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PHEROMONE INTERACTIONS AND IONIC COMMUNICATION IN GAMETES OF AQUATIC FUNGUS *Allomyces macrogynus*

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Abstract—The flagellate male and female gametes of the aquatic fungus *Allomyces macrogynus* are each attracted to a sexual pheromone produced by the opposite gamete type. The sperm attractant, sirenin, causes chemotaxis to female gametes. Examination of sperm chemotaxis shows that the pheromone influences the frequency of directional changes and the duration of a chemotactic run. Physiological experiments using tertiary amine local anesthetics or calcium chelators such as EGTA demonstrate that sirenin stimulates the influx of calcium ions (Ca^{2+}) into the sperm cytoplasm. Radiological experiments with $^{45}CaCl_2$ have demonstrated this calcium flux directly. Structurally, sirenin is an oxygenated sesquiterpene that consists of a cyclopropyl ring attached onto an isohexenyl side chain. The pheromone displays a threshold concentration for attraction at 10 pM in chemotaxis bioassays. Structure-activity relationships with racemic sirenin and sirenin analogs indicate that biological activity requires a terminal hydroxymethyl group on the side chain. In addition, a hydrophobic group must be present at the other end of the sirenin molecule. Besides sirenin, the sperm cells of *A. macrogynus* produce a female attractant, parisin. While the molecular nature of this attractant is not completely resolved, some general features of the molecule suggest it may be similar structurally to sirenin.

Key Words—*Allomyces macrogynus*, sex pheromones, sesquiterpene, sirenin, parisin, analogs, calcium ions, chemotaxis, fungus.

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INTRODUCTION

Overview of the Organism. Chemical communication is an important process in both prokaryotic and unicellular eukaryotic organisms (Taylor and Panasenko, 1984). In the fungi and algae, chemical communication between motile gametes can involve sexual pheromones that are produced to attract mating partners. Although several algal pheromones in the division Phaeophyta have been described (see Maier and Müller, 1986), few sexual pheromones have been discovered in the aquatic fungi (division Chytridiomycota). This paper discusses the sexual pheromones of the aquatic fungus *Allomyces macrogynus* and describes what is known about the chemical composition of these pheromones. For the sperm-attractant sirenin, its possible mode of action will be discussed.

The genus *Allomyces* (class Chytridiomycetes) is a subtropical water mold that grows as a saprophyte on decaying plant and animal matter (Emerson, 1941). In the sexual phase of *A. macrogynus*, multinucleate male and female reproductive structures, called gametangia, are formed at the tips of the growing vegetative hyphae. The two types of gametangia are easily discerned with a dissection or compound microscope. The male gametangia are terminal, orange, and smaller than the subterminal, colorless, female gametangia. Under conditions of nutrient starvation, each female and male gametangium undergoes cytoplasmic cleavage (Sewall and Pommerville, 1987) and differentiates approximately 50 female gametes and 100 sperm. Both types of gametes are uninucleate and swim using a posterior whiplash flagellum (Pommerville and Fuller, 1976). These motile cells also are easily identified with a compound microscope. The sperm cells are orange, highly motile, and about half the size (5 μm) of the colorless, sluggishly motile female gametes. Cell communication is exhibited only during this motile stage in the life cycle. Communication is important for efficient gamete mating and zygote formation (Pommerville, 1982; Pommerville and Fuller, 1976).

Overview of Chemotaxis. The sperm and female gametes of *A. macrogynus* each generate a specific pheromone to attract the opposite gamete type (Pommerville and Olson, 1987a). The effects of the female attractant, sirenin, on the sperm have been intensively studied, and the findings show that the sperm require a sensory system coupled to transduction processes (Carlile and Machlis, 1965a,b; Machlis, 1973; Pommerville, 1977, 1981).

Based on quantitative chemotaxis assays, Machlis (1973) suggested that sperm attraction required a pheromone gradient. However, the nature of the attraction process become clearer when sperm motility was studied by dark-field microscopy (Pommerville, 1978). In brief, sperm motility without sirenin is characterized by short, bending runs. Each run is interrupted by a brief period (<1 sec) when the sperm cell temporarily stops swimming and the cell body

pitches an average angular distance of 60°. Another run then occurs but in the direction determined by the pitch of the cell body (Pommerville, 1978).

Sperm undergoing chemotaxis in a gradient of sirenin (toward female gametes) exhibit longer runs and fewer pitches of the cell body. Furthermore, the addition of sperm to a uniform distribution of attractant results in continuous pitches of the cell body. These findings demonstrate that sirenin influences the frequency of directional changes (chemoklinokinesis) and duration of runs.

In the majority of eukaryotic microbes that exhibit chemotactic or phototactic behavior, the cells can undergo at least a temporary reversal of flagellar beat (backwards swimming). This usually results in negative chemotaxis when sensing potentially harmful compounds. The reversal of flagellar beat is due to the influx of Ca^{2+} (Holwill, 1980; Gibbons, 1982). With *Allomyces*, experimental analysis has shown that sirenin stimulates the influx of calcium ions ($[Ca^{2+}]$) into the sperm cytoplasm (Pommerville, 1981). This finding indicates that pitches of the cell body are due to increased levels of cytosolic Ca^{2+}. Removal of extracellular Ca^{2+} with EGTA results in circular swimming even when sirenin is present (Pommerville, 1981). Thus, the equivalent of flagellar reversal in sperm of *Allomyces* is pitching of the cell body. Once excess Ca^{2+} is sequestered or pumped out of the cell, another run can occur. Thus, the motile cells of *A. macrogynus* can undergo negative chemotaxis (Pommerville and Olson, 1987b), but this requires the cells to undergo several pitches of the cell body to turn around.

When sperm are placed in a solution containing tertiary amine local anesthetics (procaine or tetracaine), the sperm swim in circles without pitches of the cell body (Pommerville, 1981). The addition of sirenin to such a solution of sperm does not change the behavior of the cells. Removal of the anesthetic leads to recovery of normal motility and gamete attraction. These results can be understood based on known actions of procaine and tetracaine. These compounds displace Ca^{2+} from membranes (Low et al., 1979), interfere with normal membrane electrical properties, and act as antagonists to the normal functioning of calcium-binding proteins, such as calmodulin (Volpi et al., 1981). In summary, physiological and pharmacological experiments with chelators and anesthetics suggest indirectly that Ca^{2+} is important to the regulation of sperm chemotaxis in *A. macrogynus*.

Experiments with $^{45}CaCl_2$ have been used to demonstrate directly the influx of Ca^{2+} in *A. macrogynus*. The addition of sperm to a $^{45}CaCl_2$ solution results in the binding of $^{45}Ca^{2+}$ to the exterior of the plasma membrane (Figure 1). After washing these cells in distilled water or initially using lower concentrations of $^{45}CaCl_2$, the levels of radioactivity are reduced (Figure 1b). However, when sirenin is added to a suspension of sperm in a $^{45}CaCl_2$ solution, there is a 2.5-fold increase in radioactivity above that detected before sirenin was added (Figure 1a). The simultaneous addition of sirenin and $^{45}CaCl_2$ to a suspension

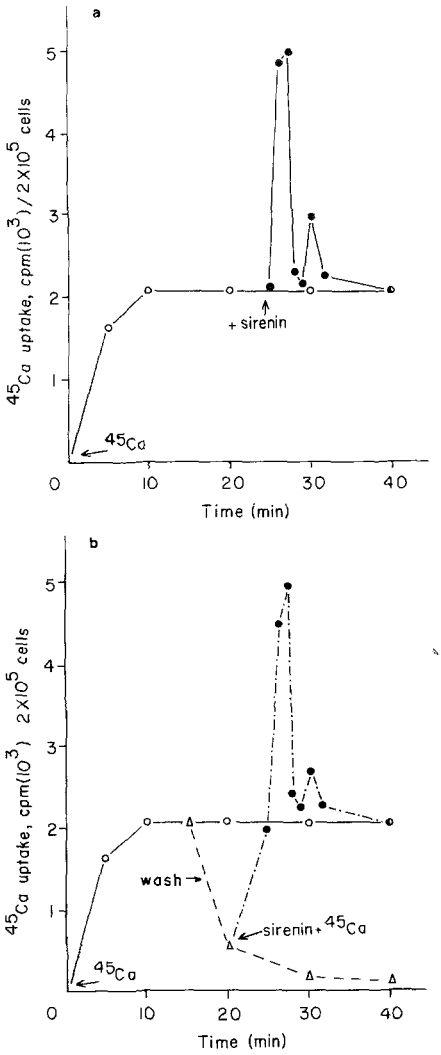

FIG. 1. Measurement of ^{45}Ca activity with and without sirenin addition. (a) Addition of sirenin to sperm incubated in ^{45}Ca resulted in a 2.5-fold increase in cell radioactivity. For these experiments, sperm in Eppendorf tubes were mixed with $^{45}CaCl_2$ in distilled water (○ —— ○) to give 2×10^5 sperm/0.4 ml (5 μM $CaCl_2$). The tubes were incubated for 24 min, at which time sirenin (● —— ●) or distilled water (○ —— ○) was added. At 1-min intervals, the solution from one tube was removed and layered over 12.5% sucrose, centrifuged for 30 sec, and the tube frozen. After a complete set of experiments, the tips of the tubes were cut off and the cell pellets placed in scintillation fluid. (b) Simultaneous addition of sirenin and ^{45}Ca to sperm that had been incubated in ^{45}Ca (○ —— ○) and washed with distilled water (△ -- △) before sirenin/^{45}Ca addition (● - · - ●). Note that addition of sirenin and $^{45}CaCl_2$ to washed cells still stimulated the same level of ^{45}Ca uptake. Radioactivity was determined as described in (a).

of washed gametes also results in a rapid influx of $^{45}Ca^{2+}$ (Figure 1b). When zoospores are substituted for sperm in these experiments, influx of $^{45}Ca^{2+}$ above membrane-bound levels is not detected.

SIRENIN, THE SPERM ATTRACTANT

Machlis et al. (1966) examined the molecular nature of sirenin by producing large quantities of the attractant from pure female strains of *Allomyces*. This provided sufficient material to isolate and purify the pheromone (Machlis et al., 1966), and to determine the structure of sirenin (Machlis et al., 1966, 1968; Nutting et al., 1968).

Sirenin is a sesquiterpenediol (Figure 2) of low molecular weight (236 daltons). Following the synthesis of *dl*-sirenin, a few isomers and analogs were generated (Bhalerao et al., 1970). When these synthetic products were tested for their ability to attract sperm cells (Machlis, 1973), only the synthetic *l*-enantiomer of sirenin was found to be active (Figure 2). The ability to exclude inappropriate synthetic sirenin analogs (the *d*-enantiomer and the *exo*-methyl diastereomer; Figure 2) was shown by their inability to attract sperm in chemotaxis bioassays (Machlis, 1973). When *d*-sirenin was mixed with *l*-sirenin, there was no decline in or interference with chemotaxis. These findings provided the first indirect evidence for pheromone receptors in *Allomyces*.

To understand receptor recognition further, we have synthesized several

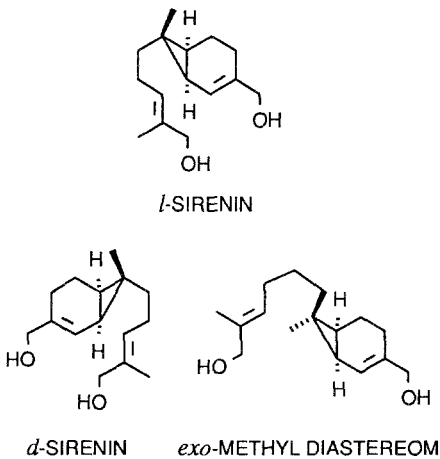

FIG. 2. Structure of *l*-sirenin and two of the analogs tested by Machlis (1973). Both the *d*-enantiomer and the *exo*-methyl diastereomer were inactive in attracting sperm in a chemotaxis bioassay.

active sirenin analogs and tested them for their ability to attract sperm in a bioassay (Pommerville, 1987; Pommerville et al., 1988). The most potent of these compounds is a monohydroxy analog (Figure 3A), which exhibits identical biological activity to the natural pheromone. Sperm respond to the monohydroxy analog and to racemic sirenin with threshold concentrations (i.e., the minimal concentration needed to stimulate male chemotaxis in a bioassay) of 10 pM. The other analogs we synthesized were either inactive or exhibited much higher threshold concentrations (10 μM or higher). However, sperm attraction at these higher analog concentrations still varied with their structural resemblance to sirenin (see Pommerville et al., 1988). Indeed, the benzyl ether of racemic sirenin (Figure 3B), which has a threshold activity of 10 μM, again supports the need for receptor recognition.

Important structure–activity relationships can be discerned by comparing the relatively inactive compounds (Figure 3B–E) with the monohydroxy analog (Figure 3A) and *l*-sirenin (Figure 2). First, the hydroxymethyl group attached on the bicyclic ring of sirenin is not necessary for activity (compare Figure 2 and Figure 3). The monohydroxy analog was as potent as sirenin. Second, replacement of the hydroxymethyl group on the ring with a large hydrophobic group (benzyl ether) reduces activity substantially (Figure 3). Thus, chemotactic activity in the 10 pM range requires a structure having a terminal hydroxy-

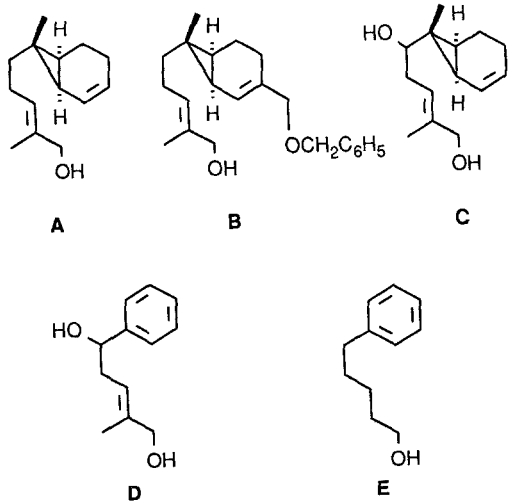

FIG. 3. Structures for several active analogs of sirenin. The monohydroxy analog (A) had a threshold concentration at 10 pM, identical to racemic sirenin. Analogs B–E all had threshold concentrations at 10 μM. See text for details. (Adapted from Pommerville et al., 1988.)

methyl group on the side chain and a large hydrophobic group at the other end of the molecule. Any structural change that leads to improper fit at the receptor's recognition site requires an attractant concentration of at least 10 μM for a chemotactic response—if indeed any response occurs (Pommerville et al., 1988).

Sirenin must bind in a conformation that distinguishes it from the *d*-enantiomer and other analogs. We postulate that when *l*-sirenin is bound, the hydroxymethyl group on the ring is located in a nonspecific hydrophilic region (Figure 4A). Thus, the monohydroxy analog is a potent attractant. *d*-Sirenin lacks activity because its hydroxymethyl group intrudes into a region of the receptor that must be occupied by the hydrophobic ring of *l*-sirenin (Figure 4b).

PARISIN, THE FEMALE ATTRACTANT

When sperm and female gametes are physically separated from one another in a bioassay, the sperm cells attract female gametes (Pommerville, 1977). With the generation of pure male and female strains of *A. macrogynus* (Olson, 1984), parisin can be isolated. A series of general chemical tests have been completed on parisin (Pommerville and Olson, 1987a).

In chemotaxis bioassays, sperm cells or a supernatant solution from these cells stimulated attraction of female gametes. Sperm cells and supernatant were without effect using zoospores in the bioassay (Pommerville and Olson, 1987a). The stability of parisin was examined using chemical tests originally designed to study the stability of sirenin (see Machlis, 1958, for methods). The activity of parisin is maintained after freezing, boiling for 10 min, or autoclaving for 20 min (Pommerville and Olson, 1987a). However, the product of hot acid or alkaline conditions had no chemotactic activity.

Machlis (1958) demonstrated that sirenin could be extracted with ether. Following this protocol, parisin activity also remained associated with the ether fraction (Pommerville and Olson, 1987a). Thus, in these preliminary tests, the pheromone behaves similarly to sirenin.

PERSPECTIVES

The previous research activities point to the next stage in understanding the role of sirenin during chemotaxis in *Allomyces*. It is now possible to determine if pheromone receptors are present on the plasma membrane or in the cytoplasm of the sperm cells. In addition, we can identify the pathway(s) responsible for the influx of $[Ca^{2+}]$ into the sperm cytoplasm.

The studies discussed above demonstrate the ability to synthesize sirenin and a variety of active sirenin analogs. Therefore, we now can measure the

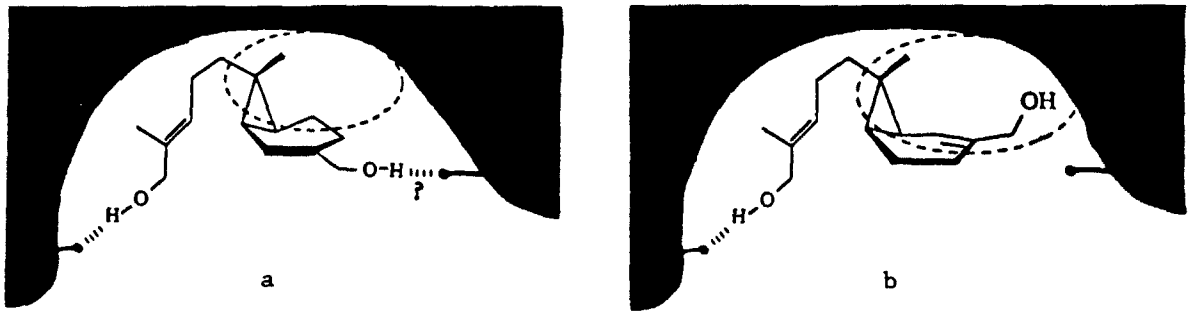

FIG. 4. A hypothetical model for the binding of *l*-sirenin (a) and the absence of binding for *d*-sirenin (b) with a presumptive sperm chemoreceptor. The *l*-enantiomer (a) would bind to the receptor by one or two hydrogen bonds (dashed lines) at the primary alcohol and the ring hydroxymethyl group, respectively. The bicyclic ring lies in a hydrophobic binding site of the receptor (dashed circle). *d*-Sirenin (b) would not bind due to inappropriate stereochemistry of the hydroxymethyl group.

binding of tritiated pheromone ([^{3}H]sirenin) or analogs to potentially small numbers of receptors. By using these radioligands, we can distinguish between actual receptors (specific binding sites) and nonspecific binding sites. In addition, since we have a better idea of the structure–activity relationships for sirenin, photoaffinity labeling can be carried out. Sirenin can be prepared with both a radiolabel and a photosensitive functional group to link covalently pheromone and receptor.

[^{3}H]Sirenin can be used to determine if the attractant enters the sperm cytoplasm. Uptake could involve the intact molecule or a labeled fragment. Absence of cytoplasmic radioactivity would suggest that stimulation of chemotaxis by sirenin requires Ca^{2+} as a second messenger system at the cell surface.

One process resulting from the binding of pheromones, hormones, or neurotransmitters to cell-surface receptors is the transduction of these signals into a change in cellular function or behavior. Ca^{2+} very often acts as a second messenger to transduce these signals (Rasmussen et al., 1985; Carafoli, 1987). How sirenin generates a transient rise in cytoplasmic Ca^{2+} needs to be determined. Because Ca^{2+} most often crosses the plasma membrane through channels, the existence of such channels on the sperm cell surface needs to be examined. Ligand binding to receptors can trigger potential-dependent channels that are responsible for the slow Ca^{2+} current or receptor-operated (mechanical) Ca^{2+} channels (Godfraind, 1985). Calcium and calmodulin antagonists can be used to identify the presence and types of calcium channels in *Allomyces*. Similar procedures have been used with other eukaryotic microbes, including the green alga *Chlamydomonas* and the protozoan *Paramecium* (Nultsch et al., 1986; Ehrlich et al., 1988), for the examination of calcium channels.

Finally, our findings point out some similarities between parisin and sirenin. Studies are in progress to determine the complete structure of parisin and to examine female gamete behavior in the presence of this attractant.

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