A Transient Intracellular Coupling Explains the Facilitation of Responses in the Bioluminescent System of Scale Worms

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Abstract. Isolated elytra of polynoid worms emit a flash of bioluminescence when stimulated by an electric shock. With repeated stimulation, hundreds of flashes can be elicited which, in typical series, exhibit large and progressive variations. The amount of luminescence emitted by each flash first increases during a period of facilitation and then decreases exponentially during a longer period of decay.

Through a microscope and image intensifier, the activity of individual microsources or photosomes was observed, using their fluorescence as a natural probe, in that its intensity is a function of the amount of luminescence previously emitted. Sequential observation showed a progressive and basically intracellular recruitment that correlated with facilitation. Facilitation and/or recruitment depended on the frequency of the stimulation. Recruitment proceeded among the photosomes of each photocyte, beginning with those of the cell periphery and progressing to those of the center. When the repetitive stimulation was interrupted and then resumed, the refacilitation was a function of the duration of the pause, and the pathway of recruitment duplicated that of the preceding sequence.

It therefore appears that, within a given cell, individual photosomes can be either coupled and respond to stimulation or uncoupled and quiescent, that the coupled state has a basic lifetime of about 1 s which can be lengthened by reinforcement, and that this state must be established in a matter of milliseconds as a result of the stimulation. In preparing an increased response to a forthcoming stimulation, coupling acts as a short-term memory.

In the study of excitable systems, cells are usually considered to be the units of activity that react in an "all-or-nothing" way, increased responses occurring when more cells are involved. The present study will demonstrate that excitation and recruitment may occur at the level of organelles; it will analyze the stimulation-dependent mechanism by which intracellular units of activity are progressively coupled, coupling being an absolute requirement for these units to react to stimulation. The system investigated is bioluminescent as well as fluorescent. These two natural indicators not only allow photometric recording of the system's activity, but also, under the microscope, reveal its microsources, i.e., the photosomes (I). These paracrystals of endoplasmic reticulum are easy to observe individually in vivo in the photogenic epithelium, a preparation which is practically two dimensional. This opens up the possibility of correlating the overall and the individual activity of the units participating in the luminous events (4, 10). In this cytophysiological sense, the system of the scale worm is undoubtedly the most favorable of all the bioluminescent systems (28).

Scale worms (Annelida, Polychaetes, Aphroditidae, Polynoinae) bear a double row of dorsal scales or elytra, which are parapodal cirri modified as flat, discoidal expansions 2–8 mm in diameter (14, 21, 27). They can be isolated by autotomy or by section of the elytrophore. When stimulated by an electric shock, their basic response is a single flash of green light (19, 23), lasting 50–300 ms. When these shocks are repeated, long series of unitary responses can be elicited (22). These emission series exhibit large and remarkably progressive variations, even though the repeated stimuli are maintained at the same threshold value (10). The intensity of the successive flashes first increases during a period of facilitation and then decreases exponentially during a longer period of decay, until complete exhaustion of the photogenic reserves.

Such typical series of emissions have been recorded from elytra of all the luminous polynoid species so far studied (2–5, 7, 10, 17, 21, 22). Whether they comprise a few score or several hundred responses, they always begin with facilitating responses. Facilitation increases with the frequency of stimulation (2). After a pause in the repetitive stimulation, a new sequence begins again, with facilitating responses. Facilitation is thus clearly stimulation dependent. How and at which step of the excitation–bioluminescence process is it regulated with such amazing progressivity?

Our observation of flashing scales of Acholoe astericola during the period of facilitation provided a first answer (4). At low magnification the zone that illuminates itself during the very first flash of a typical emission is much smaller than
the photogenic area. This zone of activity expands centrifugally, step by step, flash after flash, and finally reaches the boundaries of the photogenic area. Territorial recruitment is thus correlated with facilitation. However, it was not possible with our system of image intensification to perform sequential analysis of this recruitment at a magnification sufficient to distinguish the individual photosomes, because of the relative weakness and shortness of the flashes.

Fortunately, photosomes are also specifically identifiable under UV or deep-blue illumination at 360 or 480 nm by a fluorescence of the same green (520 nm) as the bioluminescence (5, 13, 14). This fluorescence is attributed to oxidized flavin (25, 26). Its overall intensity is a function of the amount of luminescence previously emitted and remains stable in quiescent elytra (3, 5, 7). It allows exceptionally sharp and beautiful observation in vivo and also measures the past activity of individual photosomes.

Taking advantage of this fluorescence, the present study will analyze recruitment by image-intensified microscopy with a better resolution than in luminescence and show that the mechanism that couples individual photosomes is basically intracellular, fast, and labile.

**Materials and Methods**

The experiments were performed on Harmothoe lunulata, a scale worm commensal on other invertebrates, which is common on the shores around Roscoff, in Brittany. Worms were anesthetized by chilling to near freezing point or with 10 M tricaine (MS 222, Merck, Darmstadt, Federal Republic of Germany [FRG]). Elytrophores were cut with fine scissors. Isolated elytra (rinsed several times in seawater when tricaine was used) were individually checked under a fluorescence microscope. Because fluorescence is a permanent record of previous luminescence, only elytra devoid of green fluorescence in the photogenic area were used and referred to as virgin elytra.

Electrical stimulation was usually applied via two tungsten or platinum electrodes inserted into the elytrophoral disk, or by suction electrodes. The stimulation was a 5-ms square pulse delivered by an SD5 stimulator (Grass Instrument Co., Quincy, MA). The voltage was progressively increased until the first luminous response appeared and was then maintained at this threshold value (usually 1-5 V). In standard experiments, the pulse was repeated every 1 or 2 s.

Bioluminescence, conveyed by a light guide, was measured with a photomultiplier (model IP21, RCA, Lancaster, PA) and recorded on a storage oscilloscope (model 5103 ND 15, Tektronix, Inc., Beaverton, OR) or a chart recorder (model 7402, Hewlett Packard Co., Palo Alto, CA). Concomitant or alternate observations of the preparation, as well as measures of fluorescence, were made through an inverted microscope built with parts of

**Results**

Typical emission series were almost always obtained in hundreds of experiments with repeated stimulation. The total number of flashes and relative duration of the periods of facilitation and fatigue differed greatly, depending on the individuals (Fig. 1). Nevertheless, in a given individual, the emissions obtained from successive elytra were usually very similar. The relationship between facilitation and the fre-
Figure 3. Refacilitation. (A) An elytrum was stimulated at 1 Hz and the stimulation interrupted for periods of 2–60 s. The sequences of responses always began by a period of facilitation. (B) The rate of this period of refacilitation, expressed as the ratio of the first flash of a sequence ($I_o$) to the maximal flash ($I_m$) is a function of the duration of the pause.

Figure 4. Conventional fluorescence photomicrographs of elytra of *H. lunulata*, after repeated stimulation at 1 Hz limited to 10 flashes. (A) Part of the photogenic area extending around the disk of insertion of the elytrophore, which is shown on the left. The number of apparent photosomes and their fluorescence intensity decrease from the epicenter to the periphery of the photogenic area. The mosaic pattern of the photosomes outlines the photogenic cells. Bar, 100 µm. (B) The individual photosomes are sharply indentified at higher magnification. The photosomes which outline the cells are called outer ring photosomes and are brighter than those deeper in the cells. Exposure time: 40 s. Bar, 20 µm.

Frequency of the repetitive stimulation could thus be clearly ascertained (Fig. 2). The slope of facilitation was nil or insignificant below 0.05 Hz and maximal at 2 or 3 Hz. Above 3 Hz, up to 15–18 Hz, the frequency limit for unitary responses, summation occurred in addition to facilitation. The relation between refacilitation and the duration of the preceding period of rest was also established in a given elytrum (Fig. 3). This relation was similar whatever the time at which the series of flashes were interrupted, even when the flashes became very weak at the end of the emission, and was thus obviously independent of the level of endogenous photogenic substrate. Temperature affected facilitation. At room temperature (18–20°C), the period of facilitation was shorter and progressed with less regular increments than at 5 or 10°C. Alternate measures of luminescence activity and of the overall fluorescence of the photogenic area confirmed the rigorous relationship between the two phenomena in this species (7). The curves for fluorescence intensity and bioluminescence integration were superimposable. Fluorescence intensity, which was nil in a "virgin" elytrum, rose quickly during the period of facilitating responses, allowing accurate measures during that time, but reached saturation asymptotically long before the end of the emission series. The level of fluo-
Fluorescence pictures of a field of the photogenic area taken through a microscope and image intensifier after every 2 of the 12 first flashes of a typical series emission stimulated at 0.5 Hz. A 20-ms pulse of excitatory UV light allowed imaging fluorescence. The average level of fluorescence increased rapidly as a function of the amount of bioluminescence previously emitted. Nevertheless, the fluorescence enhancement of the individual photosomes was not synchronous. Flash after flash, more and more photosomes ignited. The first modality of recruitment was territorial. The zone of activity extended progressively in a centrifugal direction, towards the limits of the photogenic area, on the lower left of the field. The other modality was intracellular and centripetal. The photosomes of the outer ring of photocytes were first ignited and formed a mosaic pattern of increasing brightness. The inner photosomes appeared later. As their fluorescence increased, they progressively filled up the meshes of the pattern. These processes explain the progressively changing aspects of the photogenic area along its radials in Fig. 4. Bar, 30 μm.

However, fluorescence remained stable when stimulation was interrupted, and measured the past activity even when it happened several hours previously. Nevertheless, an exponential photochemical decay occurred under continuous excitatory illumination. Such decay was of course considerably reduced by short pulses of excitatory light (10–20 ms) and by a dimmer beam.

**Imaging of Recruitment**

There were 30–50 photosomes, each measuring 2–8 μm in diameter, in each photogenic cell of the single-layered photogenic epithelium which constituted the photogenic area on the lower surface of the elytrum (25). The photogenic area extended asymmetrically around the insertion disk of the elytrophore. Photosomes were sharply defined when they were fluorescent and were lying in a single focal plane at the periphery of the photogenic area (Fig. 4). Fluorescence was much easier to record photographically through a microscope and image intensifier than the dimmer and short flashes of luminescence, and good pictures were repeatedly obtained with 10- or 20-ms exposure between each flash of an emission stimulated at 0.5 or 1 Hz. By comparison, conventional microphotographs such as Fig. 4 required exposures at least 1,000 times longer. Figs. 5, 6, and 7 show, frame by frame, the general increase in fluorescence intensity during the period of facilitation. Examination of these sequences reveals that individual photosomes have different time relations in the enhancement of their fluorescence. Two modalities of recruitment appear to combine: territorial extension throughout the photogenic area (Fig. 5), and intracellular propagation within each photocyte (Figs. 6 and 7).

(a) The first modality, which was expected from previous observations of the luminescence of *Acholoe* (4), is the territorial enlargement of the zone of activity. This enlargement was indeed observed in *Harmothoe*, but was much less striking. Here, the zone of activity corresponding to the first flash was already quite large and the territorial extension was completed by large jumps in a few flashes, well before the end of the period of facilitation (Fig. 5).

(b) The intracellular modality of recruitment, which requires a magnification high enough to distinguish the individual photosomes, can be considered as fundamental. The process itself revealed that photosomes are not scattered randomly but are arranged in two concentric rings inside...
Figure 6. (A–I) Similar sequence at higher magnification. The fluorescence pictures (exposure time: 10 ms) were taken between each of the nine first flashes of a typical emission stimulated at 1 Hz. Arrows indicate the photosomes which are newly revealed after each of the five first flashes (A–E), in a cell of the center of the field. They progressively mapped out an outer ring, which was completed in F. However, recruitment did not proceed via neighboring photosomes, as shown by those which were inserted between two others in C or D. The inner photosomes appeared progressively after the sixth flash (F–I). Meanwhile the outer ring photosomes further increased their fluorescence and apparent size. Bar, 25 μm.

Each photocyte. This was particularly clear for the photosomes of the outer ring, which were aligned like beads along the edges of a hexagonal pattern outlining the cell boundaries. However, the cell walls were not distinct and these outer ring photosomes belonged either to one cell or to its neighbor. Inside the outer ring, the photosomes were sometimes linearly arranged along an inner ring surrounding the central nuclear region, and sometimes more randomly dispersed.

In virgin elytra, all the photosomes were equally devoid of green fluorescence but were fortunately identifiable by a faint, brownish fluorescence which allowed them to be focused. The photosomes revealed during the first flashes of an emission always belonged to the outer rings, of course, within the limits of the zone of activity. Recruitment then spread exclusively through the outer ring photosomes. They were apparently triggered in random order in their rows, so that the edges of a mosaic pattern were defined progressively and with increasing brightness. It was only when the outer rings were fully illuminated that recruitment reached the inner photosomes. With the maximal flashes at the peak of the emission, all the photosomes were active, but their fluorescence was of uneven intensity and depended on the order of their recruitment. Finally, at the end of the typical emission, all the photosomes displayed equal maximal fluorescence, and the mosaic pattern was no longer distinguishable in their densely packed population (Figs. 5–7).

This process of intracellular recruitment was combined
with the territorial extension described above. The enlargement of the zone of activity progressed along a network of outer ring photosomes, and did not seem at all affected by the cell boundaries. The combination of the two modalities of recruitment explains all the different aspects previously observed in fluorescence microscopy (5) and the order of their evolution from the epicenter to the periphery of the photogenic area (Fig. 4).

It should be noted that no movement of a photosome was ever observed. Once the photosomes had been recruited and were under sustained repeated stimulation, they flashed repeatedly and their size apparently increased, so that fully fluorescent photosomes were almost coalescent and filled up the photogenic cells except for a central dark spot which constituted the nucleus. This homothetical enlargement of the photosomes was well demonstrated by the technique of image subtraction (Fig. 7).

After a pause that interrupted a series of emissions, a new sequence began again with facilitating responses, as already explained. Observation of recruitment during this period of refacilitation was more difficult than during the initial period since the fluorescence level was already high, with a smaller relative variation. Nevertheless, it was clearly observed that the pathway of recruitment of the new sequence duplicated the preceding one and involved the same photosomes in the same order (not illustrated). Thus, during the pause between two sequences of emissions, the recruitment process was exactly reversed.

Discussion

The modulation of a flash is governed by three factors only. The first is the wave of triggering. As a visible concomitant of the action potential, it spreads over the photogenic area flash after flash, at the same speed and with the same trajectory. It determines the duration and shape of the response. The second factor is the charge of the system in terms of potential photogenic material. This charge decays progressively after each response and does not recover in isolated elytra. The third flash modulation factor is the number of
In the present study, the number of photosomes that can be coupled or uncoupled.

Photosomes in these two states can coexist within the same cell. In response to stimulation, uncoupled photosomes remain quiescent, whereas coupled photosomes flash and acquire the increased fluorescence which distinguishes them. Coupling starts, not from zero, but from the number of precoupled photosomes that react to the very first efficient stimulation, and progressively extends to the total population of photosomes. As a result, the successive flashes display facilitation until the overall decay of the photogenic charge counterbalances the gain of the recruitment. However, each flash except for the first integrates the firing of photosomes with different levels of photogenic charge, because they become active at different times.

**Duration of the Coupled State**

In the resting system, coupled photosomes tend to return to the uncoupled state in that, after the repeated stimulation is interrupted, the new sequence begins with a weaker flash and with a new period of facilitating responses which progressively reach the level attained by the last flash of the preceding sequence. Therefore during such an experiment, a given photosome can be successively coupled, decoupled and re-coupled, as shown by its reactivity or non reactivity to stimulation. The relationship between facilitation and stimulation frequency also points to the conclusion that the coupled state has a limited lifetime. This lifetime is of the order of a second, because below 2 Hz, facilitation decreases significantly.

**Reinforcement of the Coupled State**

We observed that, after a pause, the recruitment that correlated with refacilitation followed the same topographic pathways as during the preceding facilitation. This means that the decoupling which occurs during resting periods is not synchronous for all photosomes but exactly reverses the order in which they were coupled. The reason for this differential stability might be that the photosomes of the outer rings are not only easier to couple than those of the inner rings, but also able to remain more strongly coupled, for the same constitutive topologic reasons. Another possible explanation is that the lifetime of the coupled state is basically the same for all photosomes, but might be lengthened by condensation, extension or duplication of the coupling device under further sustained stimulation.

**Rapidity of Coupling**

The transition from the uncoupled to the coupled state is necessarily very fast. For instance, in a typical emission stimulated at 10 or 15 Hz, facilitation (in addition to summation) is usually achieved in less than 20 flashes, so that each step in the gradual process of recruitment must occur in <100 ms. Such rapidity is amazing. Nevertheless, a clear distinction must be made between coupling and the functional effect which reveals it. Coupling could well be a progressive construction requiring repeated stimulation both for its full establishment and for further reinforcement. Its functional effect, on the contrary, has an all-or-nothing character.

**Propagation of Coupling**

The present observations confirm that recruitment is not random but spreads according to two major modalities: centrifugal territorial extension throughout the photogenic area and centripetal propagation within each photocyte. In addition, an apparent enlargement of the size of individual photosomes was noted. Both the territorial and the intracellular progression initially recruit photosomes of the outer ring, revealing a mosaic network which appears to be superimposed on the cells that it outlines, as if this pathway were the easiest to follow. It is only when this network is completed that recruitment acts on the photosomes of the inner ring. Both modalities might involve the same mechanism and result from the action of a similar coupling-inducing factor able to diffuse within the cells as well as from cell to cell.

The apparent size increase of individual photosomes seen by direct observation and demonstrated by image subtraction cannot be interpreted only as a halo artifact owing to light diffusion that would increase with the brightness of the microsources. It could also indicate the actual establishment of the coupling apparatus.

**Precoupled Photosomes**

The main problem relating to precoupled photosomes, which respond to the first threshold stimulation, concerns their permanence or possible induction by preliminary subthreshold stimulation. It will be difficult to solve as long as luminous activity is the only criterion for recognition. The localization of precoupled photosomes in a limited territory around the epicenter of the photogenic area and inside the luminous cells, in the outer ring of photosomes only, cannot be solely explained by constitutional factors, because precoupled photosomes are fewer when the temperature is lowered. In this connection it should be noted that the initial flash of an emission resulting from the firing of the precoupled photosomes usually differs from the subsequent flashes emitted during facilitation, in that it is not only the weakest, but its delay, ascending slope, and duration are also quite unrelated to the graded variations that start with the second flash.

Similar observations of cellular or subcellular units of activity have been made with image intensification in several bioluminescent systems, such as Noctiluca (12), the hydroid Obelia (20), or the siphonophore Hippopodius (2, 6). In this last example, the illumination of the exumbrellar epithelium, which is both conducting and luminous as in scale worms, began at precise sites and extended territorially with further flashes of increasing brightness. In the dinoflagellate Pyrocystis, the first response to a mechanical shock was surprisingly stronger than the succeeding and facilitating ones and involved all the microsources, so that Widder and Case (29) suggested a "precharging" mechanism. In the scale worm system, we prefer the term "precoupled" to maintain a clear distinction between the photogenic charge and the priming or coupling mechanism.
Determinism of Coupling

The relationship between stimulation frequency and facilitation is the key to the analysis of the mechanism which induces coupling in the scale worm system. Electrophysiological studies have demonstrated that stimulation elicits an action potential which spreads over the entire epithelium of the elytrum and which is Na\(^+\)-Ca\(^{2+}\) in its photogenic part, Ca\(^{2+}\) being essential to trigger the flash response (8, 9, 16). However, these studies completely omitted the process of facilitation. What is more, calcium does not trigger in vitro the luminous reaction of polynoidin, the membrane photoprotein of the photosomes, which reacts specifically to superoxide radicals (25, 26). The luminous reaction in situ is obviously linked to the oxidation of the flavin responsible for the increased fluorescence of the photosomes. The link between these processes and the action potential is not known.

Whatever it is, the possibility should now be considered that some ionic influx of the action potential might also induce coupling. Assuming that the action potentials elicited by constant repeated stimulation are themselves constant, it is clear that acceleration of the stimulation frequency would proportionally increase the ionic fluxes per unit of time. As will be shown elsewhere, both flash amplitude and coupling are enhanced when the external Ca\(^{2+}\) concentration is raised.

To sum up, the notion of coupling in the elytral system covers an intracellular mechanism which builds up and destroys itself at one and the same time and which, functionally, links excitation and bioluminescence at the level of the individual photosomes. As a function of the frequency of stimulation, the level of coupling builds up and reaches a maximum when all the photosomes populating the photogenic area are functional. Thereafter, the decrease of the photogenic charge only acts on the amount of light emitted during a flash, so that the decay of the flash intensity during the second part of a typical emission is exponential (10).

Evolutionary and Functional Implications

The fast process of coupling–uncoupling can be considered as an optional switch-in device, localized at the level of the photosomes along the excitation–bioluminescence pathway. It confirms their individuality by giving them a degree of freedom. It implies a distinction between two parts of the luminous system, which could have been fundamentally independent in their evolution before becoming associated: one is the conducting system of the elytral epithelium, thought to be present in the scales of all polygonid worms, bioluminescent or not (Bilbaut, A., personal communication). The second part consists of the paracrystals of endoplasmic reticulum containing the photoprotein polynoidin (25, 26). It might have appeared later in the evolution, as suggested by the late photosome differentiation during the regeneration of the elytrum (24).

Functionally, the progressive cooperation between the photosomes results from the coupling mechanism. The procedure of recruitment not only increases the magnitude of the response with the number of reacting photosomes, but also apparently lengthens the life-time of the coupled state and thus the readiness to respond to possible further stimulation. Along with precoupled photosomes, which are always ready to respond, these cellular strategies allow economical management of a limited photogenic charge for a maximum of bright responses.

In terms of signal response coding, it is particularly interesting to see that the monotonous repetitive stimulation is translated into responses of increasing intensity because the recruitment is stimulation-dependent, and that the degree of this facilitation depends on the stimulation frequency because the coupled state has a limited lifetime. This simple mechanism leads to a new degree of complexity and integration. Theoretically, it might allow great diversification of the types of response, depending on the number and rhythm of the stimuli. It would indeed be interesting to know which characteristics of the flashing activity are significant enough for a scale worm to trigger a behavioral response which would itself eventually represent a selective advantage. Unfortunately, the behavioral function of bioluminescence in polygonid worms, be it mating or predator avoidance, remains completely hypothetical (11, 14, 17).

A fascinating aspect of the coupling mechanism, which is revealed by the elytral system but might be much more general, lies in its characteristics of memory and anticipation. The coupled state can be considered as a short-term memory. As with every kind of memory, it is revealed as such only by subsequent activity, be it movement, thought, or light emission. It improves the efficiency of the response and at the same time improves itself with repetition, the improvement being expressed here by the visible spatial propagation of the recruitment and by the reinforcement of the coupled state. The notion of anticipation, usually debated in complex neurobiological or psychological systems, is exemplified at a basic subcellular level in the photocytos. Its elementary manifestation of preadaptation rests on the optional coupling of photosomes, a process that prepares a network of cooperation in the event of further excitation. Compared with the cellular mechanism discovered by Kandel and his group (15, 18) in specific neurons of Aplysia, which increase their spike duration after training, the mechanism revealed in the scale worm system appears to be basically different because it occurs in the effector cells themselves, is established faster, and has a much shorter lifetime.

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