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CALCIUM AND CALMODULIN ANTAGONISTS DELAY ORAL REGENERATION IN THE CILIATE *STENTOR COERULEUS*

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Summary

The regeneration of a new oral apparatus in *Stentor coeruleus* (oral regeneration) has been shown to be sensitive to events that inhibit calcium uptake and calmodulin function. Removal of extracellular calcium delays oral regeneration significantly. The calcium channel antagonist verapamil also delays oral regeneration, as does lanthanum, which is known to block calcium uptake. Both inhibitors are active in the concentration range 10^{-7}–10^{-6} mol l^{-1}. Verapamil acts primarily in the earliest stages of regeneration (prior to stage 5) though some minor delays occur in the later stages as well. In addition, verapamil caused an apparent 'clumping' of the pigment granules in the interior of the cell similar to the effects of high concentrations of theophylline and caffeine. The effects of verapamil on oral regeneration were not reversible in the presence of excess extracellular calcium but those of lanthanum were. The calmodulin antagonists trifluoperazine and W-7 were also shown to delay oral regeneration, but the dechlorinated analogue of W-7, W-5, had no effect even at concentrations 10 times those of W-7. The effects of W-7 were not reversed by excess extracellular calcium. These results suggest that calcium uptake is necessary for oral regeneration and that calmodulin is involved in the control and/or formation of the oral apparatus.

Introduction

One of the more dramatic morphological features of the blue-green ciliate *Stentor coeruleus* is the large oral feeding apparatus located at the broad end of this cone-shaped cell (Fig. 1). The oral apparatus consists of a ciliated membranellar band, a buccal cavity and a frontal field. *S. coeruleus* has the unique ability to regenerate a new oral apparatus if the old one is lost, a process known as oral regeneration (Tartar, 1961). This complex morphogenetic event begins with the assembly of thousands of basal bodies and associated structures at a precise location on the ventral surface of the cell (Fig. 1) at a considerable distance from

Key words: *Stentor coeruleus*, oral regeneration, calcium, calmodulin.

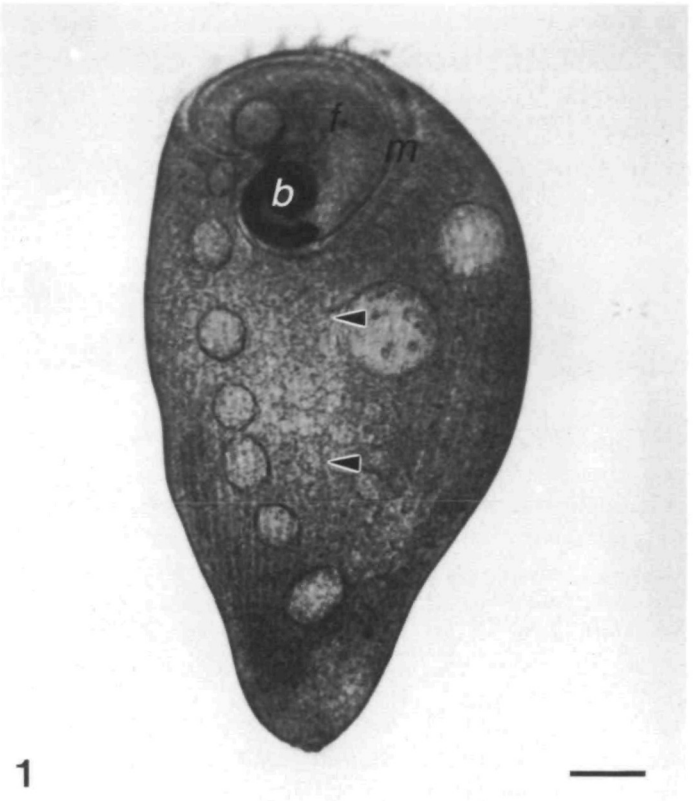

Fig. 1. Morphological features of *Stentor coeruleus*. The oral apparatus consists of the membranellar band (*m*), frontal field (*f*) and buccal cavity (*b*). The region between the arrowheads is the site where the new membranellar band forms during oral regeneration. Scale bar, 50 μm.

the position of the old oral apparatus. The collection of basal bodies eventually assumes the form of an oral primordium that will regenerate the membranellar band of the new oral apparatus. Successive events in oral regeneration involve the formation of a new buccal cavity at the posterior end of the oral primordium and the movement of the primordium up and around the anterior end of the cell until it assumes the normal position occupied by the membranellar band (for a review, see Tartar, 1961). The whole process takes approximately 8–10 h at room temperature and has been divided into a series of eight morphological stages by Tartar (1961; modified by Burchill, 1968).

The cellular and molecular events involved in controlling this complex morphological event are largely unknown. It has been well established that RNA and protein synthesis are necessary for the development of the oral primordium (Burchill, 1968; Pelvat and de Haller, 1984). Pelvat and de Haller (1984) have

begun to analyze the proteins synthesized during oral regeneration and have shown that the tubulins utilized in regeneration come from a pre-existing pool of tubulin. Complex microsurgical manipulations have shown that the signal for the initiation of regeneration travels over the cortex (de Terra, 1971, 1973, 1975). This has been supported by experiments suggesting that this process may be partly controlled by cell surface proteins that can bind plant lectins (Maloney, 1984, 1986, 1988). Additional studies have also provided evidence suggesting that calcium ion fluxes and membrane perturbations may also be involved (Maloney, 1980). These studies showed that various local anesthetics known to bind to membranes and disrupt calcium ion fluxes could inhibit oral regeneration and that the effects of one of these, dibucaine, could be reversed by excess extracellular calcium. Interestingly, the inhibitory effects of the plant lectins Concanavalin A (Con A) and phytohemagglutinin (PHA) could also be reversed by excess calcium (Maloney, 1984, 1986).

Although these last studies on the effects of calcium are important, the results are only suggestive of an involvement of calcium ions in oral regeneration. That calcium ions might be a part of the control mechanisms involved in the initiation and/or elaboration of an oral primordium would not be surprising, based on the widespread involvement of calcium as a second messenger in metazoan cells and in the protozoa. In protozoans, calcium fluxes have long been known to be associated with ciliary reversal, as first established in *Paramecium*, where an inward calcium current in the cilia triggers the reversal of ciliary beating (Eckert, 1972). Moreover, in the case of ciliary reversal, most of the major components necessary to translate the inwardly directed calcium current into a mechanochemical event are present in *Paramecium tetraurelia* and *Tetrahymena pyriformis*, including calmodulin, adenylate and guanylate cyclases and a cyclic-GMP-dependent protein kinase (Muto *et al.* 1983; Nagao *et al.* 1981; Ohnishi *et al.* 1982; Schultz and Klumpp, 1988). Calmodulin has also been implicated in the process of flagellar surface motility in *Chlamydomonas* (Bloodgood, 1990). More directly related to this study, calcium currents like those in *Paramecium* are known to occur in *Stentor* (Wood, 1982) and calcium ions have been shown to be involved in the contraction of *Stentor* (Huang and Pitelka, 1973) and in photosensory events (Kim *et al.* 1984).

If calcium ions are involved in controlling oral regeneration at some level, one would like to know whether the calcium ions involved pass across the cell membrane or whether they are released from intracellular stores. Once the intracellular calcium concentration has been increased, it becomes crucial to know how this affects the process of oral regeneration: is calmodulin involved? In an attempt to determine more specifically whether calcium ions are involved in oral regeneration, specific inhibitors of either calcium fluxes or calmodulin were used to determine whether these compounds could affect oral regeneration. The results suggest that calcium fluxes through the cell membrane occur during oral regeneration and that the calcium ions may affect regeneration, in part by interacting with calmodulin at some point in the process.

Materials and methods

Culture methods

Two strains of *Stentor coeruleus* were used in these experiments. Most of the experiments utilized cells of the 'stella' strain of *Stentor coeruleus* (Tartar), the kind gift of Dr Noel de Terra, while some experiments used cells originally obtained from Carolina Biological Supply Co. Both strains were cultured as previously described (Maloney and Burchill, 1977). Briefly, cells were cultured in a synthetic salt solution composed of 0.55 mmol l^{-1} $CaCl_2$, 0.15 mmol l^{-1} $MgSO_4$, 0.75 mmol l^{-1} Na_2CO_3 and 0.33 mmol l^{-1} $KHCO_3$ made up in deionized water, with the pH adjusted to 7.5 using HCl. Boiled wheat grains were added to stimulate bacterial growth and the cells were fed *Tetrahymena pyriformis* every 3–5 days.

Oral regeneration

Oral regeneration was initiated by exposing cells to a 10% (w/v) sucrose solution for 1–2 min, which caused the cells synchronously to slough off their oral apparatuses. The cells were then transferred through four depression slides containing either filtered 'conditioned' medium drawn from the culture or fresh *Stentor* medium. Cells were transferred from the last wash to the wells of nonwettable plastic spot plates (Falcon Plastics, Los Angeles, CA) containing either *Stentor* medium or *Stentor* medium plus a drug for the duration of regeneration. Spot plates were stored in moist Petri dishes to limit evaporation. The progress of the cells through oral regeneration was observed and recorded at hourly intervals with a Zeiss stereomicroscope using the staging sequence of Burchill (1968). Once exposed to any drug, the cells remained in that drug for the duration of the experiment. When 50% or more of the cells in each treatment group had completed regeneration (stage 8), that time was taken as the time for completion of development. When data from several experiments had been accumulated, the progress of all the cells from several experiments (at least three) was then recorded and summarized. The data are generally expressed as the percentage of all the cells from several experiments that had regenerated by 9 h, when most of the control cells had finished regeneration. In some experiments, an overall median was determined and the experimental data were expressed in this fashion. In this format, cells were labeled as 'blocked' if the majority of the cells in that treatment group had failed to form an observable primordium by 24 h.

Verapamil was dissolved in 95% ethanol and then diluted to a stock concentration of 10 μg ml^{-1}. This stock solution was then diluted in place in the spot plate wells to achieve the desired concentration.

Trifluoperazine, W-7 and W-5 were dissolved in deionized water and initial solutions were diluted to stock solutions of 10 μg ml^{-1}. In some of the experiments involving W-5, the first stock solution was diluted to 100 μg ml^{-1} and then diluted in the wells.

In the verapamil and W-7 experiments with additional calcium, the calcium was

prepared as a 100 mmol l^{-1} Ca^{2+} solution in *Stentor* medium and this was then added to the wells to create the 10 mmol l^{-1} Ca^{2+} solution.

Lanthanum experiments

Since the presence of dissolved phosphates, sulfates or carbonates in the medium can precipitate lanthanum, the normal *Stentor* medium had to be modified to eliminate these anions. For the lanthanum experiments, a modified *Stentor* medium containing 0.55 mmol l^{-1} $CaCl_2$, 0.15 mmol l^{-1} $MgCl_2.H_2O$, 0.75 mmol l^{-1} NaCl and 0.33 mmol l^{-1} KCl was used so that the molar concentrations of the cations were identical to those in normal *Stentor* medium. All lanthanum solutions, in the form of lanthanum nitrate, were prepared in this modified medium. Since it was not known whether the nitrate ion itself had any effect on oral regeneration, excess calcium nitrate was included as a control for the effects of this ion.

Chemicals

Lanthanum nitrate was obtained from Electron Microscopy Sciences, Fort Washington, PA. Verapamil was the kind gift of Knoll Pharmaceutical, Whippany, NJ, and trifluoperazine the kind gift of Smith, Kline & French, Philadelphia, PA. W-7 and W-5 were obtained from Sigma. All other chemicals were reagent grade.

Results

As a first step in examining the role of calcium ions in oral regeneration, we looked at the effects of modifying the extracellular calcium ion concentration by removing calcium from the medium. As seen in Table 1, the absence of calcium significantly delayed oral regeneration. This was seen in both the stella and the Carolina strains of *S. coeruleus*. Addition of excess calcium up to 10 mmol l^{-1} (normal calcium concentration of *Stentor* medium is 0.55 mmol l^{-1}) had no effect on the time necessary to complete oral regeneration.

Table 1. *The effects of modifications in the extracellular calcium concentration on the time required to complete oral regeneration*

Treatment	Percentage regeneration at 8 h	Number of cells
Control	69.3	75
Calcium-free medium	14.1	142
10 mmol l^{-1} calcium	>50	25

The values are medians and represent the percentage of the number of cells indicated that completed regeneration by 8 h.

Table 2. *The effects of the calcium antagonist verapamil (VRL) on oral regeneration in* Stentor

Treatment	Percentage regeneration at 9 h	Number of cells
Control	83.1	83
0.29 % ethanol	64.5	31
2 $\mu g\,ml^{-1}$ VRL	10.0	70
3 $\mu g\,ml^{-1}$ VRL	0.0	25
4 $\mu g\,ml^{-1}$ VRL	0.0	14

Cells were placed in the drug at the beginning of regeneration and remained in the drug for the duration of the experiment (24 h).

The values represent the percentage of the number of cells indicated that have completed regeneration by 9 h.

These results suggested that calcium ions enter the cell during oral regeneration, probably through calcium channels in the membrane. To verify this, we tested the calcium channel antagonist verapamil on regenerating cells. As shown in Table 2, verapamil concentrations of 2 $\mu g\,ml^{-1}$ and above significantly delayed oral regeneration. At concentrations above 2 $\mu g\,ml^{-1}$, the majority of the cells did not form an oral primordium even by 24 h. Notice in Tables 2 and 3 that the ethanol concentrations employed did not generally influence the time course of oral regeneration except for a slight effect at the higher concentration (0.29 %). This effect was less than the delays caused by verapamil at 3 $\mu g\,ml^{-1}$, the verapamil concentration at which this concentration of ethanol would be encountered.

The effects of verapamil on *S. coeruleus* go beyond inhibiting oral regeneration. Cells in the presence of verapamil undergo a pronounced 'bleaching' of their cortical pigmentation that becomes more pronounced at higher concentrations of verapamil. This bleaching is accompanied by the appearance of a dark, greenish ball in the center of the cell. As bleaching proceeds, the large, diffuse greenish mass becomes progressively smaller but darker and the cells become progressively lighter at the periphery. Eventually there appears to be a clear area around a greenish pigment mass in the interior. A similar response has been noted in cells exposed to theophylline (Maloney and Burchill, 1977) and caffeine (Burchill *et al.* 1979) but at much higher concentrations (millmolar compared to micromolar). By 24 h, however, the cells partially recovered their pigmentation and the central mass became less concentrated.

One point of interest in these experiments is the stages at which most of the verapamil-induced delays occur. As seen in Fig. 2, at 2 $\mu g\,ml^{-1}$ verapamil, the majority of the delays occur prior to stage 5, though there are some smaller delays in stages 5 and 6. A similar pattern has been observed with other drugs influencing calcium metabolism, such as dibucaine (Maloney, 1980), except for the delays in stages 5 and 6. This emphasizes how sensitive the early stages of regeneration are. However, one alternative interpretation of these results would be that the cells are

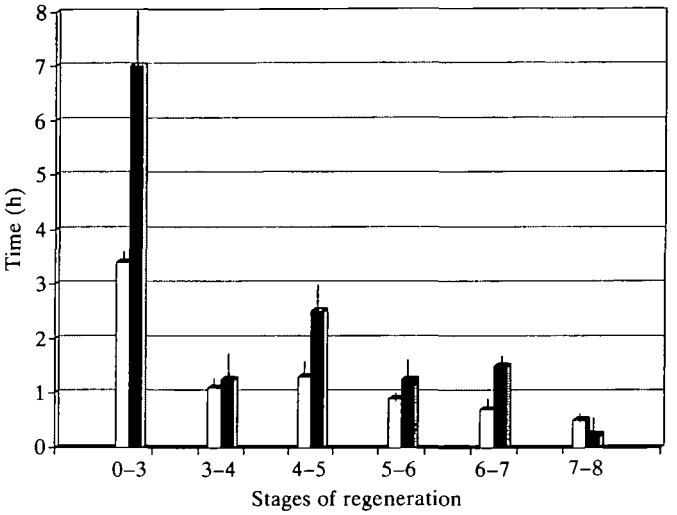

Fig. 2. The effects of verapamil on the various stages of oral regeneration. The *x*-axis indicates the stages of oral regeneration and the *y*-axis shows the mean time that the cells spent in each stage. Each stage transition represents the time needed for 50 % of the cells to progress through a stage (first one shown) and into the next one (second one shown). Open columns, control (N=10; 87 cells); filled columns, 2 $\mu g\,ml^{-1}$ verapamil (N=4; 37 cells). Lines represent S.E.M.

Table 3. *The effects of verapamil and verapamil plus excess extracellular calcium on oral regeneration in* Stentor

Treatments			Hours required to reach stage 8	Number of cells
Verapamil	Ethanol	Calcium		
–	–	–	9.0	23
–	0.19 %	–	10.0	22
2 $\mu g\,ml^{-1}$	0.19 %	–	>10.0	36
2 $\mu g\,ml^{-1}$	0.19 %	10 $mmol\,l^{-1}$	>10.0	32

Verapamil was added at the beginning of regeneration and was present throughout. The data are medians from three experiments.

blocked in regeneration for 8–10 h but then recover and become resistant to the drugs.

The effects of verapamil in some systems can be reversed by increasing the extracellular calcium concentration and it seemed possible that this might be true in *S. coeruleus* as well. To test this, we performed a series of experiments in which cells were exposed to verapamil in the presence or absence of excess extracellular calcium. Table 3 shows that verapamil alone delays regeneration to the same extent regardless of whether excess calcium is present.

Table 4. *The effect of lanthanum on oral regeneration in* Stentor

Treatment	Hours to reach stage 8	Number of cells
Stentor medium	7.0	28
Modified *Stentor* medium	7.0	32
8×10^{-6} mol l^{-1} calcium nitrate	7.0	32
2×10^{-7} mol l^{-1} lanthanum	>24.0	48
4×10^{-7} mol l^{-1} lanthanum	Blocked	23

The time is the median time for the number of cells indicated to complete regeneration.

As a further test for calcium influx during oral regeneration, we examined the effects of the calcium influx inhibitor lanthanum on oral regeneration. As seen in Table 4, low concentrations of lanthanum dramatically delay oral regeneration. Concentrations of lanthanum as low as 2×10^{-7} mol l^{-1} delay oral regeneration, while 4×10^{-7} mol l^{-1} lanthanum completely blocked regeneration. Neither the modified *Stentor* medium nor the presence of the nitrate ion had any effect on oral regeneration.

Lanthanum has long been known to displace calcium from membrane binding sites by competing with calcium ions for these sites (Weiss, 1974). If the effects of lanthanum on *S. coeruleus* are due to this phenomenon, it should be possible to reverse the effects by increasing the extracellular calcium ion concentration. To test this, regenerating *S. coeruleus* were exposed to either lanthanum or lanthanum plus 10 mmol l^{-1} calcium at the start of regeneration. The presence of excess extracellular calcium reversed the delays caused by lanthanum (data not shown), suggesting that lanthanum is displacing membrane-bound calcium. Since lanthanum does not penetrate the cell membrane (Langer and Frank, 1972) and so must exert its effects at the cell membrane, it is even more plausible that lanthanum and calcium compete for the same binding sites.

In many other cells where changes in intracellular calcium concentration are involved in cellular regulation, the calcium regulatory protein calmodulin is also involved. Since calmodulin has been isolated from other protozoans, particularly *Tetrahymena pyriformis* and *Paramecium tetraurelia*, we thought it might be present in *S. coeruleus* and involved in controlling oral regeneration. To examine this, we tested a number of calmodulin antagonists on oral regeneration. Trifluoperazine, added at the beginning of regeneration and present throughout, significantly delayed regeneration at concentrations of 4 μmol l^{-1} and above, as seen in Fig. 3. The response was dose dependent. Similar results were seen with another potent calmodulin inhibitor, W-7 (Fig. 3). Experiments with the inactive analogue of W-7, W-5, resulted in no inhibition of regeneration even at concentrations 10 times those of W-7 (data not shown).

Since the effects of W-7 and trifluoperazine can often be modified by changing the concentration of extracellular calcium, we tried increasing the extracellular calcium concentration in the presence of W-7. As seen in Table 5, 10 mmol l^{-1}

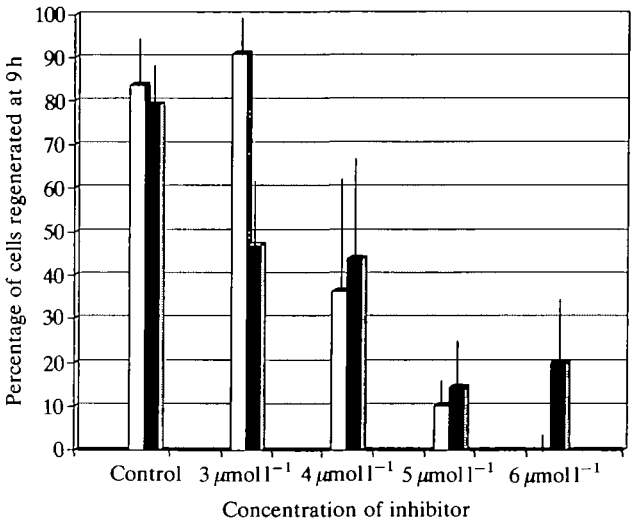

Fig. 3. The percentage of cells that completed oral regeneration in the presence or absence of trifluoperazine or W-7. Bars represent the means from 2–5 experiments (14–48 cells) and the lines are the S.E.M. for each experiment. Open columns, trifluoperazine; filled columns, W-7.

Table 5. *The effects of the calmodulin antagonist W-7 on oral regeneration in the absence or presence of 10 mmol l^{-1} extracellular calcium*

	Percentage of cells completing regeneration by 9 h	
W-7 concentration	*Stentor* medium	*Stentor* medium+Ca^{2+}
Control	80.5% (36)	–
4 μmol l^{-1}	62.5% (8)	37.8% (8)
5 μmol l^{-1}	9.7% (31)	3.1% (32)
6 μmol l^{-1}	0.0% (22)	0.0% (24)

The data are based on the results of 1–4 experiments.
The values in parentheses are the number of cells tested in each category and the percentage is the percentage of these cells that complete regeneration by 9 h.

calcium was unable to reverse the effects of W-7 at any concentration tested. In fact, the combination may have slightly inhibited regeneration compared to that in the presence of W-7 alone.

Discussion

The essential premise of our studies is that calcium ion fluxes occur during oral regeneration and that, by specifically inhibiting calcium uptake, one can thereby

inhibit oral regeneration. Implicit in this is the availability of specific inhibitors that block only calcium uptake during regeneration without nonspecific side effects. Verapamil is considered to be a specific calcium antagonist known to inhibit calcium uptake by binding to and inhibiting those calcium channels by which calcium passes down its electrochemical gradient into the cell (for a review see Janis and Scriabine, 1983; Schramm and Towart, 1985). Although verapamil does not have as high an affinity for these channels and, therefore, is not as specific for calcium channels as some other drugs, such as the dihydropyridines, it is still classified as a 'calcium channel blocker' (Nayler, 1982; Schramm and Towart, 1985).

Even with specific calcium channel inhibitors available, it is also necessary to have reason to believe that calcium channels are present in *S. coeruleus* for these inhibitors to act upon. Wood (1982) has shown that calcium channels that carry a depolarizing inward calcium current in response to mechanical stimulation of the cells are present in *Stentor*. This calcium channel is voltage dependent (Wood, 1982), as are the majority of the calcium channels in mammalian cells. Whether calcium antagonists can inhibit the calcium current in *Stentor* is unknown but tubocurarine can inhibit and bind to a calcium-dependent mechanoreceptor channel found in *Stentor* (Wood, 1985). The possibility that verapamil does inhibit calcium channels such as those in *Stentor* and other ciliated protozoans was enhanced by the recent demonstration of verapamil binding sites in both the plasma and intracellular membrane fractions, as well as in isolated flagella, of the protozoan *Chlamydomonas* (Dolle and Nultsch, 1988).

With this established, our results clearly show that verapamil delays oral regeneration at concentrations within the range normally used to inhibit calcium fluxes and that its effects occur primarily in the early stages of regeneration. The previous discussion suggests that it is reasonable to assume that any effects of verapamil on *S. coeruleus* would be due to an inhibition of calcium uptake. However, other interpretations are also possible.

Additional support for the blocking of calcium uptake by verapamil in *S. coeruleus* is based on the results with lanthanum, which is known to displace calcium ions from the cell membrane, inhibit calcium uptake (Weiss, 1974; Langer and Frank, 1972) and depress the calcium-dependent action potential in *S. coeruleus* (Wood, 1982). In *S. coeruleus*, lanthanum delays oral regeneration much as verapamil does, though regeneration is much more sensitive to lanthanum. The effects of lanthanum are not due to the modified *Stentor* medium employed or to the added nitrate ion (Table 4). The extreme sensitivity of oral regeneration to lanthanum (at concentrations as low as 2×10^{-7} mol l^{-1}) is surprising. Lanthanum has been shown to affect numerous calcium-dependent events in mammalian cells but usually at concentrations of 0.5 mmol l^{-1} or above (Weiss, 1974). Even when lanthanum was utilized in *S. coeruleus* to show the calcium dependency of the photophobic response or inhibition of the action potential, concentrations of 30–100 μmol l^{-1} were used (Kim *et al.* 1984; Wood, 1982). Although it is not certain why oral regeneration is so sensitive to lanthanum, this sensitivity may

reflect the necessity for calcium ions for oral regeneration, a point supported by the effects of the removal of calcium ions from the medium (Table 1).

In an earlier paper (Maloney, 1980), it was reported that calcium-free media did not affect oral regeneration, while we report here that it does. Both studies involved large sample sizes (over 100 cells) and numerous repetitions so the explanation for the differences in the two studies is uncertain. However, we believe the different results are probably due to differences in the strains of *Stentor* used and to differences in the sources of distilled water used in culturing the cells. It should also be pointed out that, while there were no delays observed in regeneration in the earlier study, the cells in calcium-free media were abnormal in appearance, so that the lack of calcium was having some effect on the cells although it was not enough to slow regeneration (Maloney, 1980).

In other systems, the effects of both verapamil and lanthanum can often be reversed by increasing the extracellular calcium concentration (Janis and Scriabine, 1983; Weiss, 1974). Excess extracellular calcium did not reverse the effects of verapamil (Table 3) but it did reverse the effects of lanthanum (data not shown). If lanthanum was displacing calcium from membrane binding sites, this is precisely what one would expect (Langer and Frank, 1972; Weiss, 1974). Since the effects of verapamil were not reversed by calcium, it would appear that verapamil does not bind to the calcium binding site of the suggested calcium channel in *Stentor* and that calcium does not function as a competitive inhibitor of verapamil.

The experiments with trifluoperazine and W-7 strongly suggest the involvement of calmodulin in some aspect of oral regeneration. Both W-7 and trifluoperazine are classic calmodulin antagonists and they both inhibit oral regeneration at concentrations that are below those used to demonstrate the involvement of calmodulin in swimming behavior and secretion in *Paramecium* (Otter *et al.* 1984; Garofalo *et al.* 1983) and the inhibition of guanylate cyclase in *Tetrahymena pyriformis* (Nagao *et al.* 1981). The specificity of these results was verified by the use of the dechlorinated analogue of W-7, W-5, which, despite this minor structural change, was unable to inhibit oral regeneration at concentrations 10 times those of W-7. This also suggests that the inhibitors used are not cytotoxic to the cells, even though the cells are exposed to the drugs for 24 h. To our knowledge, this is the first demonstration of the possible involvement of calmodulin in a complex morphogenetic event such as the elaboration of a complete oral apparatus in *S. coeruleus*.

Although the results of these last experiments are important, their significance would be enhanced if calmodulin were known to be present in *S. coeruleus*. Calmodulin has been isolated from two other ciliates, *Tetrahymena pyriformis* and *Paramecium tetraurelia* (Suzuki *et al.* 1981; Maihle *et al.* 1981; Schultz and Klumpp, 1988), and from the flagellated alga *Chlamydomonas reinhardtii* (Van Eldik *et al.* 1980). In the two ciliates, calmodulin has been found in the cilia, where it is associated with the membrane-bound guanylate cyclase in *Paramecium* (Schultz and Klumpp, 1988) while in *Tetrahymena* (Suzuki *et al.* 1981) it has been reported to be a component of the interdoublet links (Ohnishi *et al.* 1982). In

addition, Bloodgood (1990) has used inhibitors of calmodulin to suggest an involvement of calmodulin in flagellar surface motility in *Chlamydomonas reinhardtii*. The association of calmodulin with cilia and flagella may be particularly significant for our observations in *S. coeruleus*, as the process studied here involves the coordinated assembly of thousands of cilia, as well as the assembly of other components of the membranellar band (Paulin and Bussey, 1971). We think it possible that calmodulin may be involved in the control of the initial events of oral regeneration and/or as a physical component of the ciliary apparatus of *S. coeruleus*, as it is in *Tetrahymena pyriformis* and *Paramecium tetraurelia*. Interference with its function by trifluoperazine or W-7 could inhibit the control mechanisms of regeneration and/or the proper assembly of calmodulin into the ciliary apparatus. In the latter context, Hirano and Watanabe (1985) have identified 36 calmodulin-binding proteins in *Tetrahymena* cilia, most of which do not bind calmodulin in the presence of trifluoperazine. Also, in both *Tetrahymena* and *Paramecium* (Schultz *et al.* 1983), lanthanum has been shown to remove calmodulin from guanylate cyclase and it may have a similar effect in *S. coeruleus*, i.e. causing calmodulin to dissociate from its binding sites or interfering with the functioning of calmodulin in some manner.

All the results discussed above depend on the specificity of the inhibitors used, which, while reasonably specific, do have some nonspecific effects, particularly in the case of verapamil (Norris and Bradford, 1985; Fairhurst *et al.* 1980; Mori *et al.* 1980; Schlondorff and Satriano, 1985). Even trifluoperazine and W-7 are not necessarily specific calmodulin antagonists and may have other effects unrelated to calmodulin (Ross *et al.* 1985; Wulfroth and Petzelt, 1985; Corps *et al.* 1982). However, these nonspecific effects of verapamil, trifuoperazine and W-7 are generally observed at concentrations higher than those employed here (Norris and Bradford, 1985; Mori *et al.* 1980; Corps *et al.* 1982; Ross *et al.* 1985), or in the case of the calmodulin antagonists, the effects were noted with trifluoperazine and not with W-7 or *vice versa* (Ehrlich *et al.* 1988). For these reasons, we believe that the effects we have observed here are specific effects of these inhibitors and not the result of nonspecific side effects. Some of these 'nonspecific' effects deserve further attention, however, because for both the calcium channel blockers and the calmodulin antagonists the side effects involve reciprocal aspects of calcium metabolism. Verapamil has been shown to interact with calmodulin (Schlondorff and Satriano, 1985), while calmodulin antagonists can inhibit calcium channels (Ehrlich *et al.* 1988). Although these could be considered as nonspecific effects of the inhibitors in some studies, in ours these are complementary effects in that they support our general thesis that calcium fluxes and calmodulin are involved in oral regeneration. Still, some caution is warranted in the interpretation of the calmodulin antagonist and verapamil results.

The implication of calcium fluxes and calmodulin involvement in oral regeneration is consistent with earlier studies. The local anesthetics dibucaine, tetracaine and procaine were all shown to inhibit oral regeneration, presumably by interacting with the cell membrane and inhibiting calcium fluxes, since they have

been shown to do this in other systems and since the effects of dibucaine were reversed by excess extracellular calcium (Maloney, 1980). Interestingly, local anesthetics have also been shown to interact with calmodulin in *Tetrahymena* (Muto *et al.* 1983) and, if calmodulin is membrane bound in *S. coeruleus*, as is the case in *Tetrahymena* and *Paramecium*, the local anesthetics could have been interacting with calmodulin in this earlier study.

The ability of plant lectins to inhibit oral regeneration, presumably by perturbing membrane glycoproteins (Maloney, 1984, 1986, 1988), may also be related to calcium metabolism. Both Con A and PHA delay oral regeneration and excess extracellular calcium can reverse the delays caused by these lectins (Maloney, 1984, 1986). Con A and PHA have been shown to bind only to membranellar and somatic cilia in exerting their effects (Maloney, 1988; M. S. Maloney, unpublished results). Furthermore, Con A also inhibits one of the two membranellar calcium currents observed in *Stylonychia mytilus* (Deitmer *et al.* 1986), suggesting that a similar phenomenon may occur in *S. coeruleus*. Studies in several mammalian systems in which putative calcium channels have recently been isolated also support this possibility, in that at least one of the proteins in the channel is glycosylated and binds to wheat germ agglutinin (Leung *et al.* 1987). Recently, we have shown that wheat germ agglutinin (WGA) also delays regeneration in *Stentor* (M. S. Maloney and P. R. Walsh, unpublished observations). The identification of putative calcium channels in the flagella of *Chlamydomonas reinhardtii*, based on the binding of calcium antagonists (Dolle and Nultsch, 1988) and the evidence that there are calcium channels in *Stentor* (Wood, 1982), combined with these other results provides circumstantial evidence that calcium channels are located in the cilia, that they may be glycosylated and that they are affected by verapamil as well as by Con A, PHA and WGA. What is clearly necessary is a direct demonstration of calcium uptake during oral regeneration and its inhibition by these various agents.

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