

Lidocaine Reversibly Inhibits Fertilization in *Chlamydomonas*: A Possible Role for Calcium in Sexual Signalling

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ABSTRACT A flagellar adhesion-induced signal sent during the mating reaction of the biflagellate alga, *Chlamydomonas reinhardtii*, initiates release of cell-wall-degrading enzymes, activation of mating structures, and cell fusion. The nature of this signal is unknown, but it may be mediated by an adhesion-induced change (activation) of flagellar tips. The studies reported here show that lidocaine, a local anesthetic that is reported to interfere with the movement of divalent cations across cell membranes, reversibly blocks cell wall loss and gametic fusion without blocking adhesion or flagellar tip activation. In these experiments lidocaine inhibited both the initial rates and the extent of wall loss and zygote formation. Studies with gametes of a paralyzed flagellar mutant, pf 17, revealed that lidocaine also blocked flagellar surface motility (visualized as movement of polystyrene beads) at concentrations of the inhibitor which also prevented gametic fusion. The concentration of lidocaine required to block cell fusion was dependent on the concentration of calcium or magnesium in the medium. In the absence of added calcium, 0.5 mM lidocaine inhibited fusion by 70%. In 0.5 mM calcium, 0.5 mM lidocaine had no effect on fusion and 2 mM lidocaine was required for 90% inhibition. The results suggest that divalent cations may play a critical role in sexual signalling in *Chlamydomonas*.

Fertilization in the biflagellate alga *Chlamydomonas* is a highly complex process beginning with a cell surface recognition and adhesion event and ending with gametic fusion and zygote formation. In *Chlamydomonas* several intervening steps between adhesion and fusion have been identified (see references 9 and 10 for reviews). When first mixed together, gametes of opposite mating type adhere to each other via their flagella. The points of adhesion, which are initially randomly located along the length of the flagella, become localized at the flagellar tips. Although the mechanism for the realignment of flagellar adhesions is poorly understood, it may involve the same surface motile system (flagellar surface motility) that has been shown to permit paralyzed flagellar mutant cells to glide over their substratum (15) or to rapidly move ($2 \mu\text{m/s}$) latex microspheres up and down the surface of the flagellum (2, 3). Concomitant with the localization of adhesion points at flagellar tips, the tips undergo a change from a tapered to a bulbous morphology (16). At this point in the process a signal sent to the cell bodies initiates release of cell-wall-degrading enzymes (lysin) and activation of mating structures. In the case of the mating type

(mt^+) gametes, an acrosomelike mating structure containing actin filaments is formed (6, 8). The activated mating structures of cells of both mating types fuse and the cells merge to become quadriflagellated zygotes.

Although the sexual signalling event is an especially interesting feature of the mating process, little is known about how the signal is generated, transmitted, or received. Colchicine and vinblastine are reported to block cell wall loss, mating structure activation, and cell fusion without blocking adhesion. These agents, however, also prevented localization of adhesion points at the flagellar tips as well as flagellar tip activation, and it was concluded that the signal was not being generated (11).

In the present investigation we report on experiments with the local anesthetic, lidocaine, which we have found reversibly blocks cell wall release and cell fusion without affecting flagellar adhesiveness, cell aggregation, or flagellar tip activation. Experiments with calcium and magnesium indicate that these ions are able to modulate the effect of lidocaine and suggest that divalent cations may play a critical role in sexual signalling in *Chlamydomonas*.

MATERIALS AND METHODS

Cultures

Stock cultures of *C. reinhardtii* strains 21gr, (mt⁺), 6145c (mt⁻) (originally obtained from Dr. Ruth Sager, Sydney Farber Cancer Center, Boston, MA) and a paralyzed flagellar mutant, pf 17 (mt⁻), (obtained from the *Chlamydomonas* Culture Collection, Duke University, VA) were grown axenically on 1.5% agar slants supplemented with 2 g/liter of sodium acetate and 4 g/liter of yeast extract in medium I of Sager and Granick (17) at 12°C in light as previously described (21). Axenic liquid cultures were inoculated from agar slants and grown in 250-ml Erlenmeyer flasks in 150-ml quantities of medium I at 25°C with continuous aeration on a cycle of 12 h of light and 12 h of dark.

Materials

Lidocaine, octyl glucoside (OG), HEPES, EDTA, dithiothreitol (DTT), and Tris(hydroxymethyl)aminomethane (Tris) were all from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade.

Induction of Gametes

To obtain gametes, vegetative cells were grown to a density of $2-4 \times 10^6$ cells/ml. After 6-10 h in the light, the cells were washed into and incubated in nitrogen-free (N-free) medium in continuous light with aeration at 25°C as previously described (21).

Assays

In the assays described below mt⁻ gametes (wild type or pf 17) were mixed with mt⁺ gametes (each at 1.6×10^7 cells/ml) at 13°C or 23°C. The cells were in N-free medium or in N-free medium containing 10 mM HEPES, pH 7.4, with FeCl₃, sodium citrate, and phosphate buffer omitted (HTM). The mixtures were gently swirled every 1-3 min to keep the cells in suspension, and at predetermined times, samples were withdrawn for the measurements described below.

AGGREGATION: A Coulter model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, FL) fitted with an electrode with a 100- μ m bore aperture was used to determine cell concentration and to assay cell aggregation (as loss of single cells) as previously described (26, 27).

ZYGOTE FORMATION: Zygote formation was measured by use of a microscopic assay. Samples from a suspension of mt⁻ and mt⁺ gametes were fixed in 0.5% glutaraldehyde and the number of quadriflagellated cells (QFCs) was determined. For each time point, 200-500 cells were counted. To obtain the percent of cells forming zygotes the following equation was used:

$$\% \text{Cells forming zygotes} = 100 \times \frac{2 \times \text{QFCs}}{\text{Total cells counted} + \text{QFCs}}$$

FLAGELLAR TIP ACTIVATION: To observe alterations in flagellar tip morphology, aggregating cells were added to an equal volume of 1% glutaraldehyde in N-free medium, incubated for at least 15 min, washed twice with 10 mM Tris, pH 7.8 by centrifuging for 10 s in a Beckman microfuge B (Beckman Instruments, Inc., Fullerton, CA), and then resuspended in 40 mM OG in 10 mM Tris, 0.1 mM EGTA and 0.1 mM DTT, pH 7.4. The OG removed the membranes and made activated tips easily distinguished from nonactivated tips (16). After 10 min at room temperature the samples were washed into N-free medium, negatively stained with 1% uranyl acetate and viewed with a Philips 300 electron microscope. Flagella (200 per time point) were scored as having bulbous (activated) or nonbulbous (nonactivated) tips as previously described (16, 22).

CELL WALL RELEASE: To assay for wall loss 0.6-ml portions of a cell suspension were mixed with 1.0 ml of ice cold 0.075% Triton X-100 in 5 mM EDTA, pH 7.8. Cells that had released their walls were soluble in 0.075% Triton X-100, whereas cells with walls were resistant to being solubilized by this treatment (23, 25). This mixture in 1.6-ml polypropylene conical centrifuge tubes was vortexed for 5-10 s at the maximum setting on a vortex mixer (Model S8220, Scientific Products, Inc., State College, PA) and then centrifuged in a Beckman microfuge B (Beckman Instruments, Inc.) for 30 s at room temperature. The optical density (OD) at 435 nm of the supernatant was measured in a Gilford model 240 spectrophotometer (23). Information on wall release was presented either directly as AOD_{435} or as % of the nonlidocaine treated sample.

FLAGELLAR SURFACE MOTILITY: Flagellar surface motility was quantitated according to the method of Bloodgood (2) by incubating pf 17 mt⁻ gametes with 0.35- μ m diameter polystyrene monodisperse microspheres (Polyscience, Inc. Warrington, PA) and microscopically determining the percentage of the attached microspheres that were in motion at the time of observation. In agreement with Bloodgood (2), we found that ~60% of the attached beads in control samples were in motion at the time of observation. At least 200-300 attached beads were scored for each determination.

RESULTS

Lidocaine Effects on Aggregation, Cell Wall Release, and Zygote Formation

Initial observations by phase contrast microscopy revealed that aggregation of mt⁻ and mt⁺ gametes in 3-4 mM lidocaine was indistinguishable from normal aggregation by at least three criteria: the aggregates were not easily disrupted by pipetting; the cells exhibited what appeared to be normal tip-to-tip flagellar adhesions; and glutaraldehyde fixation of aggregating gametes did not disrupt the aggregates (7, 10). On the other hand, it appeared that the gametes did not release their cell walls nor did they fuse. These initial microscopic observations were confirmed by quantitative measurements. Gametes were pretreated for 5 min with various amounts of lidocaine, mixed in the continued presence of the reagent, and the extent of aggregation (which is a measure of cell adhesion) and wall loss, and the percent of cells forming zygotes, were determined. The results shown in Fig. 1 indicated that adhesion, as measured by the Coulter counter assay, was unaffected at 4 mM and was only slightly affected at 8 mM lidocaine. The inhibition at 8 mM was associated with changes in flagellar morphology to a spherical shape giving the appearance of "Mickey Mouse ears." There was also some deflagellation at these high concentrations.

Quantitative measurements of wall loss and zygote formation, however, showed that these two postsignalling events were almost completely inhibited by 4 mM lidocaine (Fig. 1). Moreover, the effect of lidocaine on wall loss was reversible. If the cells were washed out of 4 mM lidocaine into fresh medium, within 10-20 min they recovered the ability to release their walls (Fig. 2). Control experiments (not shown) indicated that the blockage of wall loss was due to an inhibition of release or secretion of the cell wall degrading enzymes (lysin). No lysin activity could be detected in supernatants of gametes aggregating in lidocaine, and when gametes incubating in lidocaine were treated with exogenously added lysin, the cells released their walls but were still unable to fuse.

To determine if the lidocaine inhibition of zygote formation was reversible, mt⁻ and mt⁺ gametes were preincubated in 3 mM lidocaine for 5 min at 13°C and the gametes were mixed together. Fig. 3 shows that 3 mM lidocaine almost completely

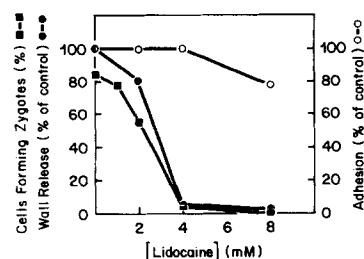


FIGURE 1 Effect of lidocaine on adhesion, cell wall loss, and zygote formation. Mt⁻ and mt⁺ gametes (2 ml each, 1.6×10^7 cells per ml in N-free medium; HTM was used for the adhesion experiments but N-free medium gave similar results) were separately pretreated for 5 min at room temperature in the indicated concentrations of lidocaine and then mixed together in the continued presence of the inhibitor. Adhesion was measured with the Coulter counter method after the cells had been mixed together for 3-5 min (open circles). In the control (0 mM lidocaine), 85% of the cells aggregated. The extent of wall loss (closed circles) and zygote formation (closed squares) were determined after the cells had been mixed together for 10 min.

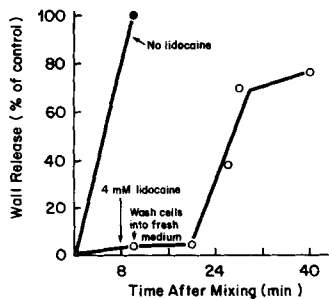


FIGURE 2 Reversibility of lidocaine inhibition of cell wall release. Mt^- and mt^+ gametes (2 ml each, 1.6×10^7 cell per ml in N-free medium) at room temperature were preincubated with (open circles) or without (closed circles) 4 mM lidocaine for 5 min and then mixed together. After 10 min 2-ml portions of the cells in lidocaine were diluted with N-free medium, washed twice by centrifugation into fresh nonlidocaine-containing medium, and at the indicated times wall release was measured.

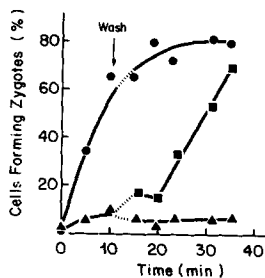


FIGURE 3 Reversibility of lidocaine inhibition of zygote formation. Mt^- and mt^+ gametes (8 ml each, 1.6×10^7 cells per ml in N-free medium) at 13°C were preincubated for 5 min with (triangles) or without (circles) 3 mM lidocaine and then mixed together and assayed for zygote formation at the indicated times. At 10.2 min after mixing, 2-ml portions of the lidocaine-treated cells were diluted with N-free medium, washed twice by centrifugation, and resuspended either in fresh lidocaine-containing medium (triangles) or lidocaine-free medium (squares). The control, nonlidocaine-treated cells were also washed into fresh medium. The stippled portion of the lines indicates the time during which the suspensions were being washed by centrifugation.

inhibited zygote formation; but, when the lidocaine-treated cells were washed out of lidocaine into fresh medium, they began to form zygotes within 15 min after the wash. Once zygote formation began, it proceeded at nearly the same rate and to nearly the same extent as did the samples without lidocaine. If gametes were washed into lidocaine-containing medium instead of lidocaine-free medium, there was no increase in zygote formation and washing the non-lidocaine-treated gametes into fresh medium had no effect on their ability to form zygotes.

Since fertilization occurred so rapidly at room temperature it was difficult to obtain information on initial rates. At 13°C , however, the processes were slowed sufficiently to reveal that the initial rates of wall loss and zygote formation were also inhibited by lidocaine (Fig. 4). As with the experiments at room temperature, aggregation was unaffected by these concentrations of the inhibitor, and the extent of deflagellation was $<5-10\%$ at all the concentrations of lidocaine tested (data not shown).

Flagellar Surface Motility

Since lidocaine blocked cell wall loss and zygote formation without affecting the ability of cells to adhere to one another,

the adhesion-induced signalling mechanism that leads to wall loss must have been sensitive to lidocaine. To learn more about the effect of lidocaine on adhesion-induced flagellar surface events thought to be involved in signalling (9, 10) the effect of the local anesthetic on flagellar surface motility was investigated. Because the normal swimming motion prevents studies on flagellar surface motility in wild type cells, a paralyzed flagella mutant, pf 17, was used. To do these studies pf 17 mt^- gametes were pretreated for 5 min in various concentrations of lidocaine, mixed with $0.35\text{-}\mu\text{m}$ polystyrene microspheres, and the extent of flagellar surface motility was assessed by phase contrast microscopy. In non-lidocaine-treated cells 60% of the attached microspheres were in motion at the time of first observation. As the lidocaine concentration was increased, there was a progressive decrease in flagellar surface motility (Fig. 5); at 1 mM lidocaine flagellar surface motility was inhibited by 90% and at 2 mM, bead movement was completely blocked. When the cells were washed out of lidocaine into fresh medium, flagellar surface motility returned (data not shown).

To determine the sensitivity of pf 17 zygote formation to lidocaine, these paralyzed flagellar mutants were mixed with wild type mt^+ gametes in various concentrations of the local anesthetic. As shown in Fig. 5, pf mutants appeared to be more sensitive to lidocaine than wild type cells (See Fig. 1), and inhibition of zygote formation was evident at concentrations of lidocaine which also blocked flagellar surface motility.

Flagellar Tip Activation

Another event that normally occurs after adhesion between mt^- and mt^+ gametes is a change in the ultrastructural appearance (activation) of the flagellar tips from a tapered to a bulbous form (10, 16, 22). Flagellar tip activation (16) may represent an active feature of signalling for subsequent steps in the fertilization process. To determine the effect of lidocaine

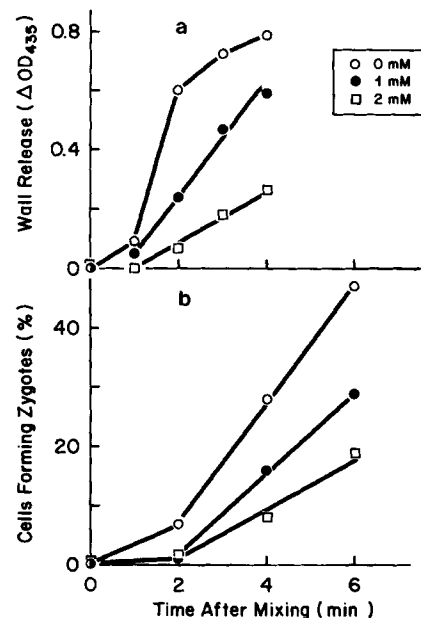


FIGURE 4 Effect of lidocaine on initial rates of zygote formation and cell wall release. Mt^- and mt^+ gametes (5 ml each, 1.6×10^7 cells per ml in HTM) were pretreated for 5 min at 13°C with 0 (open circles), 1 (closed circles), or 2 mM lidocaine (squares). The cells were then mixed together and assayed for wall release (panel a) and zygote formation (panel b) at the indicated times.

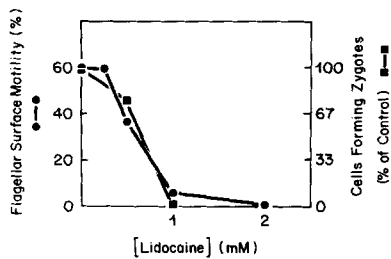


FIGURE 5 Flagellar surface motility and zygote formation in paralyzed flagellar mutants (pf 17). Pf 17, mt^- gametes (1.0 ml, 1.6×10^7 cells per ml in HTM) were pretreated at room temperature for 5 min in the indicated concentrations of lidocaine and then $10 \mu\text{l}$ of microsphere in H_2O were added. After 2 min the number of attached microspheres in motion at the time of observation was determined (circles). At 2 mM lidocaine there was some (30–40%) reduction in the extent of bead binding; but since the assay measured movement of attached beads, reduced binding did not interfere with interpretation of the data (3, 11). To determine zygote formation pf 17 (mt^-) and wild type mt^+ gametes (5 ml each, 1.6×10^7 cells per ml in HTM) were mixed together after a 5-min pretreatment in the indicated concentration of the local anesthetic and 30 min after mixing the extent of zygote formation was determined (squares). In this experiment, 30% of the control, nonlidocaine treated gametes had fused by 30 min.

TABLE I
Flagellar Tip Activation in the Presence of Lidocaine*

Lidocaine mM	Zygotes	Flagella with bulbous tips
	%	%
0.0	74 (100)‡	63 (100)‡
1.0	35 (47)	64 (100)
2.0	20 (20)	62 (100)
3.0	1 (1)	40 (63)

* Mt^- and mt^+ gametes (2 ml each, 1.6×10^7 cells per ml in HTM) were pretreated for 5 min at 13°C with the indicated concentrations of lidocaine and then mixed together; the extent of bulbous tip formation was determined at 4 min and the extent of zygote formation at 15 min.

‡ The numbers in parentheses represent the percent of control values.

on flagellar tip activation, mt^- and mt^+ gametes were pretreated for 5 min at 13°C as above, mixed, and 4 min after mixing the percentage of flagella with bulbous tips was determined. In the same experiment the extent of zygote formation was assessed. Table I illustrates that 2 mM lidocaine inhibited zygote formation by 80–100%, whereas flagellar tip activation was reduced by <40%.

Ca^{++} or Mg^{++} Ions Modulate Inhibition of Fusion by Lidocaine

Since lidocaine has been reported to effect the interaction of divalent cations with cell membranes and is thought to block Ca^{++} influx into cells (5, 19), we investigated the effects of Ca^{++} and Mg^{++} on lidocaine inhibition of zygote formation. To do this, gametes of each mating type were pretreated for 5 min with various concentrations of lidocaine at several different concentrations of CaCl_2 or MgSO_4 and then mixed together in the continued presence of the reagents. 4 min after mixing, the cells were fixed and assayed for the extent of zygote formation. The results shown in Fig. 6 indicated that the effects of lidocaine could be modulated by both Ca^{++} (Fig. 6 A) and Mg^{++} (Fig. 6 B) ions. For example, in the absence of divalent cations, 1 mM lidocaine completely blocked zygote formation;

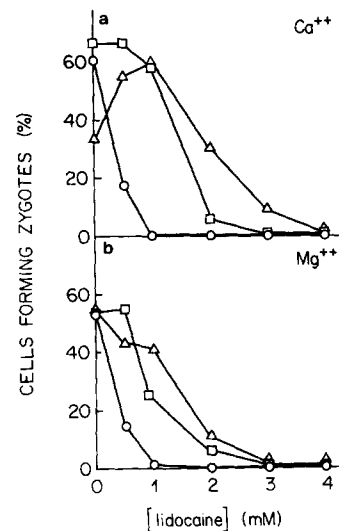


FIGURE 6 Modulation of lidocaine inhibition by Ca^{++} and Mg^{++} . Mt^- and mt^+ gametes (3 ml each, 1.6×10^7 cells per ml in 10 mM HEPES) at room temperature were pretreated for 5 min in the indicated concentrations of lidocaine with 0.0 (circles), 0.5 (squares), or 3 mM CaCl_2 (triangles) (shown in panel a) or 0.0 (circles), 0.5 (squares), 1.0 mM MgSO_4 (triangles) (shown in panel b) and the extent of zygote formation after 5 min was determined.

whereas addition of 0.5 mM CaCl_2 or 1 mM MgSO_4 along with the 1 mM lidocaine prevented the inhibition.

DISCUSSION

The results presented in this report suggest that movement of calcium and magnesium ions may be necessary for sexual signalling during the initial stages of fertilization in *Chlamydomonas*. When gametes of opposite mating type were mixed together in the presence of lidocaine, cell wall release and gamete fusion were blocked. This inhibition occurred even though the gametes were able to proceed through several of the normal stages of fertilization; motility appeared unaffected and aggregation, as measured by a quantitative Coulter counter assay (Coulter Electronics, Inc.) was indistinguishable from non-lidocaine-treated cells. Moreover, flagellar tip activation, an event which has been postulated to be necessary for signalling for release of cell wall degrading enzymes, was partially inhibited by lidocaine but still occurred at 60% of the level of nontreated gametes. The ability of the cells in lidocaine to proceed through the steps in fertilization up to wall release, and the ability of gametes to release their walls and fuse when the lidocaine was washed out, indicated that the anesthetic was not simply having a toxic effect on the cells.

Since lidocaine is reported to interfere with the movement of divalent cations across cell membranes, and to cause release of membrane-bound calcium (19), the ability of the local anesthetic to block sexual signalling in *Chlamydomonas* suggested that the divalent cations might be important in the signalling event. Ca^{++} influx has been shown to be a central feature of both sperm and egg activation during fertilization in many higher organisms (20). Of particular relevance to our studies, Collins and Epel (5) showed that lidocaine and Ca^{++} worked antagonistically in interfering with or promoting the acrosome reaction in sea urchin sperm. We also examined the effects of calcium and magnesium on the lidocaine inhibition of fertilization in *Chlamydomonas*. The result that these cations were able to modulate the effects of lidocaine suggests that

binding to the cell membrane or movement of these ions across the cell membrane may be essential for sexual signalling.

Although this is the first evidence that Ca^{++} may be necessary for signalling in *Chlamydomonas*, this ion has been shown to play an important role in several other flagellar processes in this alga. Schmidt and Eckert (18) have reported that the photo-stimulated reversal of flagellar beat from a forward to an undulating form in *Chlamydomonas reinhardtii* requires influx of Ca^{++} . Hyams and Borisy (13) using isolated, intact flagellar apparatuses and Bessen et al. (1) using reactivated flagellar axonemes have shown that Ca^{++} can control the form of flagellar beat in vitro. Based on information about the role of Ca^{++} influx in the avoidance response in *Paramecium* (14), these workers have suggested that several stimuli (e.g. light, physical contact) might depolarize the flagellar membrane and permit an influx of Ca^{++} , thereby causing flagellar undulation or twitching. Watanabe and Flavin (28) and Bessen et al. (1) demonstrated a 3S Ca-ATPase which may be localized in the flagellar membrane. Such an ATPase could serve to maintain a normally low intracellular Ca^{++} concentration.

These observations on Ca^{++} control of flagellar motility may be pertinent to mating in *Chlamydomonas* because gametes show altered flagellar motion during aggregation. Because of their adhesions to other flagella the flagella become nearly immobilized. Although not extensively studied it appears that they no longer undergo the characteristic flagellar beat but instead seem to twitch or undulate (12). Moreover, Homan et al. (12) have reported that partially purified monovalent sexual binding components from *C. eugametos* are unable to cause agglutination but induce a mating type specific twitching (undulation) only of gametes of the opposite mating type. One interpretation of these observations is that flagellar adhesion can serve as a stimulus to depolarize the flagellar membrane, allow Ca^{++} influx and thereby cause flagellar undulation or twitching. This altered flagellar movement might itself be necessary for lysin release and gamete fusion. Conversely, the twitching may simply be a result of increased Ca^{++} influx which is the actual signal. Certainly, Ca^{++} influx alone is insufficient to induce release of lysin, because gametes do not release cell wall degrading enzymes every time they undergo reversal of flagellar beat. It is possible, however, to induce lysin release by use of reagents other than flagellar adhesion molecules (e.g. anti-flagellar antibodies) (9, 10). These reagents which also cause agglutination, may act by increasing Ca^{++} influx. It will be interesting to determine if lidocaine also blocks lysin release induced by these artificial stimulators.

In other studies on the role of calcium in events which occur during mating, Claes reported (4) that treatment of vegetative or gametic cells with the Ca^{++} ionophore A23187 brought about cell wall loss but only on flagellated cells; deflagellated cells were unresponsive to the ionophore. More recently Bloodgood has found that there is a dramatic increase in the rate of Ca^{++} efflux from *Chlamydomonas* gametes which begins immediately after mixing of gametes of opposite mating types and continues for 4–6 min (personal communication). It is not yet clear if this increased efflux is due only to a change in the rate of movement of Ca^{++} out of the cell or if it reflects a change in permeability in both directions.

Bloodgood has also shown that flagellar surface motility requires Ca^{++} (3). Our results that lidocaine blocks flagellar surface motility in pf 17 gametes confirms his results but also raises a question about the relationships between flagellar surface motility, flagellar tip activation and sexual signalling.

In our experiments we found that lidocaine blocked flagellar surface motility at around the same concentration that blocked gametic fusion (Fig. 5). Thus, our data are also consistent with (but do not prove) the idea that flagellar surface motility is necessary for sexual signalling. On the other hand, it has also been suggested (10, 11) that flagellar surface motility is necessary for flagellar tip activation but in our experiments, (with wild type cells) flagellar tip activation was not blocked. Future studies on the effects of lidocaine on flagellar surface motility and flagellar tip activation in pf 17 gametes should help to resolve this point.

Finally, in addition to helping delineate new physiological steps in fertilization, these observations on lidocaine-inhibition of fusion should be useful in dissecting the molecular events which occur during fertilization. For example, we have previously demonstrated an adhesion-induced turnover of flagellar adhesion molecules in *Chlamydomonas* (26) and have also found that there is an adhesion-induced synthesis of two cell surface proteins that copurify with flagellar membranes (24). By use of lidocaine, it should be possible to determine if fertilization events before wall release are necessary or sufficient for the adhesion-induced turnover and synthesis of molecules involved in flagellar adhesion.

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