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Evidence for a Signal Transduction System Initiating Stalk Excision in *Vorticella convallaria*

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ABSTRACT

We present indirect evidence that stalk excision in *Vorticella convallaria* is controlled by a signaling system similar to that described for flagella excision in *Chlamydomonas* (Quarmby and Hartzell, 1994). Excision is initiated by micromolar quantities of two G-protein agonists, mastoparan or Compound 48/80. The effects of these agonists can be reduced significantly by pretreatment with neomycin which prevents hydrolysis of phosphatidyl inositol 4,5-bisphosphate or by polymyxin B which inhibits protein kinase C activity. Additionally, mastoparan 17, an inactive form of mastoparan has little effect on excision. Following excision induced by these compounds, the cell bodies transform into the motile telotroch form. Phosphoric acid at a pH where the protonated form of the molecule dominates, initiates high levels of excision. In cells where stalk excision is initiated by phosphoric acid few excised cells transform into telotrochs unless calcium is present.

INTRODUCTION

Vorticella convallaria, a peritrich ciliated protozoan, has been recently used to study rapid contractions initiated by spasmin contractile mechanisms and formation of extracellular matrices (Buhse, 1998). Results from the present study will facilitate examination of the synthesis of spasmin protein in synchronously transforming trophonts of *Vorticella*. Recently the biology of *Vorticella* has been published in Encyclopedia of Life Sciences in which the morphology and cell biology, reproduction, ecology, systematics and methods of culturing are described (Buhse and Clamp, 1999).

Vorticella exhibits two dominant morphological forms: a sessile feeding stage termed a trophont (Fig. 1) which transforms through a series of changes (Figs. 2 and 3) into a motile telotroch (Fig. 4). The trophont and the telotroch represent interchangeable stages which are dependent upon environmental conditions.

The trophont stage (Fig. 1) is characterized by an inverted bell-shaped cell body that tapers posteriorly (aborally) to the area where it attaches to a contractile stalk. Feeding structures, the nuclei, and most other cellular organelles are contained within the cell body. The stalk consists of a contractile structure called the spasmoneme which is bounded by a triple membrane system typical of ciliates. The spasmoneme is surrounded by extra cellularly produced material termed the matrix which is bounded by a stalk sheath (Wibel et al., 1997).

The morphological events of *Vorticella* transformation from the trophont to the telotroch have been described elsewhere (Vacchiano et al., 1992). This process always involves severing the connection between the cell body and the contractile stalk at a specialized area called the scopular region (Fig. 2), located at the aboral surface of the cell body (the region farthest away from the oral region). This semi-permanent junction consists of a ring of stereo-cilia and the last scopular ridge of the cell body called the scopular lip. Together, they form a cup into which the stalk sheath inserts. The sheath is anchored both to the cilia and the lip by groups of many small fibrils (Wibel et al., 1997). It has been postulated that during telotroch formation, these fibrils are broken and the connection between the cell body and the stalk is ruptured (Wibel et al., 1997). Following stalk excision, the scopular lip extends over the cilia forming a cupola-like structure at the scopular region which flattens out by the telescoping of the scopular ridges (Fig. 3). As the freed cell body elongates to achieve a cylinder-like shape, a band of cilia called the aboral ciliary wreath, which powers the telotroch, sprouts from the scopular region while the oral cilia are withdrawn and partially resorbed (Fig. 4). While there is much information about the morphological events that occur during the trophont to telotroch transition, little is known about the molecular events that control this process.

Vorticella is not the only organism that excises unwanted organelles in response to environmental cues. The phytoflagellate *Chlamydomonas* excises its flagella forming a non-motile stage in response to deteriorating conditions (Kudo, 1966). Two distinct calcium-mediated signal transduction pathways have been proposed to explain initiation of flagella excision. It has been demonstrated that protonated forms of acids are able to cross the plasma membrane and ionize in the cytoplasm, releasing a proton which triggers a signal transduction system (Hartzell et al., 1993). Furthermore, Quarmby and Hartzell (1994) showed that protonated benzoate crossed the plasma membrane and activated opening of external calcium channels which led to flagellar excision. In the other scenario, suspension of flagellated cells in mastoparan, a G-protein agonist (Higashijima et al., 1990), induced, through phospholipase C, a build-up of inositol 1,4,5-triphosphate (IP_3) which correlated with flagellar excision (Quarmby et al., 1992). It is known that IP_3 is heavily involved in regulation of internal calcium stores (Berridge, 1993). In addition, neomycin, an inhibitor of phosphatidyl inositol 4,5-bisphosphate (PIP_2) hydrolysis (Gabev et al., 1989), effectively blocked both mastoparan induced IP_3 build-up and flagellar excision (Quarmby et al., 1992). Using different calcium channel blockers, further supported the two distinct pathway hypothesis in *Chlamydomonas* (Quarmby and Hartzell, 1994).

The purpose of our study is to examine the effects of similar compounds on stalk excision and telotroch formation in *Vorticella convallaria*. Previous results obtained from our laboratory (De Baufer et al., 1999) showed that stalk excision can be initiated by sub-

jecting the trophonts to a monocalcium phosphate solution (MCP). Following removal of this solution and its replacement with a neutral buffer, excised cells synchronously transformed into telotrochs within 90 minutes. We speculated that one or several of the hydrolysis products of monocalcium phosphate were active in initiating stalk excision. The presence of two of the products, calcium and the protonated form of phosphoric acid suggest to us that the events leading to stalk excision may be initiated by a calcium mediated systems similar to those described for *Chlamydomonas*.

Results from the present study show that phosphoric acid, the G-protein agonists mastoparan and compound 48/80 induce excision and subsequent transformation. Treatment with neomycin, an inhibitor of IP_3 hydrolysis, and polymyxin B, an inhibitor of protein kinase C, significantly reduces the effects of mastoparan and Compound 48/80. This suggests a signal transduction system in *V. convallaria* stalk excision.

MATERIAL AND METHODS

General Cell Maintenance

The cultures of *Vorticella convallaria* were grown and maintained in 500 ml of Cerophyl medium in 2.8-liter Fernbach flasks (Vacchiano et al., 1992). Prior to each experiment, the Cerophyl medium was exchanged with fresh medium and the cells were placed on a New Brunswick G25 incubator/shaker (New Brunswick Scientific, New Brunswick, NJ) for 24 hrs at 100 rpm. This procedure produced unattached cells which could be used to inoculate Petri dishes. The content of each flask was then transferred into sterile Petri dishes where the cells reattached and grew for additional 24 hrs (De Baufer et al., 1999). These cells, called 1-day old cells, were used in all experiments.

Buffers

Studies using agonists, inhibitors and other test compounds were conducted according to methods described in De Baufer et al. (1999). Briefly, the cells were suspended in these compounds to induce excision after they were washed in the rinse buffer [0.187 mM EDTA, 3.9 mM NaCl, 0.71 mM TRIS-HCl, 0.5 mM maleic acid, pH 6.8]. Because protracted cell exposure to these stalk excision solutions proved to be toxic, the excision solutions were diluted and removed quickly by filtration and the detached cell bodies placed in transformation buffer at pH 6.8 [inorganic medium (IM) (0.24 mM KCl, 0.24 mM $MgSO_4$, 0.048 mM $CaH_4(PO_4)_2$) or calcium free inorganic medium (CFIM) (0.24 mM KCl, 0.24 mM $MgSO_4$)]. In some cases the excision solutions and/or the transformation buffer were supplemented by addition of calcium either as calcium hydroxide or calcium chloride. IM and/or CFIM at pH 6.8 were used interchangeably as a negative control in all stalk excision experiments and exclusively as transformation buffers.

Excision solutions

All excision compounds were dissolved in glass distilled water. The times of exposure and the concentrations of the excision compounds were previously determined to cause maximum excision levels with the least toxic effects on the cell populations. Attached cells were exposed to: 4.8 mM monocalcium phosphate (MCP) (Fisher Scientific, Chicago, IL) for 20 minutes (De Baufer et. al., 1999); 24 mM phosphoric acid (PA) (EM Science, Gibbstown, NJ) at pH 3.2 for 15 min; the G-protein agonists 8 μM mastoparan and 8 μM mastoparan 17, an inactive analog of mastoparan (Sigma Chemical Co., St.

Louis, MO), for 2.5 min; the G-protein agonist 25 mg/ml Compound 48/80 (Sigma Chemical Co., St. Louis, MO) for 9 min. The pH of PA was adjusted to 3.2 by addition of either NaOH or $Ca(OH)_2$. In some cases, the attached cells were exposed to inhibitors 20 μM neomycin (Sigma Chemical Co., St. Louis, MO) and 20 μM polymyxin B (Calbiochem, La Jolla, CA), for one minute prior to exposure to mastoparan or compound 48/80.

Determination of the number of attached and non-attached cells

The number of attached cells in a Petri dish (100×15 mm) was determined using a Bausch and Lomb stereozoom microscope. The bottom of the Petri dish was divided into eight randomly determined nonadjacent 0.25 cm^2 areas and the number of cells in each area was directly counted. From this the total the number of the cells in a Petri dish was extrapolated by the following formulation:

Number of cells in eight areas ÷ 8 x 254.5 = Total number of cells in a Petri dish

Where 254.5 is the number of squares fitted into the bottom area of the Petri dish (Vacchiano, at el., 1992).

Electron Microscopy

For scanning electron microscopy, the cells were fixed with 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) mixed with 1% osmium tetroxide. The fixed cells were attached to poly-L-lysine coated aluminum disks (Sigma Chemical Co., St. Louis, MO). The attached cells were critical point dried and coated with 20 nm gold. Observations were done using ISI-DS-130 scanning electron microscope at 10 kV. TEM specimens were embedded in either Spurr's or Epon 812 resin and examined with a JOEL-1200-EX transmission electron microscope at 60 kV.

RESULTS

We investigated the ability of phosphoric acid in the presence and absence of calcium to initiate excision (Fig. 5). Cells suspended in inorganic medium or in calcium free inorganic medium (the negative controls (A)) exhibited little excision, while those suspended in MCP (the positive control (B)) exhibited levels of excision in excess of 80%. This was followed by 82% telotroch formation when the freed cell bodies were washed into the transformation buffer. Cells subjected to phosphoric acid buffered to pH 3.2 with $Ca(OH)_2$ (C) exhibited high levels of excision (61%) when compared to the negative control and transformed into telotrochs at equally high values. In both cases, the levels of excision and transformation were about 20% lower that the positive control. Interestingly, buffering phosphoric acid to pH 3.2 with NaOH (D) initiated 74% stalk excision, but the level of transformation into telotrochs was reduced over tenfold when compared to the calcium-buffered cells. When calcium was added to the transformation medium following excision induced by a calcium free solution, the level of transformation was increased four-fold in a dose dependent manner to 26% of the calcium-buffered cells. In summary, the presence or absence of calcium appears to have little effect on acid-induced stalk excision. However, the presence of an exogenous supply of calcium applied to the excision medium appears critical for attaining high levels of transformation following excision.

Next, we examined the effects of G-protein agonists, mastoparan (Fig. 6) or Compound 48/80 (Fig. 7) and the effect of pretreatment with inhibitors, neomycin or polymyxin B on induced stalk excision (Figs. 6 and 7). The G-protein agonists mastoparan and Compound 48/80 induced high levels of stalk excision. In both cases, when the freed cell bodies were washed into transforming buffer equally high levels of transformation occurred. Pretreatment with either neomycin or polymyxin B followed by treatment with either mastoparan or Compound 48/80 significantly decreased the levels of excision. Mastoparan-17, an inactive analog of mastoparan, had little effect on the excision level. Taken together these results are consistent with an hypothesis favoring a role for a phopholipase C signaling pathway initiating stalk excision in *Vorticella convallaria*.

DISCUSSION

One interpretation of our data is that stalk excision in *Vorticella convallaria* is controlled by signaling systems similar to those described for flagella excision in *Chlamydomonas* (Quarmby and Hartzell, 1994). They suggested that two separable signaling systems control excision. One controls activation of phospholipase C signaling system through activation of G-proteins. A second system controls flagella excision by the import of calcium through proton activated external calcium gates. We have indirect evidence that both of these systems may also control stalk excision in *Vorticella*.

Support for our interpretation comes from the following experiments. Micromolar levels of mastoparan or Compound 48/80, both G-protein agonists, activate high levels stalk excision (Figs. 6 and 7). Mastoparan 17, an inactive analog of mastoparan, initiated low excision levels close to the negative control values (Fig. 6). Finally, prior exposure of mastoparan or Compound 48/80-treated cells to either neomycin (preventing hydrolysis of phosphatidyl inositol 4,5-bisphosphate), or to polymyxin B which competitively inhibits protein kinase C activity (Raynor et al., 1991) significantly reduced the levels of stalk excision.

The results from experiments using phosphoric acid (PA) to induce stalk excision suggest the presence of a second signaling pathway in *Vorticella*. High levels of stalk excision are induced by PA at a pH value of 3.2 where the protonated form of the acid is available. The protonated form is believed to be membrane permeable and ionized inside the cell (Hartzell et al., 1993). On the other hand, when cells are subjected to PA at higher pH, where the ionized form is not favored (Corbridge, 1990) or they are placed in solutions of ionized HCl (De Baufer et al., 1999), the levels of stalk excision were very low (data not shown). Taken together, we conclude that stalk excision may depend on lowering internal pH. If this is true, then the results are strikingly similar to results reported by Quarmby et al. (1994). In their model, the protonated acid crosses the plasma membrane of *Chlamydomonas* where it is ionized. One of the ionization products (presumably the proton) activates a pump, which allows calcium to enter the cell from the external medium. One problem with this hypothesis is the observation that stalk excision in *Vorticella* occurs when no calcium is added to the excision medium (Fig. 5). Although other explanations are possible, it might be that sufficient though low levels of calcium are present as minor contaminants in the excision solutions which are high enough to initiate

stalk excision. If this is true excision should be completely eliminated by addition of a calcium chelator to the excision media.

We have evidence that the presence of calcium is necessary for high levels of transformation to the telotroch form. Addition of calcium to the excision buffer results in high levels of transformation independent of whether or not the transforming buffer contains calcium. However, when calcium is omitted from the excision or transformation buffer, levels of transformation are very low (compare Fig. 5C with 5D). Furthermore, in support of the need for calcium, attempts to rescue cells excised in the absence of calcium by adding calcium to the transformation buffer were moderately successful. Cells excised in the absence of calcium transformed in progressively higher levels as more calcium was added (compare Fig. 5D, E and F). The amount of exogenous calcium added to the transformation buffer (Fig. 5F) was one tenth the concentration of values calculated for the positive control (Fig. 5B) or for cells excised in the presence of phosphoric acid plus calcium (Fig. 5C). Therefore, it is highly probable that had we used higher levels of calcium, higher transformation levels, equivalent to those of the control values, would have been obtained. The exact nature of the calcium requirement for successful transformation to the telotroch form remains to be resolved.

Although the exact mechanism of stalk excision is not presently known, we propose two hypotheses to explain control of stalk excision in *Vorticella*. The starting point for both is hydrolysis of PIP_2 by PLC. One involves a role for calcium controlled by phosphoinositols, the other suggests a role for phosphorylation of proteins controlled by PKC.

Calcium has been shown to initiate spasmonemal and cellular contraction in *Vorticella*; however, microinjection of IP_3 into the cell body of *Vorticella* does not initiate contraction (Katoh and Naitoh, 1994). It is possible that IP_3 regulates stalk excision by releasing calcium from some specialized stores. It is equally possible that the release of calcium is governed by generation of other phosphoinositols. In support of this possibility, we have observed that there is an increase in IP_5 or IP_6 following treatment with mastoparan (data not shown). However, these phosphoinositols have a slow response time (unlike IP_3) and are considered to control calcium house keeping functions (Berridge, 1993).

On the other hand, it is equally possible that phosphorylation of proteins plays an important role in stalk excision. In support of this possibility, we had shown that the protein kinase C inhibitor, polimyxin B, significantly reduces the levels of stalk excision in *V. convallaria*.

Furthermore, it is tantalizing to imagine that the calcium-binding contractile protein(s) spasmin may play a role in excision. It has been suggested (Wibel et al., 1997) that the stalk is anchored to the cell body by small fibrils that connect the scopular cilia with the scopular lip. They showed that rupturing these fibrils lead to stalk excision. It is tempting to postulate that either flexing or contracting the scopular lip could lead to stalk excision through rupture of these fibrils. In an analogous system, it has been suggested that contraction of the stellate body found in the flagella-basal body transition zone of *Chlamydomonas* leads to flagella excision (Sanders and Salisbury, 1989). Interestingly, the stellate body is known to contain the calcium-binding contractile protein centrin that belongs to the same superfamily of proteins as spasmin.

In support of phosphorylation, Martindale and Salisbury (1990) found that the level of centrin phosphorylation changes rapidly in response to the cells external environment, and more interestingly, phosphorylation of centrin is correlated with flagella excision in *Chlamydomonas*. Maciejewski et al. (1999) has shown that spasmin like centrin contains several putative phosphorylation sites.

Stalk excision does not automatically lead to formation of the motile telotroch form. When stalk excision is induced by PA in the absence of calcium most of these treated cells fail to transform, although they are clearly alive as indicated by movement of the peristomal whirls. This effect can be ameliorated by the presence of calcium either in the excision mediums or transforming buffer. One possible explanation is that internal cellular pH induced by PA is not regulated sufficiently following excision and prevents activation of cellular processes that underlie transformation. Support for this hypothesis comes from the observation that stalk excision induced by mastoparan or compound 48/80 at pH 6.8 always leads to telotroch formation even in the absence of an exogenous calcium supply.

We propose the following model (Fig. 8) to explain our results and to act as a framework for future work. This model is based on the similar model proposed for flagellar excision in *Chlamydomonas* (Quarmby and Hartzell, 1994). A calcium dependent pathway is shown on the left and PLC activated pathway is shown on the right. Both pathways can be activated by G-protein agonists or protonated phosphoric acid through activation of phopholipase C producing two secondary messengers: IP_3 and DAG. Phosphoinositols regulate release of internal calcium stores (Berridge, 1993). It has been shown that calcium regulates stalk and cell body contraction in *Vorticella* (Katoh and Naitoh, 1994) presumably by binding to spasmin and initiates flagella excision in *Chlamydomonas* by presumed binding to centrin (Sanders and Salisbury, 1994). DAG activates PKC so it is equally possible that phosphorylation of proteins is involved in stalk excision. The protonated form of phosphoric acid, presumed to be membrane permeable, diffuses into the cell body of *Vorticella* where protons are released. Protons open external calcium channels leading directly to stalk excision or indirectly through PLC signaling. We plan to test this model through future experiments which will determine changes of cellular pH in response to treatment with phosphoric acid. We also plan to examine changes in the pattern of protein phosphorylation and changes in phosphoinositols in response to mastoparan treatment. Results from these experiments should verify or disprove this model.

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Figures 1 – 4

1) SEM of the trophont-form of *Vorticella convallaria* showing the stalk (sk) attached to the substrate and to the scopular region (arrow) at the aboral end of the cell body (cb). The peristomal cilia (ps) are found at the oral surface are used for feeding. Bar = 10 μm.
2) SEM of the scopular region of *V. convallaria* during the transition to the motile telotroch stage. During this transition, the stalk is jettisoned. Adhesion between the scopular cilia (sc) and the scopular lip (sl) is broken as the trophont transforms into a telotroch. Bar = 0.5 μm.
3) TEM of the scopular region of an early telotroch. Here the scopular lip (sl) extends over the scopular cilia (sc) and will form a closed cupola-like structure entombing these cilia. The cupola-like structure ruptures when the telotroch docks and releases material which will form the adhesion disc and the stalk matrix. Bar = 0.5 μm.
4) SEM of two mature telotrochs. The one on the left has not attached and is tilted showing a wreath of aboral cilia (ac) that encircle the flatten scopula (arrow). The ciliary wreath powers the telotroch in a forward direction. The cell on the right has attached to the substrate. The peristomal cilia (arrowhead) seen at the oral cell end are partially resorbed. Following attachment, the ciliary wreath is resorbed, the peristomal cilia reextend and the stalk begins to grow. Bar = 10 μm.

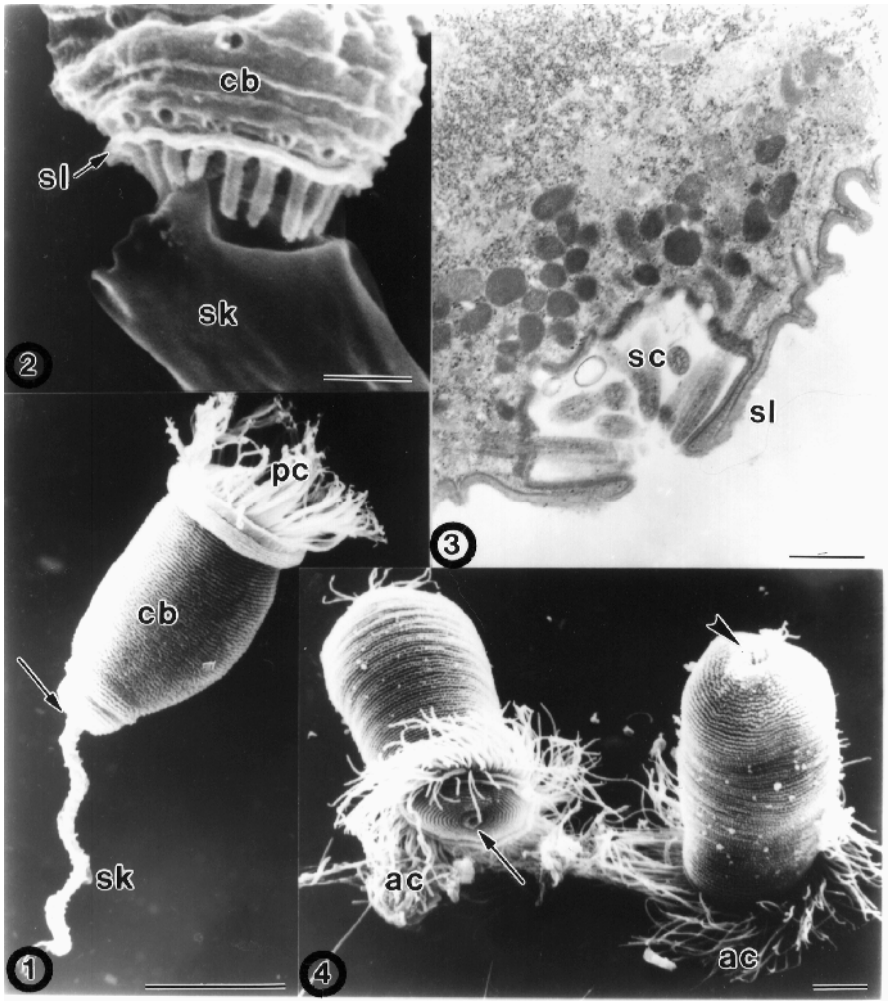

Figure 5. The effects of phosphoric acid in the presence or absence of calcium on stalk excision and telotroch formation in *Vorticella convallaria*. The pH of the excising medium was 3.2 in all cases except where IM or CFIM was used. The pH of the transforming medium was 6.8 in all cases. In columns A-F, for each treatment, the percentage of excision (black bars) and the percent of transformation (gray bars) are shown in tandem. The cells were exposed to excision solutions and transformed in buffers as follows: (A) inorganic medium (IM) or calcium free inorganic medium (CFIM), IM; (B) 4.8 mM monocalcium phosphate, CFIM; (C) 24 mM H_3PO_4 buffered with $Ca(OH)_2$, CFIM; (D) 24 mM H_3PO_4 buffered with NaOH, CFIM; (E) 24 mM H_3PO_4 buffered with NaOH, IM; (F) 24 mM H_3PO_4 buffered with NaOH, IM + $CaCl_2$ (final 0.5 mM Ca concentration). In experiments where transformation bars (gray) are not shown, the number of excised cells was too low to accurately assess telotroch formation. Error is expressed as standard deviation of the mean.

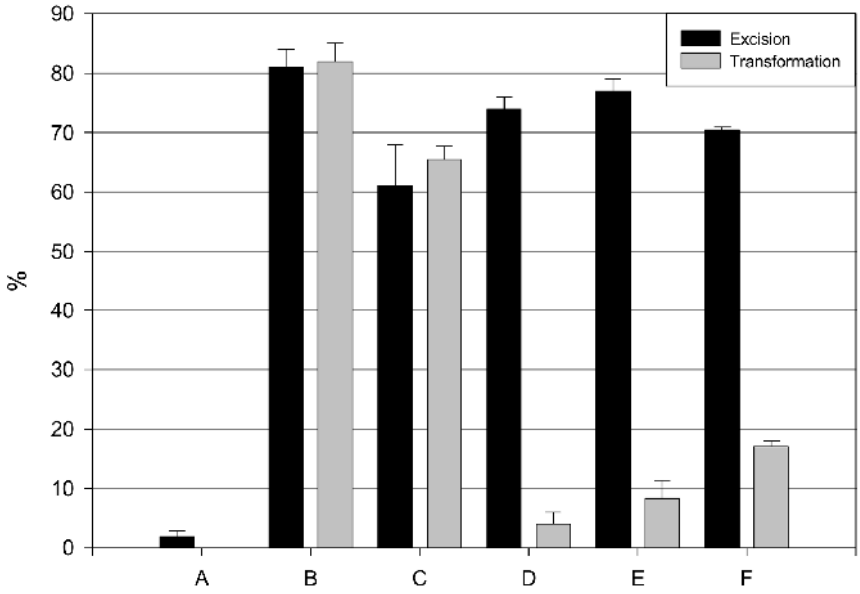

Figure 6. The effects of mastoparan on stalk excision and telotroch formation. Columns A-D represent various treatments and the resulting percentage of cells undergoing stalk excision and the percentage of those cells that formed telotrochs are shown in tandems. Black bars represent the fraction of total number of cells that undergo excision and gray bars represent the fraction of excised cells which formed telotrochs. The following solutions were used to induce stalk excision: (A) 8 μM mastoparan; (B) pre-treatment with 20 μM polymyxin B prior to 8 μM mastoparan treatment; (C) pre-treatment with 20 μM neomycin prior to 8 μM mastoparan treatment; (D) 8 μM mastoparan-17. Transformation was carried out in inorganic medium. In experiments where the percentage of transformation is not shown, the number of excised cells was too low to accurately assess telotroch formation. Error is expressed as standard deviation of the mean.

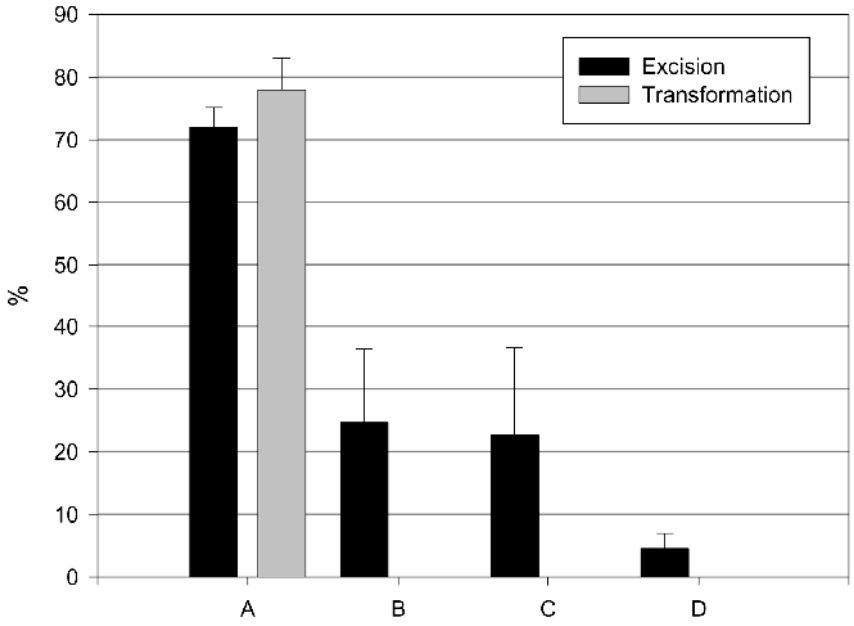

Figure 7. The effects of Compound 48/80 on stalk excision and telotroch formation. Black bars represent the fraction of total cells that excised their stalks. Gray bars show the fraction of excised cells that formed telotrochs. The following solutions were used to excise stalks: (A) Compound 48/80 (25mg/ml); (B) pre-treatment with 20 μM neomycin prior to treatment with Compound 48/80; (C) pre-treatment with 20 μM polymyxin B prior to treatment with Compound 48/80. Recoveries for all experiments were carried out in IM. In experiments where transformation bars (gray) are not shown, the number of excised cells was too low to accurately assess telotroch formation. Error is expressed as standard deviation of the mean.

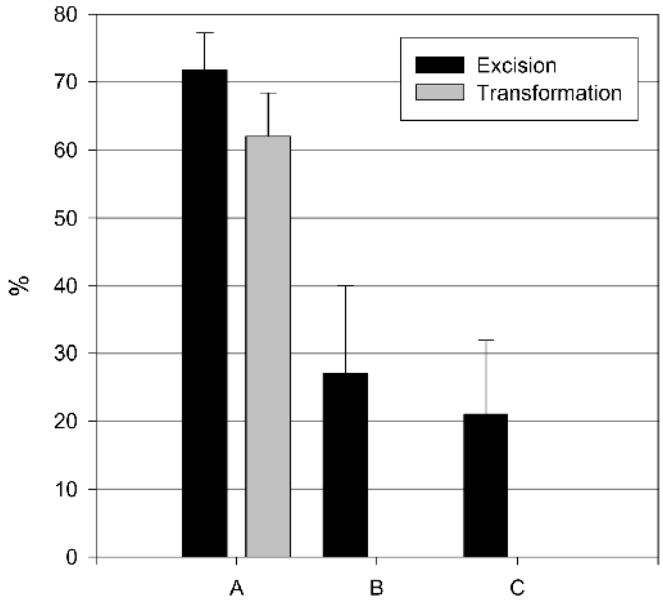

Figure 8. Proposed model for acid- and G-protein induced stalk excision based on Quarmby and Hartzell (1994). Activation of phospholipase C (PLC) by G-protein agonists leads to production of IP_3 and diacylglycerol (DAG). IP_3 or other phosphoinositols (IP_4, IP_5 and IP_6) mobilize internal Ca which leads to stalk excision and cell body contraction. DAG through protein kinase C (PKC) phosphorylates proteins which also leads to stalk excision. N.B. A single arrow in the diagram is not meant to imply a single step in the pathway.