A similar dose–response inhibition is also observable for labetalol only in *A. squamata*. All these results suggest that these drugs can induce nonspecific and specific effects.

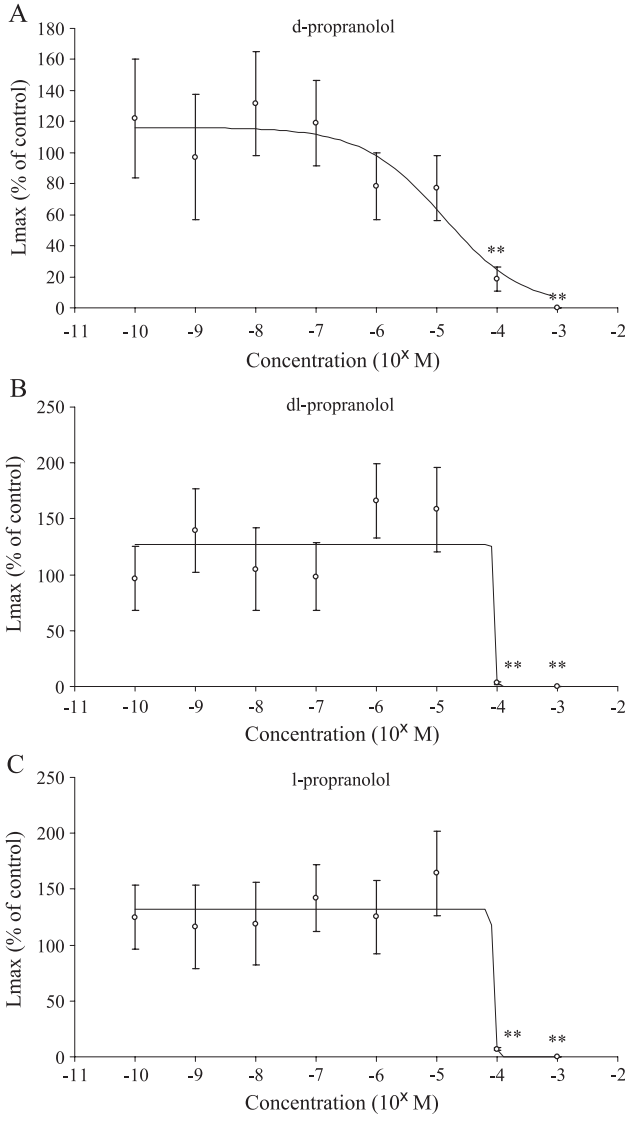

Fig. 2. Relation between different concentrations (10^{-10}–10^{-3} M) of propranolol forms [(A) D-propranolol, (B) DL-propranolol, and (C) L-propranolol] and maximal light intensity (L_{max} in % of control; mean ± S.E.M.) of KCl-induced luminescence in *A. filiformis*. **Significant difference between control and treated arms ($p < 0.01$, Dunnet test); $n = 12$ for each treatment.

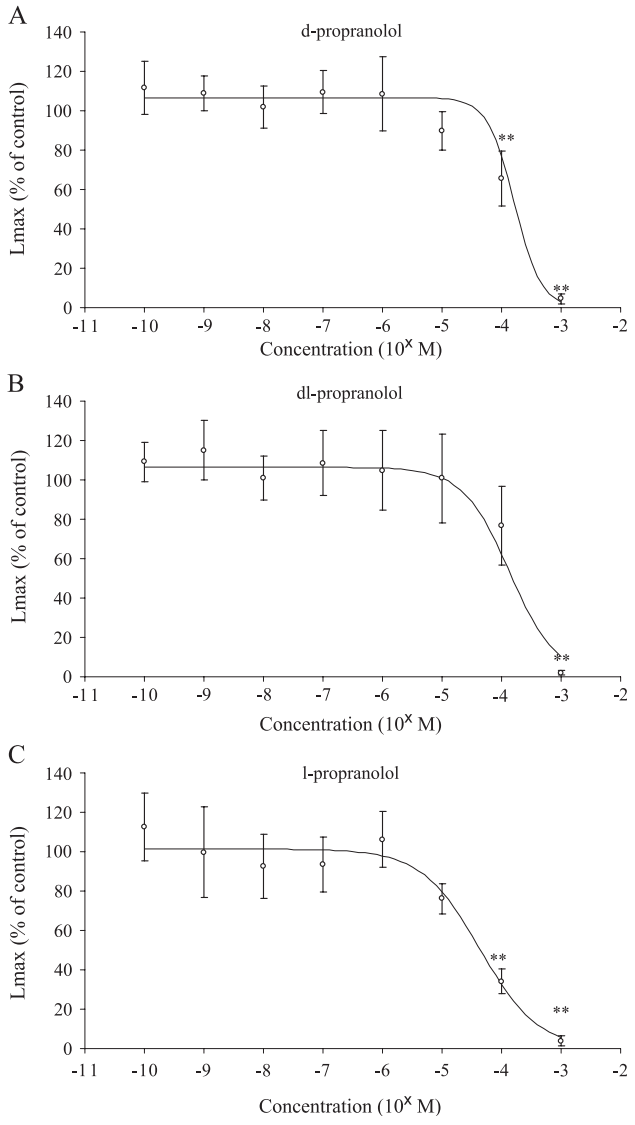

Fig. 3. Relation between different concentrations (10^{-10}–10^{-3} M) of propranolol forms [(A) D-propranolol, (B) DL-propranolol, and (C) L-propranolol] and maximal light intensity (L_{max} in % of control; mean ± S.E.M.) of KCl-induced luminescence in *O. aranea*. **Significant difference between control and treated arms ($p < 0.01$, Dunnet test); $n = 12$ for each treatment.

4.1. Nonspecific effects

Propranolol is often used at high concentrations (>10^{-4} M) to reveal the involvement of β-adrenergic receptors in the nervous control of bioluminescence (Baguet and Marechal, 1978; Anctil et al., 1982; Christophe and Baguet, 1981, 1985; Rees and Baguet, 1990). While some authors interpreted inhibition as having physiological significance (Christophe and Baguet, 1981, 1985), according to others it could result from a nonspecific action of this drug (Anctil et al., 1982; Rees and Baguet, 1990). These conclusions were derived on the basis of three types of results. (i) An unexpected light emission was often observed after propranolol treatment at high concentrations (Baguet and Marechal, 1978; Anctil et al., 1982). (ii) Different stereoisomeric forms of propranolol showed similar effects on luminescence triggered by a stimulating agent (Rees and Baguet, 1990). (iii) No dose–response curve was obtained to characterize the inhibitory effect of propranolol on luminescence (Anctil et al., 1982). Assuming that an antagonist should not trigger any activity in the tissue, the luminescence induced by the treatment with propranolol (β-adrenergic antagonist) might be the result of a nonspecific action of this substance. Moreover, since the L-enantiomer is 100 times more effective in blocking β-receptors than the D-form (Tripathi, 1993), a specific effect should appear more clearly for the active forms. Our results are consistent with the hypothesis of a possible nonspecificity of propranolol, since all forms of propranolol at high concentration (>10^{-4} M)

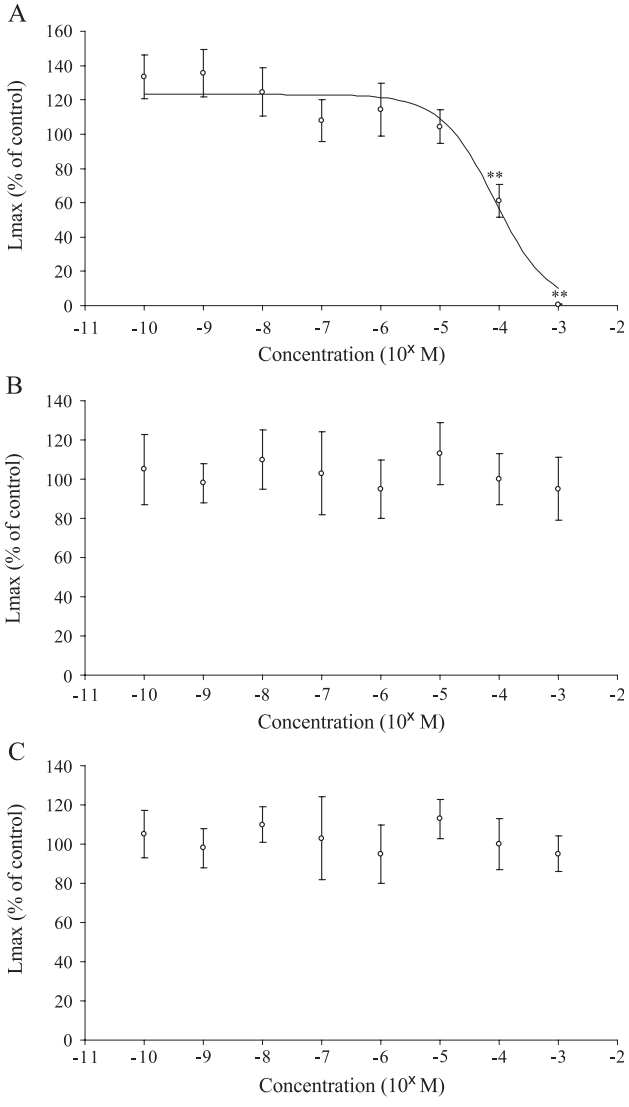

Fig. 4. Relation between different concentrations (10^{-10}–10^{-3} M) of labetalol and maximal light intensity (L_{max} in % of control; mean ± S.E.M.) of KCl-induced luminescence in the brittlestars *A. squamata* (A), *A. filiformis* (B), and *O. aranea* (C). **Significant difference between control and treated arms ($p < 0.01$, Dunnet test); $n = 12$ for each treatment.

frequently induced luminescence in *A. squamata* and *A. filiformis* before showing an inhibitory effect of KCl-induced luminescence.

The nonspecific effects of propranolol observed at high concentrations can be explained on the basis of some of its properties. (i) Interactions of propranolol with phospholipids induce various nonspecific membrane actions (Kelliher and Roberts, 1974). Consequently, high propranolol concentrations inhibit several membrane-associated functions, like enzymatic activity (Godin et al., 1976; Lacko et al., 1979) and ionic conductance (Stagg and Wallace, 1974). (ii) Holmgren and Nilsson (1974) have highlighted that propranolol also blocks other kinds of receptors, for instance, α-adrenoreceptors in the cod celiac. (iii) Finally, it was hypothesized that propranolol could interact with the luminous molecules (luciferin/luciferase system or photoproteins) and inhibit the luminous reaction (Strum, 1969; Anctil, 1979; Rees and Baguet, 1990). Our results do not allow us to determine whether nonspecific effects of propranolol act on the luminous system, on the photocyte membrane or on nerve terminals.

The pharmacological nonspecificity of propranolol is not unique and other drugs, mainly adapted for vertebrates, show similar nonspecific effects on bioluminescence. The inhibitor of phospholipase C, U-73122 used to investigate the IP_3/diacylglycerol (DAG) pathway in mammals (Yule and Williams, 1992) is another example in invertebrate bioluminescence. Application of this drug induces a strong decrease of KCl-induced light emission in *A. filiformis* and *O. aranea*. Nevertheless, the use of an inactive analogue of U-73122 (e.g., U-73344, Smith et al., 1990) and other inhibitors (e.g., GF109203X, Martiny-Baron et al., 1993) prevent a misinterpretation of initial results since they show that the IP_3/DAG pathway is not involved in the nervous control of light emission in these brittlestar species (Vanderlinden et al., 2003).

4.2. Specific effects

Since adrenaline on its own induces light production in *A. squamata* and in *A. filiformis*, the possible role of adrenaline in the luminous control of ophiuroids has been addressed using pharmacological methods. In *A. squamata*, propranolol inhibition of KCl luminescence observed at low concentrations (10^{-6} and 10^{-5} M) can be considered as a specific effect since they are only related to the active forms of the antagonist (L- and DL-). Moreover, sigmoidal dose response curves were obtained for those forms as classically observed for antagonist–receptor interactions. This was further stressed by the labetalol inhibition of KCl light emission since this β-antagonist does not show any nonspecific effects (Jaboureck-Bouttier et al., 1999). In the other ophiuroid species, some dose response curves showed a sigmoidal aspect, but only for the inactive form (D-) in *A. filiformis* and for all forms in *O. aranea*, and this does not seem to be consistent with the β-antagonist specific activity of propranolol. Furthermore, the lack of labetalol inhibition supports the absence of a β-adrenergic mechanism involvement in these same two species. Adrenaline-induced luminescence in *A. filiformis* can not be explained currently.

Control mechanisms of bioluminescence in ophiuroids have been mostly studied in *A. squamata* for 15 years. Pharmacological research contributed to unravel some aspects of the nervous control of photogenesis. Inhibitory (amino acids and catecholamines) and excitatory mediators (neuropeptides S1, S2 and purines) modulate a cholinergic excitatory system. Acetylcholine triggers photogenesis through activation of two muscarinic receptor subtypes located at the photocyte membrane, the first receptor using cAMP as a cofactor, the second receptor showing an activity mediated by IP_3 synthesis via phospholipase C stimulation (De Bremaeker et al., 2000b). Although this model integrates most of the pharmacological characteristics, it still raises some questions. Indeed huge differences (patterns and intensities) are observed between KCl and acetylcholine induced luminescences (De Bremaeker et al., 1996; De Bremaeker, 1999; Mallefet, 1999); moreover, the cAMP pathway is classically coupled to β-adrenergic receptors and not to cholinergic ones (Nicholls, 1995). Recent experiments have suggested that an adrenergic mechanism may contribute to the nervous control of bioluminescence in *A. squamata* (Dupont et al., 1999; Dupont, 2002) suggesting the hypothesis of two control synergetic mechanisms (cholinergic and adrenergic). Our present results support this hypothesis for *A. squamata* luminescence control.

The present study strengthens the idea that luminescence in brittlestars does not share a common nervous control in all species (Dewael and Mallefet, 2002b). Previous results pointed out numerous differences in the control mechanisms of light production in brittlestars, even in closely related species. (i) Different neuromediators and receptors are involved, such as acetylcholine through muscarinic receptors in *A. squamata* (De Bremaeker et al., 1996), acetylcholine through both muscarinic and nicotinic receptors in *A. filiformis* and a still unknown mechanism in *O. aranea* (Dewael and Mallefet, 2002b). (ii) Differences can also be observed in the complex action of neuromodulators (De Bremaeker et al., 1999, 2000a). (iii) The involvement of second messengers differs between species (Vanderlinden, 2002; Vanderlinden et al., 2003).

Our results present important variability leading to unusual observations: (i) in *A. squamata*, the inhibitory effect was more important for DL- than for L-propranolol at 10^{-5} and 10^{-6} M (Fig. 1); (ii), in *O. aranea*, 10^{-4} M DL-propranolol does not lead to a significant inhibitory effect (Fig. 3B). These results stress the need for suitable controls. It has been shown that brittlestar luminous capabilities present high variability (Dupont et al., 2001) which can be split into two levels: (a) between individuals and (b) within individuals. Even if the first source of variability appears to be very important (relevant difference in individual light

capabilities), it is eliminated by working in percentage of the control (each untreated arm or arm piece being considered as 100%) (Mallefet, 1999; Dewael and Mallefet, 2002b). The second level of variability, i.e., within individuals, is more difficult to characterize; moreover, no satisfying statistical tools are available yet to take into account this source of variability, therefore we suggest the intra-individual variability to be responsible of our unusual observations. The specific problem is currently being explored in detail in our laboratory.

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