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Cytoskeletal regulation of ion channel distribution in the tip-growing organism *Saprolegnia ferax*

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SUMMARY

Growing hyphal tips of the oomycete *Saprolegnia ferax* possess a tip-high gradient of stretch-activated ion channels permeable to calcium. These mechanosensitive channels appear to play a direct role in the polarized tip growth process. Treatment of *S. ferax* hyphae with cytochalasin E leads to the disruption of plasmalemma-associated, peripheral cytoplasmic actin populations and altered morphology of apical protoplasts, and eliminates the tip-high gradient of stretch-activated channels. Cytochalasin E did not alter the normal aggregation of stretch-activated channels. The density of spontaneous K^+ channels was decreased in all regions of the hyphae after treatment with cytochalasin E. These results suggest that the peripheral F-actin network in the growing tip of *S. ferax* hyphae establishes or maintains the tip-high gradient of SA channels, either by the delivery of channel-bearing vesicles to the apex or by the interactions between the channels and the peripheral actin network.

Key words: stretch-activated channel, cytoskeleton, tip growth, actin, hyphae

INTRODUCTION

The process of tip growth in diverse plant cells and fungi involves cellular systems such as the cytoskeleton, intracellular osmotic effectors, membrane-located transport mechanisms, cell wall synthesis and mechanisms for transporting organelles including wall vesicles (Heath, 1990). Previous studies on the oomycete *Saprolegnia ferax* (Jackson and Heath, 1989, 1990; Kaminskyj et al., 1992; Garrill et al., 1993) demonstrated that there is a close linkage between these cellular systems. The apical region of growing hyphae in *S. ferax* is cytoplasm-rich, and, adjacent to the cellular membrane, contains a high concentration of filamentous actin (F-actin) that is organized into a network termed an apical cap (Heath, 1987). This cap is considered to be important in tip morphogenesis (Jackson and Heath, 1990) and may have other roles in tip growth (Heath, 1990). Hyphal tips also contain a population of F-actin, which permeates their cytoplasm and may be important in cytoplasmic and organelle movements (Jackson and Heath, 1993a). Many actin-containing networks are well known to be regulated by diverse ions, especially Ca^{2+} (Bray, 1992) and both the actin cap and cytoplasmic contractions in *S. ferax* hyphae are sensitive to Ca^{2+} (Jackson and Heath, 1989, 1992), consequently the regulation of Ca^{2+} in hyphal tips may be of fundamental importance to the tip growth process (for a review, see Jackson and Heath, 1993b).

Recent observations using the patch clamp technique to study ion channels in the plasma membrane of *S. ferax* demonstrated the presence of spontaneous Ca^{2+}-activated K^+ channels (K^+ channels) and channels activated by stretching of the plasma membrane (SA channels) (Garrill et al., 1992). The growing tips of *S. ferax* hyphae possess a tip-high gradient of the SA channels that are permeable to Ca^{2+}, K^+ and Mg^{2+}, and are potentially important in regulating tip growth (Garrill et al., 1993). The gradient of the SA channels may be important to their functions and indicates the existence of some mechanism for it's establishment and maintenance. Such a mechanism may be via interactions between the integral membrane ion channel proteins and the actin cytoskeleton (Niggli and Burger, 1987) with the F-actin cap of the *S. ferax* hyphal tip being well placed for such a role (Heath, 1990).

In the present work we elucidate the possible role played by actin microfilaments in controlling the distribution of the ion transport systems involved in tip growth of *S. ferax* hyphae. Actin in the apical region was disrupted with cytochalasin E (CE) (Cooper, 1987) and the effects of such treatment on channel distributions were observed. The results demonstrate that the distribution of the SA, but probably not K^+ channels, is indeed affected by disrupting the F-actin network with CE.

MATERIALS AND METHODS

Protoplast production

Saprolegnia ferax (ATCC no. 36051) was grown on 3.5 cm × 0.5 cm strips of dialysis membrane overlying the agar growth medium, designated OM (Heath and Greenwood, 1970). Strips were scratched with fine sandpaper in order to improve hyphal attachment to the strip's surface. This scraping helped to stabilize the distinct linear sequence of protoplasts formed during 40-50 minutes of incubation

with digestive enzymes, so that we were able to selectively patch clamp protoplasts derived from definite regions of the hyphal tip (Garrill et al., 1992). After 20-24 hours growth, a segment of strip with mycelium was cut off at a distance of 8-10 mm from the growing edge of the colony and hyphae were left to recover for at least 1 hour before being used in experiments. Protoplasts were prepared according to methods described earlier (Garrill et al., 1992): the strip with hyphae was placed in a Falcon 35 mm × 10 mm tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ), then it was gently rinsed with washing solution (WS) (20 mM PIPES buffer (1,4-piperazinediethanesulfonic acid), 0.5 M sorbitol, 1 mM $MgCl_2$, 1 mM $CaCl_2$; adjusted to pH 6.5 with HCl). For experiments using CE, the hyphae were treated for 10 minutes with 5 μg/ml CE (Sigma Chemical Co., St. Louis, MO) in 0.1% dimethylsulphoxide (DMSO) in OM, then rinsed with WS. After washing, hyphae were mounted in 2 ml of bath solution, containing lysing enzymes (10 mg/ml Driselase (Kyama Hakh Kogyo Co Ltd., Tokyo, Japan), 5 mg/ml Novozyme 234 (NOVOINDUSTRY, Novo Biolabs, Bagsvaerd, Denmark), 20 mM PIPES, 0.5 M sorbitol, 1 mM $MgCl_2$, 1 mM $CaCl_2$; adjusted to pH 6.5 with KOH), in which all electrophysiological experiments were conducted. In some experiments we used 2.5 mg/ml Novozyme 234 and 2.5 mg/ml Pectolyase Y-23 (Seishin Pharmaceutical Ltd, Japan), which appeared to improve the frequency of successful patches.

Actin staining

In order to determine the effect of CE on actin, hyphae were incubated in OM with 5 μg/ml CE in 0.1% DMSO and then fixed and stained with rhodamine-phalloidin (RP) as described by Heath (1987). To find out whether the hyphae continue to grow during CE treatment, they were stained with 0.02% Calcofluor (Sigma) for 5 seconds (Jackson and Heath, 1990) prior to incubation with CE. Microscopy was performed on a Reichert Polyvar microscope equipped with Nomarski differential interference contrast (DIC) or epifluorescence optics (G2 filter set for RP, U1 filter set for Calcofluor); all photographs are reproduced at ×900.

Electrophysiology

The fabrication of micropipettes and patch clamping were carried out as described by Garrill et al. (1992). In some experiments the micropipettes were fire polished, but this did not improve the frequency of sucessful patches or seal resistance. Micropipettes were pulled for a bubble number of 3.8-4.8 (Lew et al., 1990). The tip diameter of each micropipette used for patch clamping was calculated on the basis of a calibration of the tip diameter (in μm) measured with a scanning electron microscope versus observed bubble number. A linear regression yielded a slope of 0.310 and a *y*-intercept of –0.684 (r^2=0.88). Seals were easier to form and were more stable with micropipettes of smaller diameter. The micropipette filling solution (20 mM PIPES, 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, adjusted to pH 6.5 with HCl) was usually isotonic to the bath solution (adjusted to 600-630 mosmol kg^{-1} using sorbitol). All solutions were filtered (Acrodisc PF 0.8/0.2 μm filter, Gelman Sciences, Ann Arbor, MI) prior to use. Usable seals (sub-gigaseals) between the micropipette and protoplast plasma membrane were obtained in approximately 10% of total attempts for both CE and control. The success rate of seal formation was not improved when 2,6-dichlorobenzonitrile (10^{-5} M) (Fluka Chemika, Caledon Laboratories Ltd, St. Louis, MO) was added to the bath solution in order to prevent cell wall regeneration (Delmer, 1987). All experiments were carried out in the cell-attached mode (Hamill et al., 1981) with seal resistances between 100 and 500 megaohms.

Before the tip of the micropipette was passed through the air/solution interface, a positive pressure of about 80 mm Hg was applied to the micropipette interior, resulting in an efflux of micropipette-filling solution which prevented contamination of the micropipette tip. The micropipette was manipulated close to the cell surface until it just touched the protoplast membrane, then the pressure was released from the pipette interior and usually a twofold increase of the input resistance was observed. A few gigaseals were achieved on protoplasts released from the cell wall during the first minutes of digestions. Manipulation of the membrane-pipette seal by further application of negative pressure was continued until no further increase in resistance could be induced. The magnitude of applied suction (usually 10-40 mm Hg) for activating SA channels was correlated with the magnitude of suction required to establish the sub-gigaseal (i.e. higher suction was needed to activate channels in patches that needed higher suction to form a seal). Under these sub-gigaseal conditions, the number of SA channels found was not correlated with the amount of suction.

The number of channels per patch was estimated by assuming that they equal the maximum number of simultaneously open channels observed during a long period (about 5 minutes) of continuous recording, either with suction (SA) or without it (K^+ channels) (Garrill et al., 1992). Even though the permeability characteristics of the K^+ and SA channels differ (Garrill et al., 1992), the variability in each population makes it impossible to differentiate reliably between them when both are present; consequently no such differentiation was made. There are two sources of error in the procedures for determining channel numbers: (1) not all channels may open simultaneously, resulting in an underestimate of channel density; and (2) when K^+ channels are present, they may be included in the count of stretch-activated channels, resulting in an overestimate of SA channel density. The first error is minimized by the fact that channels tend to open in bursts of activity (Garrill et al., 1992). If the second error was introducing a significant bias in the counted number of SA channels, then one would expect a correlation between the numbers of K^+ and SA channels per patch. Linear regression analysis of these data yielded r^2=0.086; thus it was not a significant source of error. Channel densities were calculated by dividing the channel number for each patch by the area of membrane patched, which was assumed to be the same as the micropipette tip aperture area.

In order to estimate the region of hyphae represented by each population of protoplasts, their actual diameters were converted to lengths, assuming a hyphal diameter of 8 μm, and a Gaussian curve for each population was calculated. The intersection points of these curves defined the boundaries between each population, and their peaks defined the mean distances from the tips, as show in Table 2 .

Data were analyzed using the SYSTAT statistical package (SYSTAT Inc., Evanston, IL).

RESULTS

Effect of cytochalasin E on actin and protoplast production

Incubation of hyphae in CE disrupted the normal (Fig. 1a) pattern of actin in hyphal tips. After 5 minutes in CE there were some tips that still contained normal apical caps and had a normal shape, whereas others were more swollen and contained plaques in the cap region (Fig. 1b-e). After 10 minutes in CE, all tips contained plaques, fibrillar caps were absent and many tips were swollen (Fig. 1f-i). Tips treated with 0.1% DMSO for 10 minutes still showed normal caps and shape (Fig. 1j-k). Hyphae stopped tip elongation in the presence of CE, as indicated by the absence of increase in hyphal length forward of the cell wall previously stained by Calcofluor.

As further evidence that CE disrupted the apical cytoskeleton, we observed differences in the formation of the apical protoplasts. For control hyphae, cytoplasm usually contracted

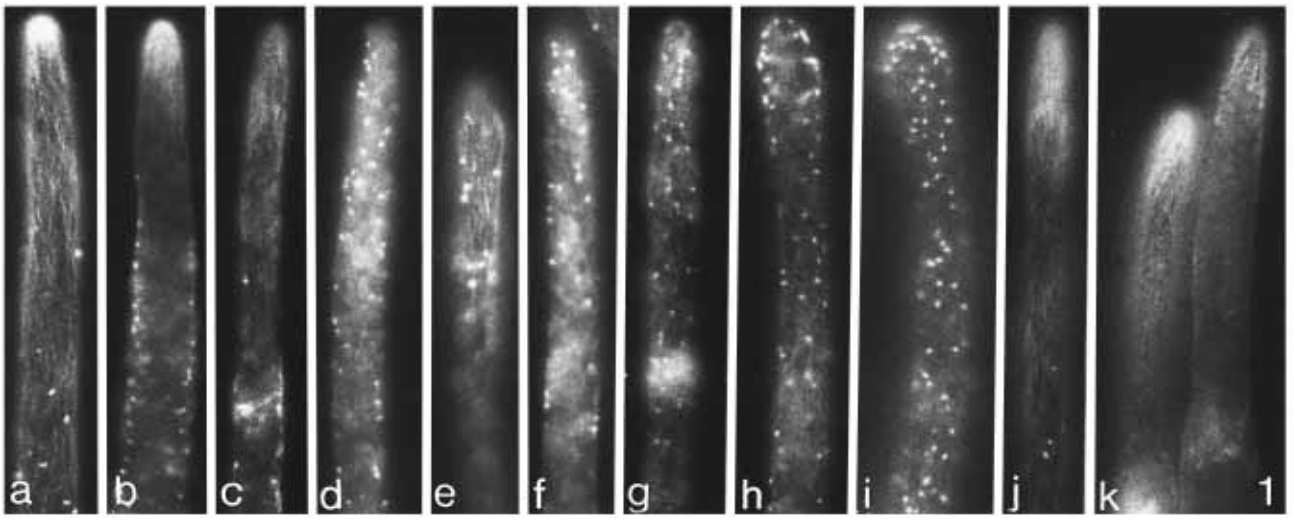

Fig. 1. Effects of cytochalasin E treatment on the actin cytoskeleton. Hyphal tips stained with RP. Control hypha (a) shows a cap of finely filamentous actin and subapical arrays of cables and plaques. (b-e) Hyphae treated with 5 µg/ml CE (final DMSO concentration of 0.1%) for 5 minutes. The actin caps were partly damaged; in some of them actin arrays were unaffected, but more plaques appeared in the cap region and some tips were slightly swollen (e), sometimes the actin remained concentrated in the hyphal apex but fibers were more diffuse (b,d). (f-i) Hyphae treated with 5 µg/ml CE (final DMSO: 0.1%) for 10 minutes. Fibrillar caps are disorganized; also, sometimes hyphae contained diffuse actin concentrated in the apical tip (f,g). All tips contained plaques among diffuse actin, and some tips were swollen (h,i). (j-k) Hyphae treated with 0.1% DMSO (v/v) for 10 minutes. The actin cap structure and the shape of the tips were unaffected; hyphal tips show morphology characteristic of control tips.

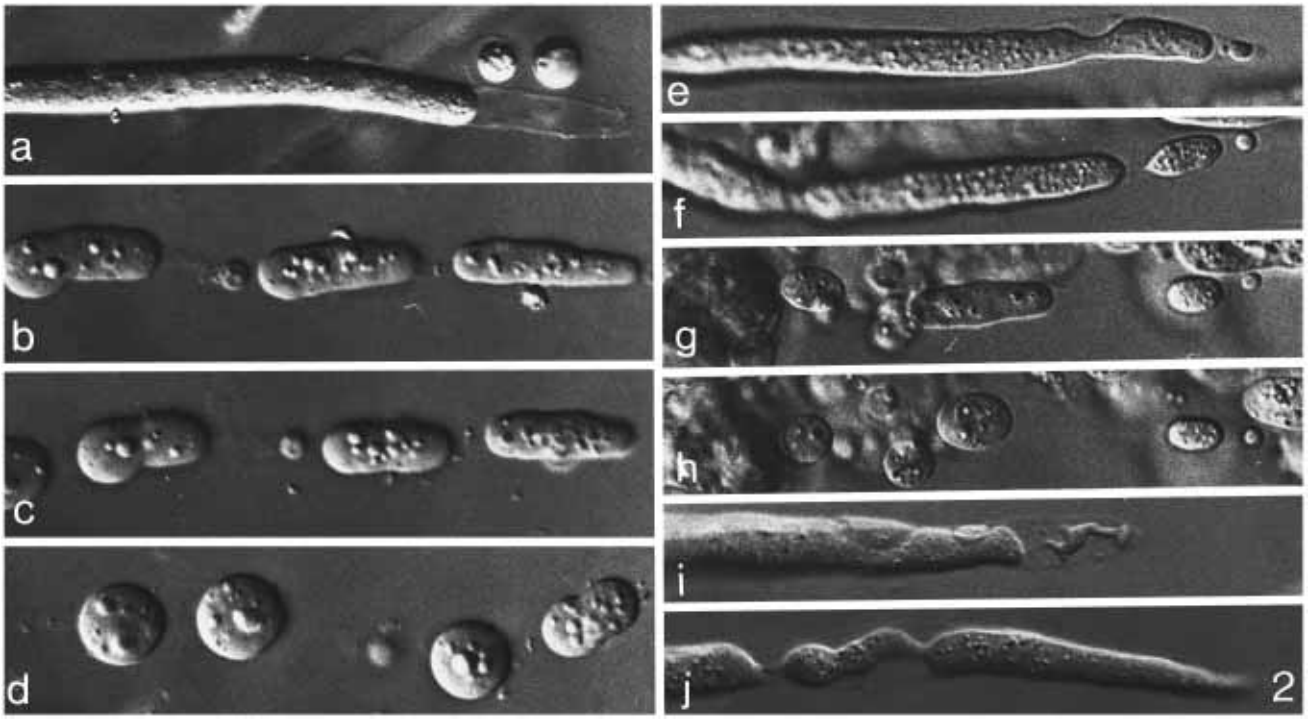

Fig. 2. Protoplast formation for control (a-d) and cytochalasin-treated (e-j) hyphae. Under control conditions, cytoplasm usually contracted from the cell wall during the first few minutes of incubation in digestion solution (DS) (a, 5 minutes in DS), then linear arrays of similar size protoplasts derived from distinct hyphal regions were formed (incubation time in DS: b, 15 minutes; c, 30 minutes; d, 45 minutes). For CE-treated hyphae, the apical cytoplasm sometimes was considerably damaged (i,j) or typically fragmented into small portions (e) during the first few minutes of incubation in DS (e, 5 minutes in DS), yielding small protoplasts ahead of the first large protoplast when linear arrays of protoplasts formed (incubation time in DS: f, 15 minutes; g, 30 minutes; h, 45 minutes).

from the cell wall during the 5-10 minutes incubation in digestive solution and after 30-50 minutes, linear arrays of protoplasts formed. The apical protoplasts were always large, being derived from portions of cytoplasm about 60 μm long (Fig. 2a-d). In contrast, for CE treated hyphae, the apical cytoplasm typically fragmented into small portions which yielded small, unpatchable protoplasts ahead of the first large protoplast (Fig. 2e-j). When this happened, the area of the small protoplast was included in the area of the next, large, protoplast, which was then considered to be the first (apical) protoplast. Because the small, unpatched, protoplasts had diameters of only about 2 μm (Fig. 2e-h), compared with the average tip protoplast diameters of about 18 μm, this procedure is unlikely to have introduced a significant error into the results. Furthermore, three of the seven apical CE-treated protoplasts that did not show SA channels (Fig. 6) came from hyphae that did not fragment into these small apical protoplasts. We did not observe apical fragmentation when hyphae were preincubated for 10 minutes in 0.1% DMSO.

Effect of CE on distribution of SA and K^+ channels

Even though patch-clamp experiments were conducted under sub-gigaseal conditions, both K^+ and SA channel activity could be resolved (Figs 3, 4) (Garrill et al., 1992).

Previously it was reported that DMSO at a concentration 3.3% (v/v) inactivated K^+ channels in *Mougeotia* protoplasts (Lew et al., 1990). In *S. ferax* the densities of K^+ and SA channels were not altered by adding 0.1% DMSO to the bath solution (Table 1), and preincubation of hyphae with 0.1% DMSO for 10 minutes prior to forming protoplasts did not inactivate either K^+ or SA channels.

The mean number of channels observed in patches (the cases

Table 1. Effect of DMSO on mean channel density

	First protoplast		Second and posterior protoplasts		Total	
	Control	DMSO	Control	DMSO	Control	DMSO
K^+ channels	8.81±3.49 (13)	7.92±2.60 (3)	5.75±3.62 (28)	9.13±2.61 (4)	6.72±3.82 (41)	8.61±2.47 (7)
SA channels	10.63±4.63 (12)	15.85±0.34 (3)	4.62±5.70 (23)	6.52±7.83 (4)	6.68±6.04 (35)	10.52±7.45 (7)

Data are number of channels/μm^2; 0.1% DMSO in bathing solution.

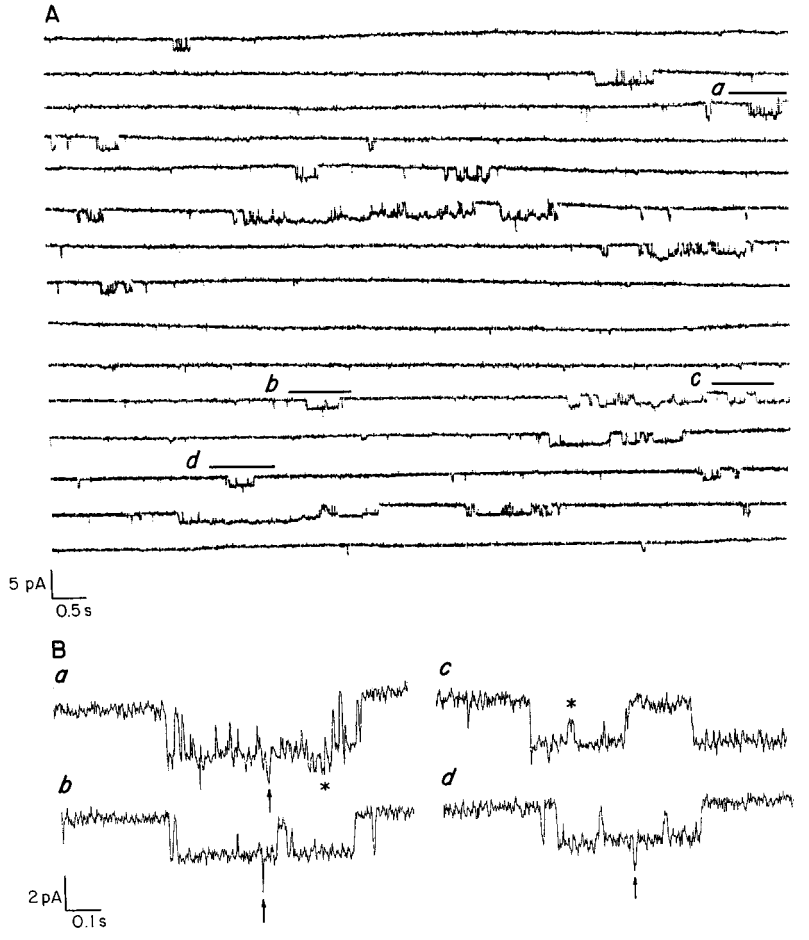

Fig. 3. Example of channel activity: spontaneous. A long trace (A) demonstrates the steadiness of the baseline and clearly discernible channel activity under sub-gigaseal conditions. The expanded view traces (B: *a-d*) show the presence of a sub-conductance state (asterisks). For this example, the channel count is two, based on transitions to a second higher current level (arrows in *a*,*b* and *d*).

Table 2. Distributions of channels in the different protoplast populations

	Tip		Second from tip		Posterior	
	Control	CE	Control	CE	Control	CE
Protoplast sizes						
Mean protoplast diameter (μm)	17.9±4.6 (6)	17.7±4.7 (18)	23.3±6.2 (7)	17.9±2.9 (14)	19.3±3.8 (13)	23.7±5.6 (24)
Mean distance from tip (μm)*	42.0±21.1 (6)	41.8±22.8 (18)	114.0±37.2 (7)	82.8±13.6 (14)	160.9±25.4 (5)	132.4±25.7 (4)
Region of hyphae (μm)†	0-75	0-65	75-135	65-105	>135	>105
K+ channels						
Frequency of patches with channels	1.00 (13)	0.72 (18)	0.82 (11)	0.75 (12)	0.88 (17)	0.68 (25)
Number of channels per patch‡	2.2±0.4 (13)	1.4±1.2 (18)	1.6±1.0 (11)	1.3±1.0 (12)	1.7±1.0 (17)	1.6±1.3 (25)
SA channels						
Frequency of patches with channels	0.92 (12)	0.21 (14)	0.63 (8)	0.55 (11)	0.40 (15)	0.50 (18)
Number of channels per patch‡	2.9±1.7 (12)	0.7±1.4 (14)	1.5±1.4 (8)	1.6±1.6 (11)	1.4±1.9 (15)	1.6±1.8 (18)

Values are given as means, followed by standard deviations, with sample sizes in parenthesis below.

*Distance calculated from the mean protoplast diameter and assuming a hyphal diameter of 8 μm. Because the apical protoplast diameters were not determined for all of the hyphae from which posterior protoplasts were used, the sample sizes for this line are lower than those from the preceding line.

†Distance along the hyphae, determined from the intersection points of the normal distribution curves for each population, as described in Materials and Methods.

‡Values include patches with no channel activity.

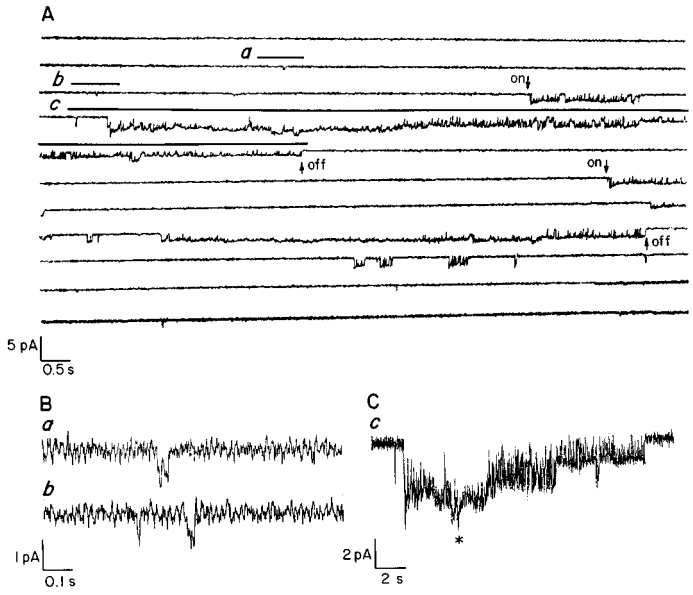

Fig. 4. Example of channel activity: stretch-activated. A long trace (A) demonstrates the steadiness of the baseline and clearly discernible channel activity activated by suction. Arrows marked the onset and removal of suction; additional stretch activation was often seen immediately after suction was removed. The first expanded traces (B: *a,b*) show spontaneous activity, which, in these examples, would not be mistaken for additional SA channels. The second expanded trace (C) (note change in scale) compresses a stretch-activated channel event. The channel count would be three; the third channel opening (asterisk) was visible as a typical steplike transition, even though it occurred during a slight change in the baseline.

in which no activity was seen are also included) and frequency of both SA and K^+ channels are shown in Table 2 for control and for CE-treated cells. Based on the area of micropipette tips and the number of channels contained in a patch, the densities of SA and K^+ channels in the plasma membrane for particular distances from the tip were estimated. The densities of both SA and K^+ channels are shown in Fig. 5. In an attempt to elucidate the gradient of channels in the very tip (0-70 μm) of the hyphae, channel densities are shown, versus the expected distance from the tip calculated from the apical protoplast diamater, in Fig. 6.

As described previously (Garrill et al., 1992), the density of SA channels shows a tip-high gradient, which is primarily due to the higher frequency of patches containing channels, as opposed to differences in the number of channels per patch (Table 2). However, the tip-high gradient is also contributed to by differences in the number of SA channels per patch, especially in the most apical protoplasts where the smallest protoplasts had a higher density of channels (Fig. 6). CE disrupted this gradient by reducing the density of SA channels in the tip protoplasts from 10.63±4.69 (mean ± s.d.) channels/μm^2 to 1.60±3.26 channels/μm^2 (Fig. 5), primarily due to a high proportion of patches lacking channels (Table 2). This lack of SA channels was more pronounced in the smallest, most apical protoplasts (Fig. 6).

In contrast to previous work (Garrill et al., 1993), there was some evidence for a tip-high gradient in the the K^+ channels too (Fig. 5). However, there was no indication of a correlation between the densities of K^+ channels and distance from the tip at the very apical region (Fig. 6). CE caused an apparent decrease in the density of K^+ channels (Fig. 5), the frequency of patches containing channels and the number of K^+ channels per patch (Table 2). The CE-induced differences were more significant for the the apical protoplasts, in which channel

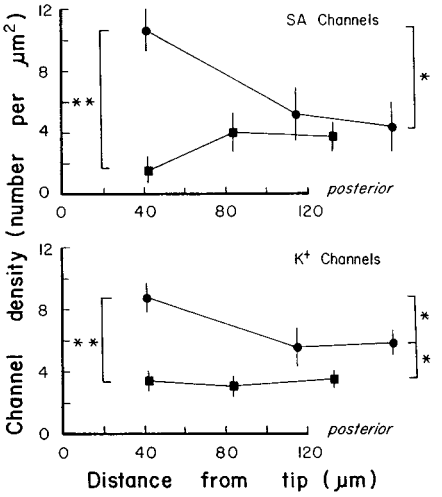

Fig. 5. Effects of CE on densities of SA (upper panel) and K^+ (lower panel) channels. Mean channel densities (channel number per μm^2) are shown versus distance from tip calculated using mean diameters of each protoplasts type and assuming a hyphal diameter of 8 μm. Circles correspond to controls; squares to CE-treated. The vertical bars are standard errors; sample sizes can be obtained from the data in Table 2. Significant differences between first and posterior protoplasts or between control and cytochalasin treatment for a particular protoplast type were determined by the nonparametric Kruskal analysis of variance and are denoted by asterisks (*P<0.05; **P<0.01).

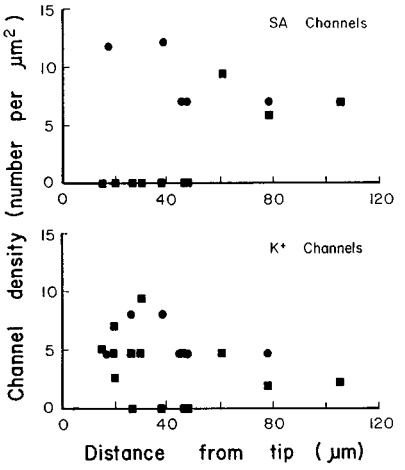

Fig. 6. Effects of CE on channel densities (channel number per μm^2) for apical protoplasts. Data are presented as density of channels versus distance from tip, calculated from the diameter of each protoplast, assuming the hyphal diameter is 8 μm. Upper panel: SA channels, control (circles) and cytochalasin (squares). Lower panel: K^+ channels, control (circles) and cytochalasin (squares).

density decreased from 8.81±3.49 channels/μm^2 in control cells to 3.43±2.73 channels/μm^2 (Fig. 5).

A comparison of the densities of SA channels versus K^+ channels reveals the presence of two populations of K^+ channels (Fig. 7). One population occurs in the absence of SA

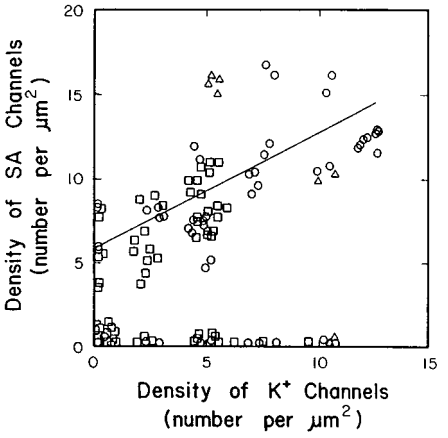

Fig. 7. Relationship between SA channel and K^+ channel densities. The number of channels per μm^2 are shown for controls (circles), CE (squares), and DMSO (triangles). The linear regression is fitted for data in which SA channels were present.

channels (mean ± s.d.: 3.76±3.70 channels/μm^2, n=40) and the other in the presence of SA channels (5.69±3.83 channels/μm^2, n=45). When K^+ channels are present with SA channels, K^+ channel density was positively correlated with the density of SA channels (r^2=0.486), indicating that these K^+ channels are to some extent associated with SA channels. The grouping of K^+ channels into two populations was found in the data for both controls and cytochalasin treatment, and comparisons of regression slopes and channel density for the compiled data were highly significant (P<0.0001).

The distributions of channel number per patch in control and CE-treated protoplasts are shown in Fig. 8. When channels were present, the number of stretch-activated channels was usually three, higher than the usual number of K^+ channels per patch (two). Treatment with cytochalasin did not influence these distributions for either K^+ or SA channels. In *S. ferax* the K^+ channels are distributed randomly in the plasma membrane, while the stretch-activated channels are distributed in non-random aggregates (Garrill et al., 1993). Cytochalasin did not influence the aggregation of SA channels. Cytochalasin did, however, cause an increase in the number of patches in which no channels were found.

DISCUSSION

In hyphae of *S. ferax*, tip-high calcium distribution, the tip-high gradient of stretch-activated Ca^{2+}-permeable channels and actin microfilaments that are organized in a finely fibrillar peripheral apical cap are associated with the process of polarized tip growth (Heath, 1990; Jackson and Heath, 1993b; Garrill et al., 1993). The existence of a tip-high gradient of Ca^{2+}, both cytoplasm-free and membrane-associated, is likely to be maintained by influx of Ca^{2+} via the stretch-activated Ca^{2+} channels localized in the apical membrane. It was speculated that the high density of SA channels in the hyphal tip, as well as their aggregation, are due to interaction between the channels and F-actin peripheral microfilaments (Garrill et al., 1993). The present study was undertaken to elucidate the

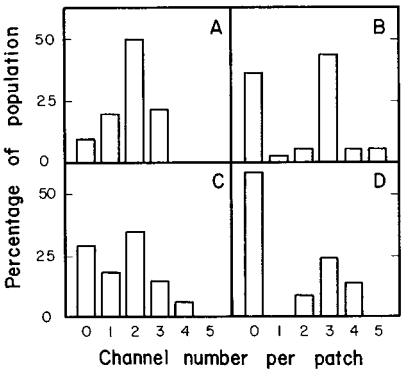

Fig. 8. Influence of CE on distribution of channel numbers per patch for K^+ channels (A,C) and SA channels (B,D). The number of channels per patch was estimated as the maximum number of concurrently open channels in a patch for all protoplast types. (A,B) control; (C,D) CE.

possible mechanism for achieving the SA channel gradient along growing tips of *S. ferax* and provides the first direct evidence for a role for F-actin in producing a non-random distribution of SA channels.

Although the cytochalasins are widely used as specific disruptors of cellular actin systems (Cooper, 1987), their mode of action is not straightforward and their effects cannot be taken for granted. However, the connection between a peripheral actin network and the plasma membrane can be disrupted with cytochalasin E in animal cells (Wessels et al., 1971). In *S. ferax*, Jackson and Heath (1990) showed that CE disrupted apical actin, altered tip morphology and accelerated growth in hyphae, and Heath and Harold (1992) showed effects on actin participating in zoosporogenesis. Our demonstration of the disruption of hyphal tip actin with rhodamine-phalloidin staining, frequently correlated with hyphal shape changes and changes in the pattern of cytoplasm behaviour during protoplast formation, together show that the regime used at present does indeed disrupt the actin in the periphery of the hyphal tips and is consistent with these previous observations. This suggests that the CE-induced changes in the distribution of the SA channels are indicative of a role for actin in generating their normal tip-high gradient.

There are two likely ways in which the peripheral actin may be involved in determining the tip-high gradient of the SA channels. The first is that channels could be directly coupled to the actin filaments of the cap and moved, or constrained, by the interaction. The filaments of the cap are well placed for this role because they lie very close to the plasmalemma (Heath, 1987). However, in this model, disruption of the filaments would be predicted to release the channels to the forces of diffusion in the membrane and lead to the concomitant reduction of density in the tip protoplasts, and increased density in the second and posterior protoplasts. This did not occur; instead CE caused a reduction in the density of SA channels in all protoplast categories, although the reduction in the non-apical categories was not as significant (Fig. 5).

The alternative model for a role for actin in SA channel gradient formation is that it is responsible for localized insertion of the channels in the tip. This model predicts that disruption of the actin would inhibit the insertion process, leading to the absence of channels in the tip. The CE-induced decrease in high SA channel density in the whole population of tip-derived protoplasts (Fig. 5) as well as the observed maximum incidence of SA channel-deficient patches in the smaller, more apical protoplasts after CE treatment (Fig. 6), are consistent with this suggestion and indicate a very localized insertion zone in the extreme tip. However, localized insertion alone will not generate a gradient of channels. Sub-apical removal of channels (presumably by turnover) or plasmalemma area expansion without further channel insertion are the most likely mechanisms for gradient generation but we have no direct evidence to differentiate between them. Because the gradients for SA and K^+ channels differ from each other (gradient of K^+ channels is flatter or non-existent; Garrill et al., 1993), either their turnover rates or their insertion patterns differ. Subapical removal is conceptually simpler and the implied greater persistence of the K^+ channels is consistent with their presumed role in osmoregulation, a process that does not demand localized channel activity (Garrill et al., 1993).

With respect to the mechanism of channel insertion, the commonly accepted view of tip growth is that plasmalemma synthesis is primarily accomplished by fusion of Golgi body-derived vesicles with the tip membrane (Heath et al., 1985), in which case one would predict that the channels would be delivered in the vesicles. Transport of the vesicles is likely to be actin-mediated, and thus CE-sensitive, in *S. ferax* (Heath and Kaminskyj, 1989), in which case CE-induced formation of channel-deficient apical protoplasts is most likely due to some expansion of the plasmalemma after interruption of vesicle supply (cf. bulbing caused by CE in Fig. 1). This might be expected if the F-actin apical cap plays a role in regulating tip extensibility, as previously suggested (Jackson and Heath, 1990).

In a previous study of the apex of tip-growing fucoid rhizoids, Brawley and Robinson (1985) showed that the normally localized inward endogenous electrical currents (which had been suggested to depend on a non-random distribution of Ca^{2+} channels; Robinson and Jaffe, 1975) could be reduced by cytochalasin-induced disruption of cortical actin arrays. They favoured a model involving actin-mediated vesicle transport of the channels to the apex, but could not rule out other models.

While F-actin appears to play a role in the production of the SA channel gradient, it is less clear that it is responsible for the observed clustering of the SA channels, since the clusters persist after CE treatment. However, it was previously suggested that the clusters correlated with the plaques of actin that form in the tips when hyphae cease growth (Garrill et al., 1993). The findings that CE did not influence this aggregation and CE-treated hyphae also contain actin plaques are entirely consistent with the model.

The observed CE induction of small protoplasts in the hyphal tips has been used as direct evidence that the CE is effective in altering the cellular F-actin. It is also an interesting indication that F-actin is important in determining the shape of the cytoplasm, independently of the cell wall, and thus supports the suggestions the F-actin plays a role in tip morphogenesis (Jackson and Heath, 1990), as well as cell volume regulation (Cornet et al., 1993; Chowdhury et al., 1992).

We conclude that the tip-high gradient of SA channels is established and maintained by an actin-dependent system.

Further work will be needed to differentiate between the anchoring and insertion models for the way in which actin is involved.

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