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The roles of Ca^{2+} and plasma membrane ion channels in hyphal tip growth of *Neurospora crassa*

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SUMMARY

Growing hyphae of the ascomycete fungus *Neurospora crassa* contained a tip-high gradient of cytoplasmic Ca^{2+}, which was absent in non-growing hyphae and was insensitive to Gd^{3+} in the medium. Patch clamp recordings in the cell-attached mode, from the plasma membrane of these hyphae, showed two types of channel activities; spontaneous and stretch activated. The spontaneous channels were identified as inward K^+ channels based on inhibition by tetraethylammonium. The stretch activated channels had increased amplitudes in response to elevated Ca^{2+} in the pipette solution, and thus are permeable to Ca^{2+} and mediate inward Ca^{2+} movement. Gd^{3+}, which is an inhibitor of some stretch activated channels, incompletely inhibited stretch activated channel activity. Both tetraethylammonium and Gd^{3+} only transiently reduced the rates of tip growth without changing tip morphology, thus indicating that the channels are not absolutely essential for tip growth. Furthermore, in contrast to the hyphae of another tip growing organism, *Saprolegnia ferax*, tip-high gradients of neither spontaneous nor stretch activated channels were found. Voltage clamping of the apical plasma membrane potential in the range from –300 to +150 mV did not affect the rates of hyphal elongation. Collectively, these data suggest that ion transport across the plasma membrane at the growing tip in *Neurospora* is not obligatory for the maintainance of tip growth, but that a gradient of Ca^{2+}, possibly generated from internal stores in an unknown way, is required.

Key words: *Neurospora crassa*, tip growth, stretch-activated channel, voltage clamp, Ca^{2+}

INTRODUCTION

Hyphal tip growth is a highly dynamic and complex process which is the fundamental growth form of the fungal kingdom and is also characteristic of specialized cells within the plant kingdom. Localized synthesis and expansion of the growing tip are regulated such that a tube (a hypha) of constant diameter is produced (Harold, 1990; Heath, 1990a; Ruiz-Herrera, 1991). The regulation of tip expansion involves cell wall extendibility (Bartnicki-Garcia and Lippman, 1969; Staebell and Soll, 1985; Wessels et al., 1983; Wessels, 1986) and the cytoskeleton associated with the plasma membrane (Allen et al., 1980; McKerracher and Heath, 1987; Heath, 1990b; Jackson and Heath, 1990; Adams et al., 1991; Barja et al., 1991), as well as turgor pressure (Kaminskyj et al., 1992; Money and Harold, 1993). However, the way in which these aspects of the process are coordinated is unclear. An attractive possibility is ionic regulation, whereby modulation of one or more ion concentrations in the apical cytoplasm would mediate the appropriate responses of the different components and thus ensure their coordinated behavior.

In common with other cellular regulatory systems, there is evidence for a role for Ca^{2+} in tip growth (Picton and Steer, 1983; Brownlee and Wood, 1986; Clarkson et al., 1988; Schmid and Harold, 1988; Rathore et al., 1991; Obermeyer and Weisenseel, 1991; Miller et al., 1992; Jackson and Heath, 1993; Pierson et al., 1994; Hyde and Heath, 1995). Given the extensibility of the hyphal tip, the recent demonstration of stretch activated (SA), Ca^{2+} transmitting channels in hyphae and yeasts (which can be viewed as showing a form of tip growth; Heath, 1990a) (Gustin et al., 1988; Zhou et al., 1991; Garrill et al., 1992; Zhou and Kung, 1992) has provided the intriguing possibility of a feedback regulatory system in which the degree of stretch of the surface of the hyphal tip determines the rate of influx of Ca^{2+} ions. This may then regulate the rate of synthesis and degree of extensibility of the tip. Evidence for such a system is most advanced in the oomycete, *Saprolegnia ferax*, in which SA channels that pass Ca^{2+} occur in an actin-dependent (Levina et al., 1994), tip-high gradient (Garrill et al., 1992). This gradient is predicted to lead to the observed tip-high gradient of Ca^{2+} ions (Garrill et al., 1993). The essential role of these ions in tip growth is shown by the disruption of both the gradient and growth following blockage of the channels (Garrill et al., 1993). If this system is of fundamental importance to the tip growth process, its essential components should occur in other tip growing cells. The presence of SA channels has been demonstrated in other fungi (Gustin et al., 1988; Zhou et al., 1991; Zhou and Kung, 1992), but their

distribution, their role in tip growth or budding, and the presence of Ca^{2+} gradients is unknown. Conversely, while the generality of a tip high gradient of Ca^{2+} is clear in pollen tubes (Rathore et al., 1991; Obermeyer and Weisenseel, 1991; Miller et al., 1992; Pierson et al., 1994), there are no data on the presence or distributions of plasma membrane channels in these cells, although K^+ channels have been investigated in pollen grain protoplasts (Obermeyer and Kolb, 1993).

We have extended our work on *Saprolegnia* to the unrelated hyphal fungus, *Neurospora crassa.* Hyphal tips do contain SA, Ca^{2+} passing channels, but not in a detectable gradient, and they are not essential for growth. Conversely, growing but not non-growing hyphae do contain a gradient of cytoplasmic Ca^{2+}, which is not abolished by Gd^{3+}.

MATERIALS AND METHODS

Cultures and handling of cells

Neurospora crassa wild-type strain RL21a (FGSC no. 2219) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kansas, USA. Mycelium was grown on 3.5 cm × 1 cm scratched strips of dialysis membrane overlying solid (2% agar w/v) Vogel's minimal medium (Vogel, 1956) plus 2% sucrose. After incubation in the dark at 28°C for 18 hours the strips with mycelium were cut off 0.5-0.8 cm behind the edge of the colony and Petri dishes with cultures were stored at room temperature (21-23°C) to allow hyphae to recover and start to re-grow for at least 1 hour before being used in experiments. All experiments were made on 19-23 hour cultures. *Saprolegnia ferax* (ATCC no. 36051) was grown as described previously (Jackson and Heath, 1990).

Ca^{2+} imaging

We acid-loaded (Bush and Jones, 1987) hyphae with Ca^{2+} sensitive fluo-3 and Ca^{2+} insensitive SNARF-1. This method has been widely used for diverse turgescent walled cells (Halachmi and Eilam, 1989; Lynch et al., 1989; Russ et al., 1991; Hahm and Saunders, 1991; Garrill et al., 1993). We used these two dyes, as opposed to a single ratio dye, because of the limited spectral lines available in our confocal microscope (Rijkers et al., 1990; Diliberto et al., 1994).

A segment of dialysis strip bearing 18 hour mycelium was cut 3-4 mm from the growing edge of a colony and placed on a slide in a drop of liquid Vogel's medium (0.2% Na-citrate buffer, pH 4.2). The strip was pulled away and the hyphae were flooded with an additional 180 μl of Vogel's medium (pH 4.2) containing 176 μM fluo-3 (pentaammonium salt) (Teflabs, Austin, TX, USA) and 118 μM 5- (and 6)-carboxy SNARF-1 (Molecular Probes, Eugene, OR, USA) with 1% DMSO. Hyphae were covered with a coverslip supported by strips of teflon tape (Heath, 1987) and placed in a moist Petri dish. These cultures were grown for 18-20 hours in the dark at 21-23°C to slow growth, so that they could incubate for sufficient time to load without growing out of the chamber (the optimal growth temperature is 28°C). By the 20th hour a few growing leading hyphae had successfully accumulated the dyes. After loading, the culture was rinsed with Vogel's medium (pH 4.2 or pH 6.0) and the slide was mounted on a thermoregulated microscope stage (Carl Zeiss, Jena, Germany) at 28°C. Living, growing hyphae were observed in the slide chambers with perfusion of Vogel's medium. When hyphae were rinsed with medium at pH 6.0, the dyes washed out of the hyphae and fluorescence decreased (cf Slayman et al., 1994), thus we routinely used medium at pH 4.2, which avoided this problem. The levels of loading and fluorescence achieved by this technique were low relative to autofluorescence levels (discussed below), but attempts to achieve more with higher concentrations of fluo-3 in the medium resulted in dye precipitation and no more dye in viable hyphae.

For observation, we used a Bio-Rad MRC 600 laser confocal microscope (Bio-Rad, Mississauga, ON, Canada), fitted with a krypton-argon laser and K1 and K2 filter blocks. The latter was modified by replacing the 585 nm barrier filter with a band pass filter transmitting at 610 nm (Omega Optical, Brattleboro, VT, USA). Fluorescence images excited at 488 nm were detected simultaneously in the fast photon counting mode via an oil immersion, 1.4 NA, 60× objective and analysed with Comos and SOM software. The number of scans used to collect the data was selected to be the fewest needed to reveal an image while minimizing total exposure and photobleaching, and varied from hypha to hypha because of the variable level of dye loading. The 522 nm filter of the K2 block selected the Ca^{2+} sensitive fluo-3 emissions (F) and the 610 nm filter selected the isobestic Ca^{2+}-independent emission of SNARF-1 (S). Ratio images (F/S) were created on an Image-1 system (Universal Imaging Corporation Media Corp., West Chester, PA, USA). Measurement of the rate of hyphal growth in these experiments used an ocular micrometer (1 μm/div.).

Mean per pixel F and S intensities were obtained from rectangles of 34×23 or 30×20 pixels (pixel size 0.09 μm square on the specimen) (selected to give the largest area exclusively over the cytoplasm) centred at '1', 3 and 25 μm from the very tip. In fact the nominally '1' μm box was positioned as close as possible to the tip, while having its entire area located over cytoplasm. Because autofluorescence (in the absence of the dyes, measured under the same regime as loaded hyphae) of growing hyphae at both the fluo-3 and SNARF wavelengths (autoF and autoS) was high (compare lines 1 and 2 with 3 and 4 in Table 1), we corrected the mean loaded values by subtracting the mean autoflourescence values (lines 5 and 6, Table 1) prior to calculating the ratios.

In order to convert the F/S ratios to intracellular Ca^{2+} concentrations, we first estimated the concentrations of the dyes inside the hyphae. We formulated a simulated cytoplasm containing 146 mM K^+, 26 mM Na^+, 1 mM Mg^{2+} (Slayman, 1965), 0.1 μM Ca^{2+} (Miller et al., 1990), 10 mM HEPES (pH 7.05, estimated to be the internal pH corresponding to the external pH of 4.2; Sanders and Slayman, 1982) and 4.3 mM EGTA. The concentration of EGTA necessary to give 0.1 μM free Ca^{2+} was calculated according to the method of Fabiato and Fabiato (1979). We did not include sucrose, commonly incorporated to simulate cytoplasmic viscosity (e.g. Miller et al., 1992; Peonie, 1990), because at the high concentrations used it will alter the water activity of the system to very non-physiological levels. This omission may have led to an approximately 15% overestimation of the Ca^{2+} concentration (Peonie, 1990). This synthetic cytoplasm was autofluorescent (similar in intensity to that of the hyphae for SNARF and about half that of the hyphae for fluo-3), the values of which were subtracted from the measured values of the

Table 1. Fluorescence and Ca^{2+} ratio data for dye-loaded hyphae

	Distance from tip (μm)		
	1	3	25
Autofluorescence (*n*=26)			
1. Mean autoF	173±9	181±9	186±8
2. Mean autoS	116±2	121±2	121±2
Loaded cells (*n*=124)			
3. MeanF	327±15	330±14	255±8
4. MeanS	223±9	212±7	197±6
5. MeanF-mean autoF (3-1)	154	149	69
6. MeanS-mean autoS (4-2)	107	91	76
7. Mean corrected ratio F/S (5/6)	1.44	1.64	0.91
8. Mean [Ca^{2+}] (μM)	0.057	0.070	<0.01

Values are means ± s.e.m. Units for lines 1-6 are photons/pixel ×100. The values in line 8 are calculated from Fig. 1 and line 7.

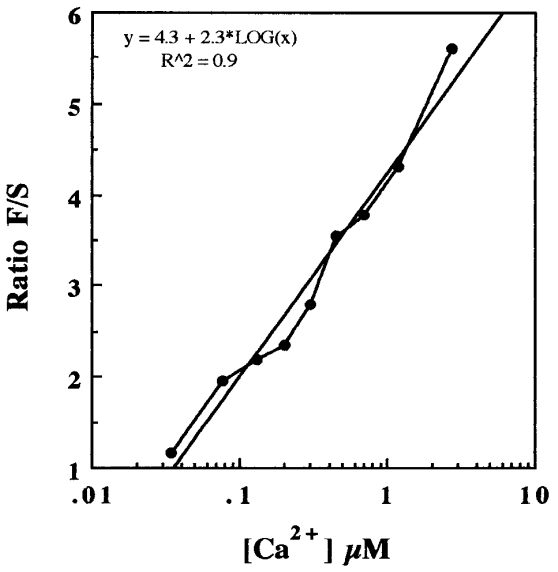

Fig. 1. In vitro calibration of F/S ratios (corrected for autofluorescence as described in the text) to Ca^{2+} concentration. The formula indicates the equation of the best fit line, used to determine Ca^{2+} concentrations in the hyphae.

dye solutions prior to use for ratio determinations. Using the simulated cytoplasm solution with various concentrations of dyes on a slide, observed under the same conditions used for the hyphae, we determined the dye concentrations corresponding to the values of F and S observed in the 25 μm region of the hyphae to be approximately 6 and 22 μM, respectively. These values indicate differential permeability of the two dyes since only 3% of the initial concentration of fluo-3 entered, compared with 19% of the SNARF-1, presumably due to the larger size and more charged nature of fluo-3. The determined concentrations were then used to calibrate the F/S ratios of the hyphae using the calcium calibration buffer kit with magnesium I (C-3721) (Molecular Probes, Inc; Eugene, OR, USA) in the slide system (Fig. 1). All calibration measurements were performed at 23-25°C, as opposed to the 27°C of the growing cells, but the temperature induced error in the free $[Ca^{2+}]$ estimations due to changes in both the Ca^{2+}/fluo-3 binding affinity and K_d for Ca^{2+}/EGTA are not significant because these values show only about a 5% change over a 15°C range (Lattanzio, 1990). Since the Ca^{2+}/fluo-3 and Ca^{2+}/EGDTA binding affinities are strongly dependent on pH (Lattanzio, 1990), we performed the Ca^{2+} calibration with special care. In *Neurospora* hyphae, the intracellular pH is 7.05 at an external pH of 4.2 (Sanders and Slayman, 1982). Thus we performed all calibrations at pH 7.05. The pH of the calibration kit is 7.2, but a change to 7.05 causes a twofold increase in the K_d of EGTA for Ca^{2+} (Tsien and Pozzan, 1989), for which we compensated by recalculating the free $[Ca^{2+}]$ when diluting the calibration kit.

Protoplast isolation

To prepare *Neurospora* protoplasts, apical fragments of hyphae attached to a dialysis strip were rinsed with bath solution (BS) containing 10 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, 100 mM KCl, 2% sucrose (adjusted to pH 5.8 with KOH and 700 mosmol.kg^{-1} with sorbitol), transferred to a Falcon 35 mm × 10 mm tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ, USA), taped to the bottom with Timemed tape (Fisher Scientific Ltd, Ottawa, Canada), and incubated for 15 minutes at room temperature in 0.5 ml of BS containing Novozyme 234 (InterSpex Products, Inc., Foster City, CA, USA) and β-glucuronidase (Sigma, St Louis, MO, USA) (2 mg each per ml of BS adjusted to 900 mosmol.kg^{-1} with sorbitol).

The procedure for protoplast isolation was similar to that of Wiley (1974) with modifications according to Garrill et al. (1992). After releasing protoplasts in the tips (Fig. 2a-c), the total volume of the solution in the experimental chamber was increased to 2 ml by addition of BS with lower osmolarity (700 mosmol.kg^{-1}). Dilution of the hypertonic lysing medium caused extrusion of protoplasts through pores along partially digested cell walls (Fig. 2f) so that by a further 10-15 minutes it was possible to get linear sequences of 3-5 protoplasts in the apical regions of hyphae (Fig. 2e) and thus to selectively patch protoplasts originating from specific areas of the apex. Branches longer than the main hyphal diameter formed separate protoplasts, while those from 'buds' (the newest initiating branches) shorter than the main hyphal diameter merged with the protoplast from the hyphal trunk (Fig. 2d). Usually seals could be obtained more easily and were more stable with protoplasts released from the tip within 2-3 minutes of incubation with digestive enzymes. Only cytoplasmically 'smooth' protoplasts (Fig. 2d-f) were used for patching within 30-40 minutes of incubation, since the quality of protoplasts began to deteriorate after this time, i.e. the frequency of seal formation began to decline and protoplasts became fragile and granulated (c.f. Peberdy, 1979; Selitrennikoff et al., 1981).

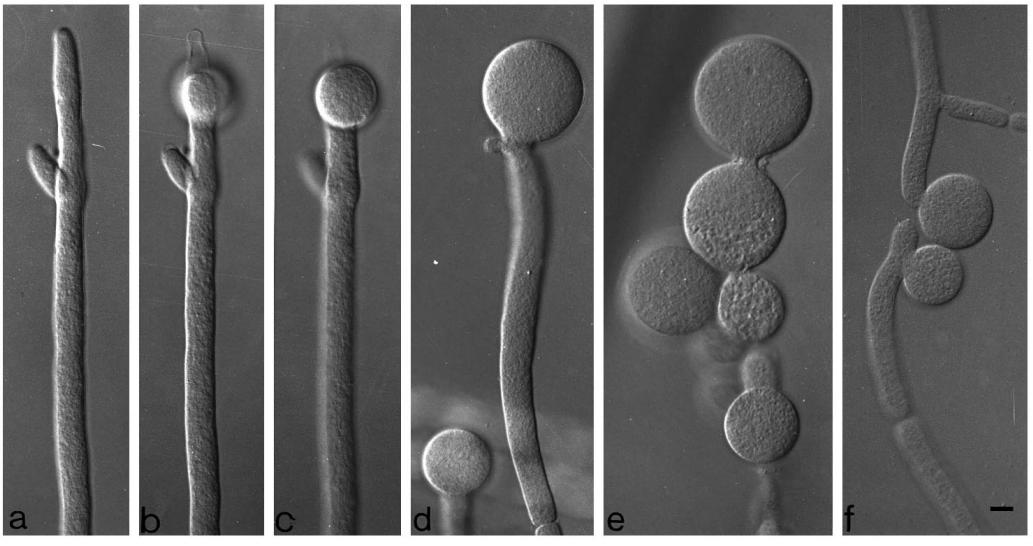

Fig. 2. Protoplast formation from *Neurospora* hyphae: (a) hypha before the addition of digestion solution (DS); (b,c) within approximately the first 2-3 minutes of incubation in DS, the apical protoplast exits from the apex of a leading hypha; (d,e) sequence of protoplasts (that from the small apical branch, or 'bud', merged with the apical protoplast) formed after 15-20 minutes in DS; (f) protoplasts exit through holes in the cell wall following decrease of osmolarity after 5-10 minutes in DS. Bar, 10 μm.

Patch clamp experiments

The fabrication of micropipettes, patch clamping, recording, data acquisition and statistical analysis were carried out as described previously (Garrill et al., 1992). Micropipettes were pulled for a bubble number of 4.0 (which corresponds to a mean tip diameter (±s.d.) of 0.56±0.04 μm). Pipettes were not fire-polished. Pipette solutions (PS) were usually 10 mM Pipes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM KCl, adjusted to pH 5.8 with KOH, with an osmolarity 10% hyper-osmolar to BS, unless otherwise mentioned. High suction (~100 mm Hg) for tens of seconds was used to form a seal. After formation of a stable seal the pressure on the patch pipette was released. The resulting observed channel activity was interpreted as spontaneous (SP). Channel openings appearing as the result of membrane stretching by further application of negative pressure on the interior of the pipette without change of seal resistance were considered as SA channels (Morris, 1990). It was usually possible to maintain a negative pressure of up to 50 mm Hg in 50% of the channel-containing patches without changing the basal seal resistance. Movement of the membrane in the pipette during suction was not observed. Pressure monitoring during the experiments was with a model DPM-1B Pneumatic Transducer Testert (Biotek Instruments Inc., Winooski, VT, USA).

Ionic currents were studied in the cell-attached configuration (Hamill et al., 1981) with seal resistances of 100-200 MOhm. The membrane potential of the protoplasts is unknown in this configuration; pipette potentials were adjusted to approximately 0 mV relative to the bath. Negative or 'inward' current is defined as positive charge directed into the cell, and presented as downward steps in the traces. Under these subgigaseal conditions we were able to obtain seals for about 3-5 minutes in approximately 10% of total attempts, and of these about 80% contained both SP and SA channels. The currents were measured with a patch-clamp amplifier (Model 8900, Dagan Corp., Minneapolis, MN, USA) and stored on a videocassette recorder (Model DAS-8900, Dagan Corp., Minneapolis, MN, USA). For analysis, saved data were digitized at 1 kHz with a Labmaster DMA data acquisition board (Scientific Solutions, Cleveland, OH, USA) after low pass filtering at 200 Hz.

The number of channels in a patch was taken as the largest number of channels to be open simultaneously. Channel densities were calculated by dividing the channel number for each patch by the area of membrane patched, which was assumed to equal the micropipette tip aperture area. This assumption is not strictly correct because after suction is applied to the interior of the pipette, the patch forms an Ω-shape which 'seals' to the tip opening. Part of the patch is sealed to the pipette wall (70%), and part faces the pipette solution (30%) (Sakmann and Neher, 1983). This error should be equal for all protoplasts. However, if protoplasts derived from different hyphal regions differentially pull into the pipette prior to seal formation, due to differences in membrane properties or associated parts of the cytoskeleton (Sokabe and Sachs, 1990; Ruknudin et al., 1991), our area measurements will be in error. Estimation of patch area by measurement of patch capacitance (Sakmann and Neher, 1983; Sokabe et al., 1991) is only possible with gigaseals, which have not been achieved. However, the general appearance of the protoplasts from the different regions of the tips, and their resistance to rupture during handling was similar, suggesting that our assumption of similar degrees of membrane entry into the pipette during patch formation is not unreasonable.

Under sub-gigaseal conditions, channel current amplitude is attenuated due to a shunt to ground between the pipette tip and the membrane (Neher et al., 1978; Lew et al., 1992). Channel amplitudes were corrected by using the attenuation factor, R_s/R_p+R_s, where R_p is the pipette resistance, R_s is the shunt resistance.

In the experiments with channel inhibitors the backfilling technique of Auerbach (1991) was used. The pipette tips were filled by capillary action with PS, the fill height (about 100 μm) was monitored using an ocular micrometer. Shanks were then backfilled by syringe with PS containing either 10 mM tetraethylammonium chloride (TEA) or 100/500 μM $GdCl_3$ (Gd^{3+}). In controls, shanks were filled with just the PS. The time between joining of the solutions after removal of the air bubble which lies between them, by gentle flicking of the pipette, and the start of channel recording was about 2 minutes for the SP channels and 4 minutes for the SA channels.

Estimation of the region of hyphae corresponding to each protoplast population was based on diameters of protoplasts, assuming a hyphal diameter of 8 μm. A Gaussian curve for each population was calculated and the boundaries between each population were defined as the intersection points of these curves; their peaks defined the mean distances from the tips, as shown in Table 4.

Data were analyzed using the SYSTAT statistical package (SYSTAT Inc., Evanston, IL, USA). Plotted data are given as mean ± s.e.m.; *n* denotes the number of independent experiments. All experiments were performed at room temperature. Data from only one cell are usually presented, but experiments were repeated at least three times, and the results were qualitatively similar.

Voltage clamping and intracellular recordings

Double-barrelled micropipettes for simultaneous recording of membrane potential and current injection for voltage clamping were fabricated as described previously (Lew, 1991) and filled with 3 M KCl. The diameter of the tip apertures was about 0.05 μm, and overall tip size was about 0.2 μm. BS contained 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Pipes (adjusted to pH 5.8 with KOH and 260 $mosmol.kg^{-1}$ with sucrose). Handling of cells was similar to that for measurements of growth. Impalements were not performed until cells had been in BS for several minutes to allow them to resume growth. Actual experiments from the time of impalement onwards did not last more than five minutes as the tips of the hyphae soon grew out of the video screen area. Impalements were performed under a water immersion objective (40×, NA 0.75) while the hyphal tip was mechanically supported by a glass micropipette. Images of the impaled growing hyphae were observed on a video monitor and simultaneously recorded on video tape. The video tape was played back and tip extension was measured every 15 seconds using an image processing system (IMAGE-1, Universal Imaging Corp., West Chester, PA, USA). The resolution of this system was such that length increases of >2 μm could be reliably measured.

Usually, an initial negative potential of about 10-20 mV was seen when the microelectrode was slightly pressed against the cell wall, which corresponded to the Donnan potential of the cell wall. From this point on, great care was required so as not to burst or cause damage to the hyphal tip while penetrating the cell wall and entering the cell. The point of impalement was usually located within 12-34 μm from the apex. Impalement was confirmed by injecting current through one micropipette and observing a voltage deflection in the other micropipette (there was no crosstalk observed between the barrels, tested before and after each impalement). When a stable potential was reached and no cytoplasmic leakage was observed, current voltage measurements and voltage clamp experiments were performed as described (Lew, 1991) via an operational amplifier configured for voltage clamping, controlled by a data acquisition system (Scientific Solutions, Cleveland, OH, USA) and compiled C programs. The current-voltage measurements were usually run after each voltage clamp, which varied in duration from 30 seconds to 120 seconds in different experiments.

Microscopical observations

To determine the branching pattern of hyphae of *Neurospora* and *Saprolegnia*, colonies were grown for 20 hours and portions cut about 1 cm behind the edge of the colony, fixed with 4% formaldehyde for 5 minutes, viewed with Nomarski differential interference contrast optics and video recorded at 90×.

To observe hyphal growth, strips of membrane with mycelium were attached to the bottom of a Petri dish with tape and immediately covered with the same BS used for the voltage clamp experiments,

with the osmolarity adjusted to 260 mosmol.kg^{-1} by sucrose. That is a 'balancing-point' in which normal extension of the hyphal apex takes place without significant changes in tip morphology (Robertson and Rizvi, 1968). After approximately 10 minutes of recovery, extension of individual hyphae was video recorded in the growth chamber under the microscope for 30 minutes (magnification 960×). After 10 minutes of persistent growth, BS was replaced by BS containing 10 mM TEA or 100 μM Gd^{3+}, or fresh BS as control. The distances between tips and branches or 'buds', and linear extension rates of hypha were measured from video recordings with the IMAGE-1 system.

To determine the effect of inhibitors on osmotic shock-induced bursting of growing hyphae, hyphal apices were cut from mycelium and immediately immersed in hypotonic medium (1:2 diluted BS, with or without inhibitors, kept at 130 mosmol.kg^{-1} with sorbitol), on a slide for 3 minutes. The numbers of burst and intact tips per slide were scored from video images. At least 60 apices of leading hyphae were counted for each slide, with a total of about 300 for each reported value.

RESULTS

Ca^{2+} distribution in hyphae

All of the hyphae used to analyse the distribution of Ca^{2+} were growing at a mean rate of 5.0±0.2 μm/min (range 1.5-12), which is lower than that observed in the open conditions used for analysis of inhibitor effects on growth rates (see below), probably due to the effects of low O_2 levels under the coverslip, the low pH and the interference of the fluo-3 with intracellular Ca^{2+}. In these hyphae, there was a consistent tip-high gradient of Ca^{2+}, which peaked at about 3 μm behind the tip (Table 1 and Figs 3, 4). The slope of this gradient can only be estimated at about 10-fold, because the concentration in the 25 μm region was below the level of accuracy of our measurements. However, the calculated concentration of Ca^{2+} in the tips was approximately 0.07 μM, similar to that recorded with intracellular electrodes (0.09 μM; Miller et al., 1990). Nine non-growing hyphae did not show a gradient of Ca^{2+} (Figs 3, 4), but, among 80 growing hyphae, we could not find any correlation between the rate of growth and the Ca^{2+} gradient, as measured by the ratio between the F/S_3 and F/S_{25} ratios (not illustrated). Hyphae treated with 500 μM Gd^{3+} showed a brief transient decrease or cessation of growth, but rapidly returned to normal rates and showed no change in the F/S_{25} to F/S_3 ratios (Fig. 5). This response did not differ from the controls perfused with normal growth medium.

We have not observed any compartmentation of dyes within hyphae (in contrast to the images shown by Knight et al., 1993; and Slayman et al., 1994), although fluo-3 did show zones of exclusion similar in shape and appearance to nuclei (Fig. 3), as might be expected if it were cytoplasmic, and SNARF did accumulate in the cell wall (Fig. 3).

Ion channels in *Neurospora* plasma membrane

Attempts to obtain giga-seals with *Neurospora* protoplasts, in the conditions which retained their linear order derived from different regions of hyphae, were unsuccessful. Modulation of osmolarity and the concentration of Ca^{2+} and Mg^{2+}, and the enzymatic composition of the digestion medium all failed to produce resistances higher than 200 MOhm. However, we were able to detect both SP and SA channels.

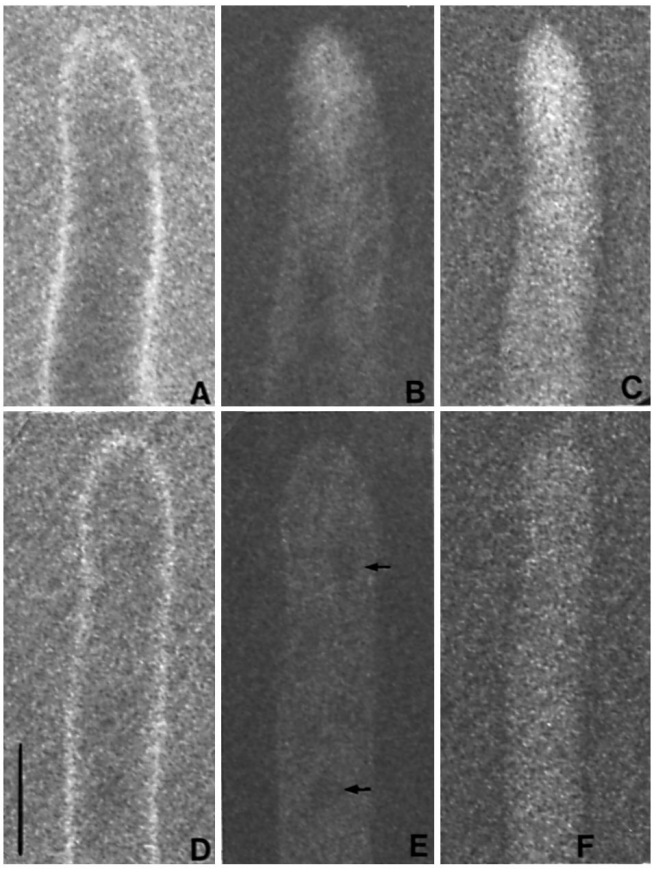

Fig. 3. SNARF (A and D), fluo-3 (B and E) and F/S ratio (C and F) images of growing (A-C) and non-growing (D-F) hyphae of *Neurospora*. Note the concentration of SNARF in the walls and its uniform distribution along the hyphae, in contrast with the gradient of fluo-3 (B) in the growing hypha (but not the non-growing one, E) and its inhomogeneous distribution in the cytoplasm, especially the nucleus sized zones of exclusion in E (arrows). Bar, 10 μm.

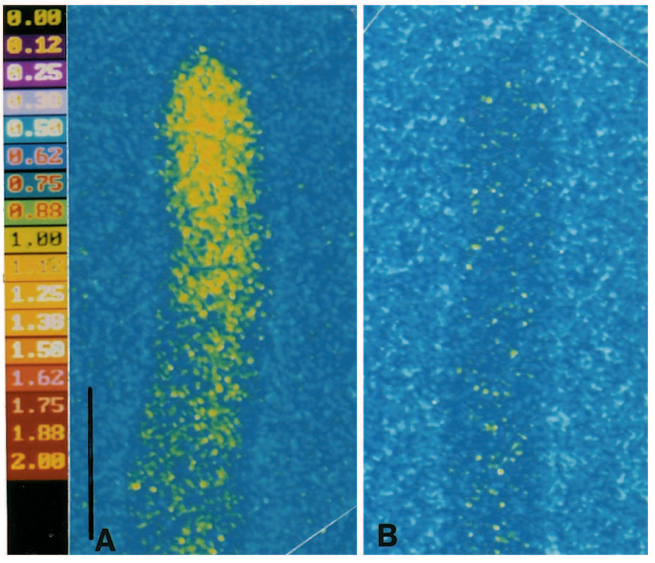

Fig. 4. F/S ratio images of growing (A) and non-growing (B) hyphae of *Neurospora*, showing the tip-high gradient present only in the growing hypha. False colour coding of the ratios is shown to the left. Bar, 10 μm.

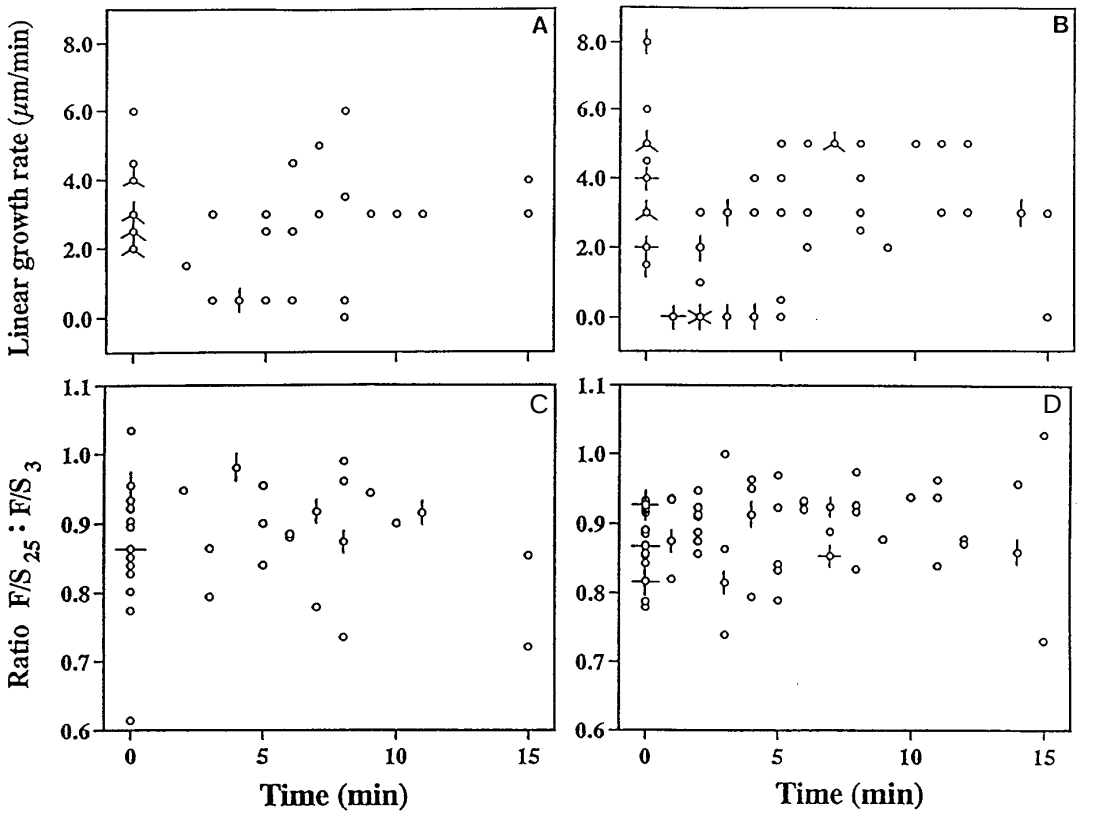

Fig. 5. The effects of control medium (A,C) and 500 μM Gd^{3+} (B,D) on the growth (A,B) and Ca^{2+} gradients (C,D) of growing hyphae. Solutions were added at *t*=0. Slight or complete transient growth inhibition was typically followed by recovery to normal rates. The shape of the Ca^{2+} gradient is indicated by the ratios between the F/S ratios at 3 and 25 μm. Because these figures are only intended to show the shape of the Ca^{2+} gradients over time, these ratios were calculated from the actual intensity values, not corrected by subtraction of the autofluorescence values. Radiating lines on data points indicate additional points which had the same values. All of the *t*=0 hyphae were included in the analysis of growth rate versus gradient mentioned in the text. The lack of congruence between sample sizes for growth and gradients at the different time points after *t*=0 is due to hyphae for which only growth or gradient was recorded and the similar variation between numbers of hyphae at one time point versus following points is due to differing sampling times for different hyphae.

SP channel activity

Typical SP channel activity from *Neurospora* protoplasts is shown in Fig. 6A. Most, but not all, patches showed the presence of channels with two distinct amplitudes. The amplitude of the smaller channels increased from −4.4±0.4 (*n*=5) to −5.2±1.2 (*n*=3) pA and that of the larger channels increased from −6.3±0.6 (*n*=5) to −8.1±1.2 (*n*=3) pA when KCl in the PS was increased from 100 to 150 mM.

SP channel activity was sensitive to the K^+ channel blocker TEA (Armstrong, 1971; Tester, 1988). We could not observe a differential effect of TEA on the large and small channels because it was difficult to distinguish between them while their activity was decreasing. Thus we shall consider them together. When the PS contained 10 mM TEA, SP channel activity disappeared approximately 70 seconds after seal formation (Fig. 7B). Data from 3 other patches showed similar results. Without inhibitor, channel activity persisted for about 5 minutes after

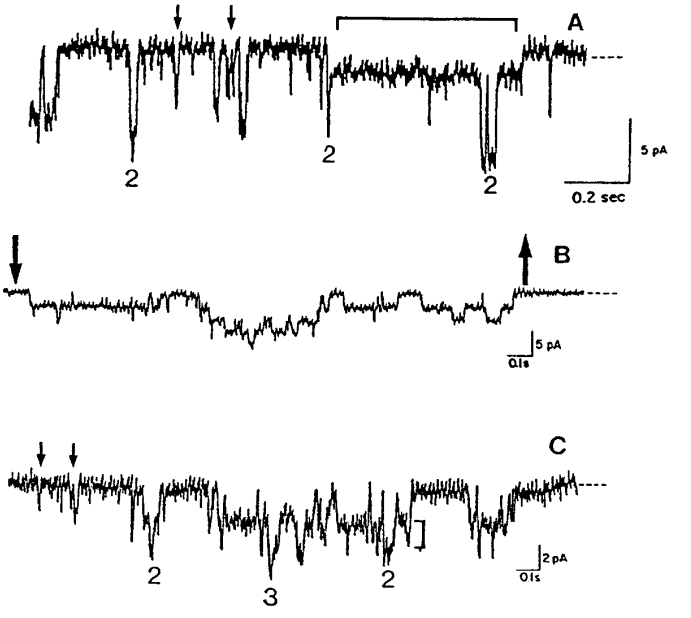

Fig. 6. Examples of channel activity. (A) SP channel activity, showing channels with different amplitudes (arrows mark brief openings and bracket shows prolonged opening of small channels) and times during which two channels were open simultaneously (numbers). This original trace is uncorrected for attenuation (see Materials and Methods), thus the amplitudes do not agree with the corrected ones in the text. (B) SA channel activity during application of negative pressure (between arrows) to a membrane. (C) An expanded trace of SA channel activity from a different recording to B, with channel openings of different amplitudes (arrows and bracket mark small channels) and periods of multiple openings (numbers). The dashed lines designate baseline (current with zero channels open). BS: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Pipes, pH 5.8. PS: 150 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Pipes, pH 5.8; 2% sucrose, 750-800 mosmol.kg^{-1}, adjusted with sorbitol.

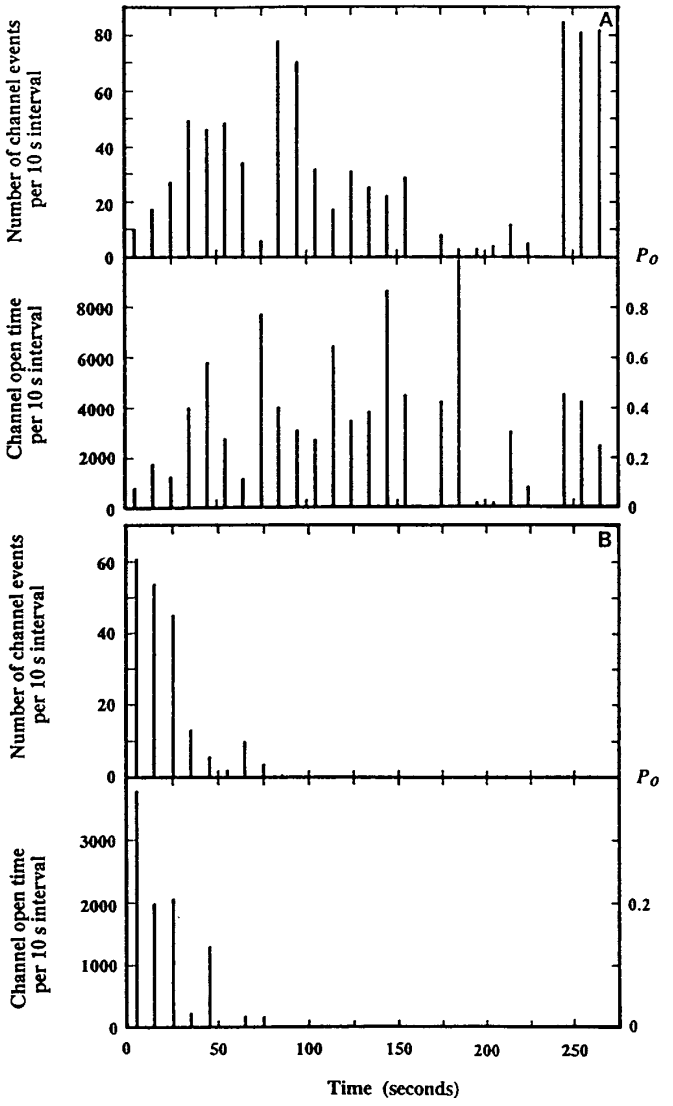

Fig. 7. Inhibition of SP channel activity by 10 mM TEA. Data represent the number of channel events and channel open times (in milliseconds) in sucessive 10 second periods in the absence (A) and presence (B) of inhibitor in the PS.

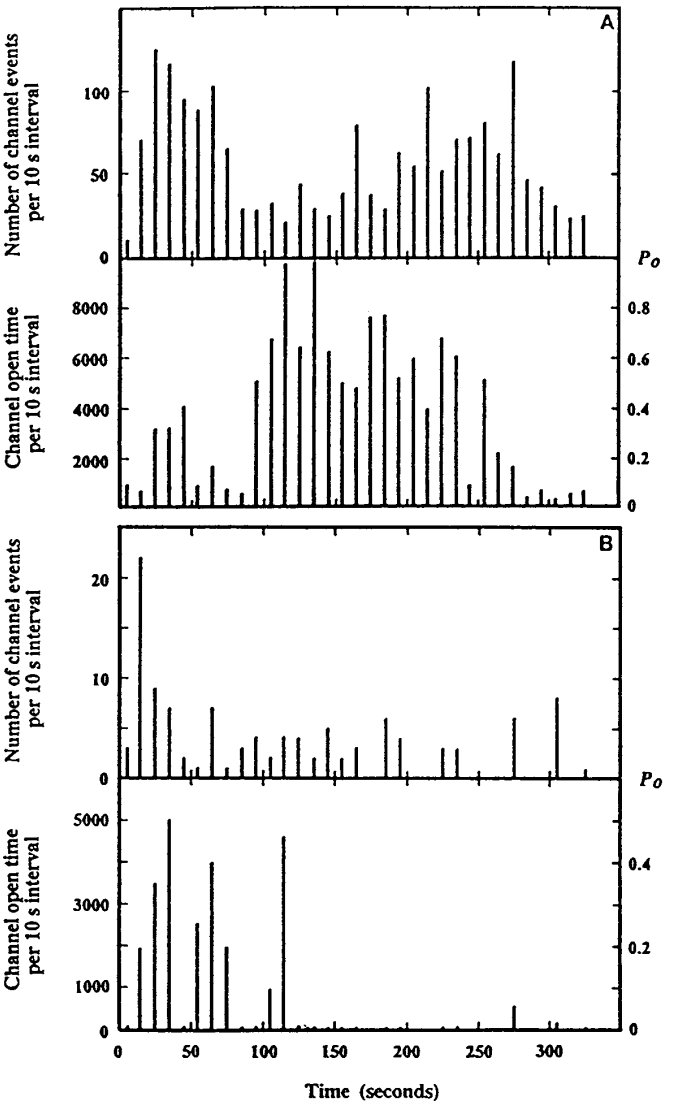

Fig. 8. Inhibition of SA channel activity by 100 μM $GdCl^3$. Data represent the number of channel events and channel open times (in milliseconds) in sucessive 10 second periods in the absence (A) and presence (B) of inhibitor in the PS.

seal formation (*n*=3) (Fig. 7A), indicating that the decrease was due to TEA and not time. TEA had no effect on SA channel activity (*n*=3, data not shown).

SA channels

The channel activity induced by suction is shown in Fig. 6B,C. Channel activity upon membrane stretching appeared in bursts. In the majority of patches we could distinguish two types of channels based on differences in their amplitude (Table 2; Fig. 6B,C). Both channels increased their amplitude with elevated Ca^{2+} concentration in the pipette solution, suggesting that SA channels are permeable to Ca^{2+} (Table 2).

To test the properties of SA channels we used Gd^{3+}, which is an inhibitor of SA channels in fungi (Gustin et al., 1988; Zhou et al., 1991; Garrill et al., 1992): 100 and 500 μM Gd^{3+} (in the PS) reduced both the open time and number of events (Table 3, Fig. 8B). The observation of SA channel activity in controls for 5 minutes after suction was applied (Fig. 8A) indicated that the decay of channel activity in the presence of inhibitor was due to the effect of Gd^{3+} and not channel rundown. Gd^{3+} had no effect on SP channel activity (*n*=3, data not shown). Due to difficulty of resolution between small and

Table 2. Effect of Ca^{2+} concentration in the PS on channel amplitudes

	Amplitudes of channel types (mean pA ± s.e.m., *n* in brackets)	
	SA channels	
PS composition*	Small	Large
1 mM $CaCl_2$	−3.9±0.6 (6)	−5.9±0.8 (6)
10 mM $CaCl_2$	−6.5±1.3 (3)	−10.3±0.6 (3)

*150 mM KCl and other constituents listed in Materials and Methods.

Table 3. Effect of Gd^{3+} on SA channel activity

	Initial†	% Change‡
Open probability		
Control	0.22±0.17 (4)	109±62$^{n.s.}$
100 μM Gd^{3+}	0.14±0.07 (3)	14±11*
500 μM Gd^{3+}	0.12±0.05 (5)	18±20**
Number of channel events		
Control	403±274 (4)	66±24$^{n.s.}$
100 μM Gd^{3+}	111±53 (3)	34±24$^{n.s.}$
500 μM Gd^{3+}	186±56 (5)	29±14*

†Values for the first 100 second interval of the recording, which, given the time elapsed between the mixing of the Gd^{3+} solution and the PS and the start of the recording (see Materials and Methods), would be likely to contain some Gd^{3+}. Mean ± s.e.m., *n* in brackets.

‡Change in values during the third 100 second interval, relative to first 100 second.

n.s., not significantly different from the initial 100 seconds, or the control, $P>0.05$.

*Significantly different from the control, $P<0.05$.

**Significantly different from the control, $P<0.025$.

large channels while channel activity declined in the presence of inhibitor, we did not distinguish between them when analysing the effect of the inhibitor.

Channel distribution

The distributions of both SP and SA channels along hyphae are shown in Table 4. SP and SA channels were evenly represented in protoplasts derived from the different regions of the hyphae. The mean number of SP channels per patch was not significantly different within each protoplast type for different concentrations of potassium in the PS or within a specific concentration for different protoplast types.

There was no correlation between the densities of SA channels versus SP channels, a comparison of the regression slope of channel densities for the compiled data ($r^2=0.123$) was not significant ($P<0.1$, Kruskul), indicating that SA and SP channels are not associated with each other. Similarily, when we compared the observed distributions of SP and SA in each protoplast type with a Poisson distribution using chi-squared goodness of fit (Goldsmith et al., 1986), their distributions were not significantly different from the Poisson distribution ($P<0.9$ for SP, $P<0.7$ for SA), suggesting that neither occur in non-random aggregates.

Table 4. Channel distributions among protoplast populations

	Protoplast populations		
	Tip	Second from tip	Posterior
Protoplast characteristics			
Diameter (μm)	24.9±6.7 (22)	24.7±5.6 (15)	22.8±7.9 (9)
Distance from tip (μm)*	81±9	155±12	227±17
Region of hyphae (μm)†	0-116	116-193	>193
SP channels			
Density (number/μm^2)	2.6±0.6 (17)	2.9±0.5 (12)	2.0±0.6 (8)
SA channels			
Density (number/μm^2)	6.3±0.4 (16)	5.9±0.7 (12)	7.6±0.8 (8)

Values are means ± s.e.m. with sample size in brackets.

*Mean distance to the centre of the region of the hyphae from which the protoplasts were derived, calculated from mean protoplast diameters translated to hyphal lengths assuming a hyphal diameter of 8 μm.

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

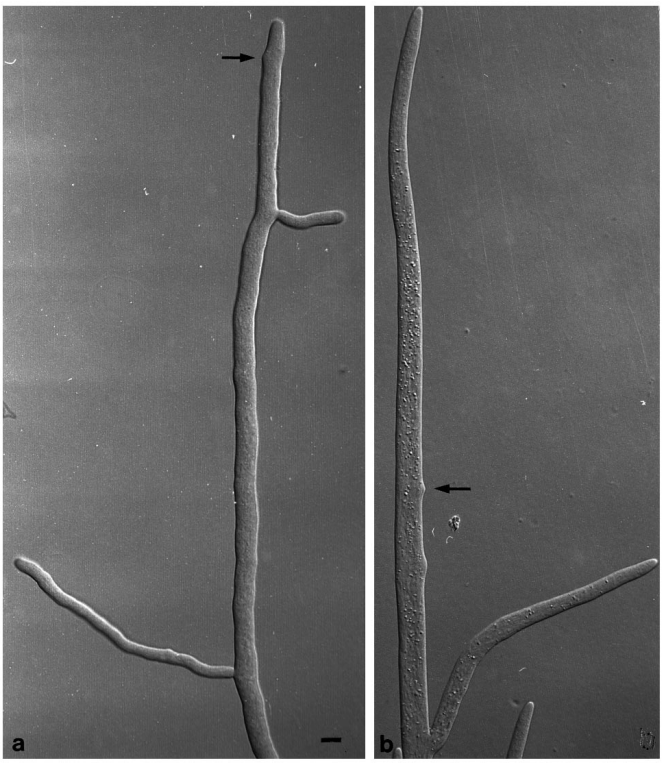

Fig. 9. Typical morphology of *Neurospora* (a) and *Saprolegnia* (b) hyphae. Arrows point to branch initiations ('buds'), which, for *Neurospora*, occur closer to the apex. Bar, 10 μm.

Apical branching in *Saprolegnia* and *Neurospora*

A possible reason for the lack of a tip-high gradient of SA channels along the *Neurospora* hyphae, in contrast to the gradients seen in *Saprolegnia* (Garrill et al., 1992), is the presence of branch initiation sites in the regions of the second and third protoplasts. If SA channels are localized in the tips, then the presence of additional tip (branch) initiation points could distort an existing gradient. To estimate the impact of branch initiation points on the observed channel distribution along hyphae we measured the distance from the tip to the nearest point of branch initiation for *Neurospora* and *Saprolegnia* hyphae (Figs 9, 10). In the estimations we distinguished between 'buds', which were integrated in the protoplasts after digestion, and 'branches', which formed separated protoplasts (see also Materials and Methods) (Figs 2, 9). To calculate the hyphal region where the initiation of new branches occurs, we followed the same procedure used to calculate the hyphal region represented by each protoplast type.

For *Neurospora* 32.4% of first protoplasts could contain membrane derived from a second tip, thus branch initiation in the apical protoplasts would lead to a 30% increase in apparent channel density over distal protoplasts (which is in the range of variability for each protoplast population). Only 0.6% of second protoplasts would include a new apex, which would not make a significant impact on the measured value of channel densities. In contrast, for *Saprolegnia*, neither first nor second

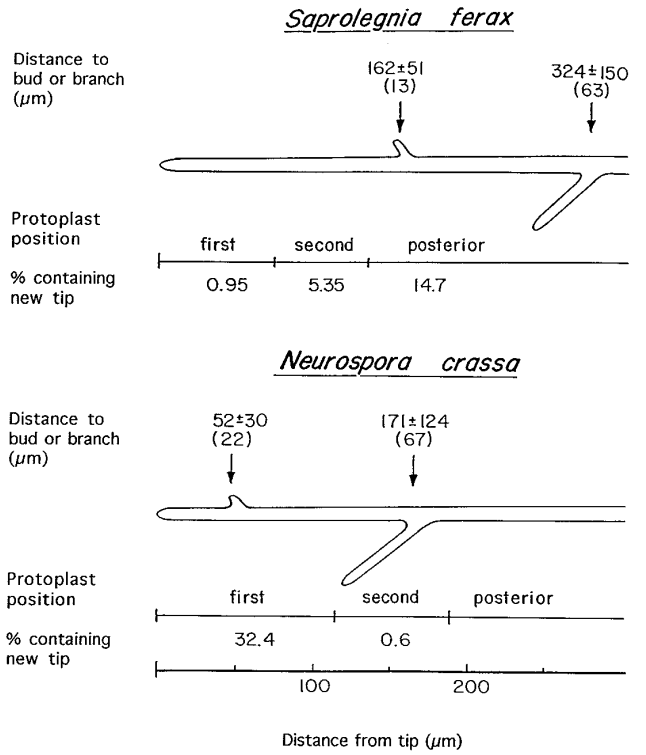

Fig. 10. Location of branches and the points of branch initiation (defined as the distance to the first detectable 'bud') for *Saprolegnia* and *Neurospora* hyphae. The regions from which the protoplasts originated, as calculated in Materials and Methods, are indicated. Distances represent means ± s.d. with *n* values in brackets. The % of protoplasts likely to contain membrane derived from a new tip, or 'bud' ('% containing new tip'), was calculated from the number of hyphae which contained 'buds' in the regions corresponding to the respective protoplast regions. Posterior protoplast regions of *Neurospora* hyphae did not contain any 'buds'.

protoplasts included a new tip at levels sufficient to significantly increase channel densities, although the 15% in the posterior population could have an impact (Fig. 10). However, this impact would tend to reduce the slope of the tip-high gradient.

Effect of channel inhibitors on hyphal growth

To elucidate whether SP or SA channels play a role in tip growth, we studied the effect of channel inhibitors on hyphal extension rates. When growing hyphae were flooded with growth medium containing 100 μM Gd^{3+}, hyphal extension transiently ceased, but resumed after about 2-3 minutes (Fig. 11C). Treatment with 10 mM TEA elicited a similar response (Fig. 11B). Similar flooding of the mycelium with fresh growth medium did not affect extension rates (Fig. 11A). Neither TEA nor Gd^{3+} induced any morphological changes such as tip swelling or proliferation of apical branches, which are frequent reactions of *Neurospora* hyphae to diverse shocks (Robertson and Rizvi, 1968).

Effect of channel inhibitors on hypotonic induced tip bursting

In order to elucidate whether the temporary blockage of SP or SA channels influenced turgor, we exposed growing tips to

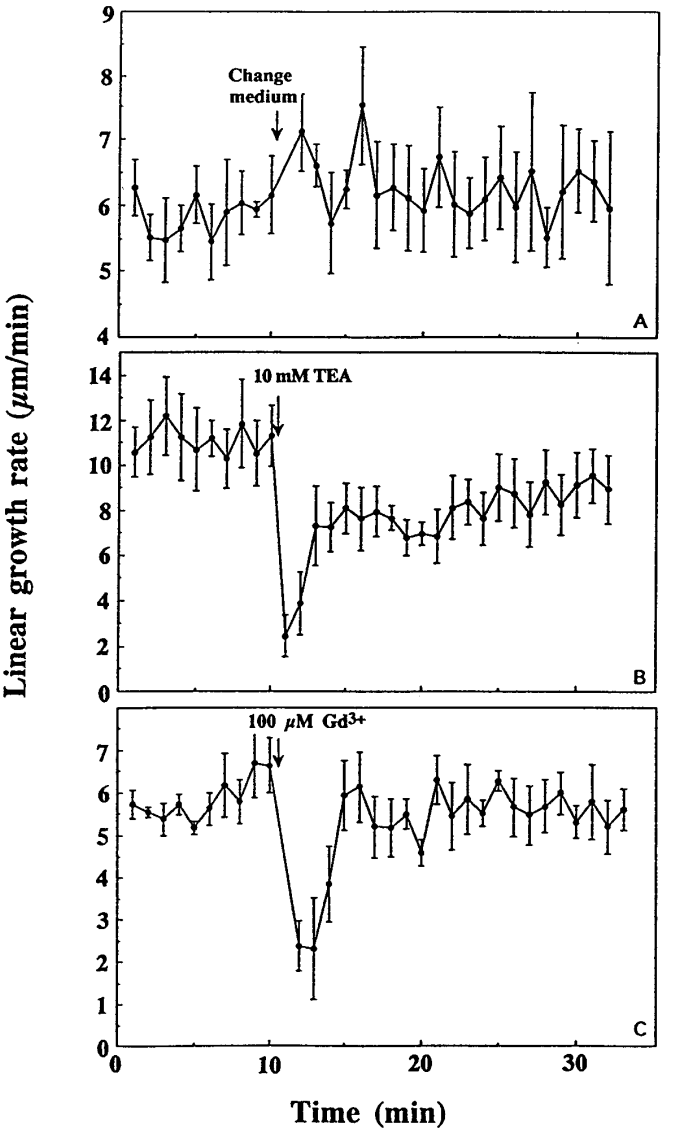

Fig. 11. Linear growth rates (expressed as the mean length of extension since the previous observation ± s.e.m.) of control (A, *n*=9), 10 mM TEA (B, *n*=6) and 100 μM $GdCl^{3+}$ (C, *n*=9) treated hyphae. The arrows point to the time of addition of inhibitors. Differences in rate values is probably due to variations of room temperature between experiments.

hypotonic solutions. Hyphal tips immediately burst when hyphae were treated with hypotonic solution and cytoplasm extruded for 2-3 minutes before the septal pores become plugged (Trinci and Collinge, 1974). Inclusion of 100 μM or 1 mM Gd^{3+} in the hypotonic solution did not affect tip bursting, but 10 mM TEA reduced the percentage (Table 5).

Voltage-clamp measurements

Since there is no tip-high gradient of either SP or SA channels in *Neurospora*, it is possible that neither these channels, nor other *trans* plasma membrane ion transport systems, function in tip elongation in *Neurospora*. We modulated the electromotive force across the plasma membrane by clamping its potential with a double-barrelled micropipette; one barrel measured the voltage while the other barrel was used to inject

Table 5. Effect of channel inhibitors on hypo-osmotic shock-induced tip bursting

Treatment	% Burst tips
Control*	58.5±10.2 (7)
0.1 mM Gd^{3+}	67.6±8.0 (5)†
1 mM Gd^{3+}	48.5±9.8 (3)†
10 mM TEA	28.3±4.5 (5)‡

*BS diluted 1:2, osmolarity 130 mosmol/kg.
†Not different from control, Student's *t*-test, $P>0.01$.
‡Different from control, Student's *t*-test, $P<0.001$.

current into the cell. This effectively changes the flux of any ionic species across the plasma membrane. Voltage could be clamped reliably between −300 mV and +150 mV. The clamping current was monitored to determine that it did not decrease as a result of the tip of the micropipette becoming plugged.

Of 25 impaled hyphae, 16 did not resume growth after impalement, which may be explained by cell injury caused by microelectrode insertion, even though they possessed normal cytoplasmic and vesicle movements, and 2 grew such that measurements could not be made.

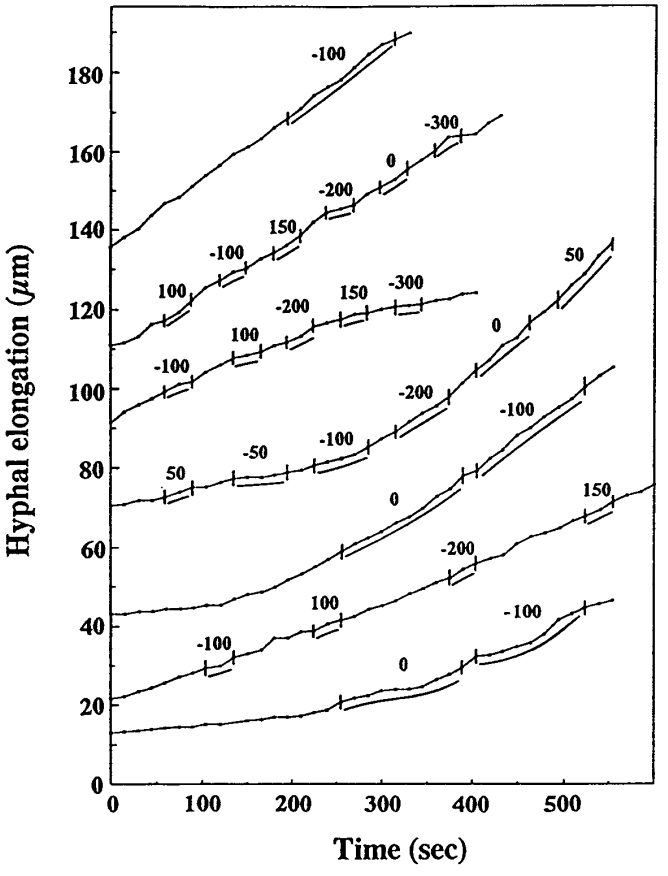

Fig. 12. Effect of clamped membrane voltages on tip elongation rates. The growth curves for 7 hyphae show steady, increasing and decreasing growth rates during the course of the experiments. Various membrane potential clamps (indicated by the numbers above the curves) were applied for different periods (indicated by the underlines along the curves) with little or no effect on the growth rates. The starting positions of the curves on the ordinate are arbitary.

Clamping of the membrane potential of growing hyphae, from +150 to −300 mV, for 30 to 120 seconds, did not influence growth rates (Fig. 12). Similarily, hyphal tips which had stopped growing, but still appeared healthy (cytoplasmic streaming could be observed), could not be induced to grow by imposed voltage clamps for up to 2 minutes (mV: +50, *n*=2; 0, *n*=3; −50, *n*=2; −100, *n*=3; 200, *n*=3).

DISCUSSION

In contrast to a previous report (Knight et al., 1993), we have been able to analyse the distribution of Ca^{2+} in hyphal tips of *Neurospora* with a ratiometric dye technique. As in other tip-growing cells (Brownlee and Wood, 1986; Garrill et al., 1993; Miller et al., 1992; Obermeyer and Weisenseel, 1991; Pierson et al., 1994; Rathore et al., 1991; Reiss and Nobiling, 1986), *Neurospora* has a tip-high cytoplasmic free Ca^{2+} gradient present only in growing hyphae. This is in agreement with the observation of a similar gradient of chlortetracycline staining reported for *Neurospora* by Schmid and Harold (1988).

The slope of the gradient is substantial. Our analysis of it is probably an underestimate of its true slope because *Neurospora* tips are likely to be more acid, perhaps by as much as 1-1.5 pH units, relative to the sub-apical regions (McGillviray and Gow, 1987). Using more sensitive methods, *Pelvetia* tips were also found to be more acid, but only with a 0.3 unit difference (Gibbon and Kropf, 1994). If the tips of the hyphae we used were more acid by 0.5 unit, our apical Ca^{2+} concentration would be underestimated by about 2-fold, and if 1.5 units lower, the factor beomes 9-fold (Lattanzio, 1990). Thus, the true Ca^{2+} concentration in the tip could be as high as 1 μM Ca^{2+}, comparable to that recently reported in lily pollen tubes (Pierson et al., 1994). Evidently further work to establish the pH profile of the hyphal tip of *Neurospora* is needed. Our procedure for loading hyphae with SNARF appears to overcome the problems encountered in dye loading by others in this organism (Slayman et al., 1994) and merits further analysis. Our finding that the gradient is higher at 3 μm behind the tip, compared with the '1 μm' value, differs from previous reports of Ca^{2+} gradients in tip growing cells. The significance of this difference is unknown, but may also relate to the putative pH gradient.

The key questions concerning the Ca^{2+} gradient in the hyphal tips are the factors which lead to its formation, and its contribution to the growth process. A similar gradient in *Saprolegnia* hyphae appears to be generated by Ca^{2+} influx across the plasma membrane via a tip high gradient of SA, Ca^{2+} transmitting channels (Garrill et al., 1992, 1993; Levina et al., 1994). A similar process seems unlikely in *Neurospora* because the SA channels do not occur in a detectable gradient and their inhibition with Gd^{3+} (at 100 and 500 μM, which is the range of concentrations used for other fungi; Zhou and Kung, 1992; Zhou et al., 1991) does not block tip growth, destroy the Ca^{2+} gradient or reduce the osmotic fragility of the hyphae. Furthermore, major changes in the electromotive force across the plasma membrane of the hyphae in the voltage clamp experiments did not alter their growth rates. Together, these observations indicate that influx of ions, especially Ca^{2+}, from the medium do not play a major role in the generation of the Ca^{2+} gradient or the regulation of tip growth.

In contrast, the fact that the Ca^{2+} gradient is always present in growing hyphae and is absent in non growing hyphae (apart from those whose growth was transiently inhibited by Gd^{3+}) indicate a significant and obligatory role in tip growth. All reports of Ca^{2+} ion gradients in tip growing cells show a strong correlation between the presence of the gradient and continued growth (Rathore et al., 1991; Miller et al., 1992; Jackson and Heath, 1993; Pierson et al., 1994). Generation and maintenance of the gradient must presumably involve an internal recycling system, possibly mediated via the vacuoles (Miller et al., 1990; Cornelius and Nakashima, 1987) or mitochondria (reviewed by Jackson and Heath, 1993). The difference with respect to the system in *Saprolegnia*, while possibly having a phylogenetic basis in such widely differing groups, may also have an ecological basis. *Saprolegnia* has evolved in an aquatic environment, whereas *Neurospora* is adapted to a terrestrial situation where the supply of *exogenous* Ca^{2+} may be very unreliable and thus unsuitable as a major regulatory factor in such a fundamental process as tip growth.

Aside from the fundamental issue of the regulation of tip growth by Ca^{2+}, the present results make a number of contributions in other aspects of fungal cell biology. For example, previous studies on the electrical properties of *Neurospora* have focused on the use of intracellular electrodes to analyse the flux of ions and the membrane potentials which they develop (Slayman, 1965; Slayman and Slayman, 1962; Slayman and Gradmann, 1975). This is the first patch clamp analysis of the membrane channels participating in some of these fluxes.

The scope of our results are limited by the sub-gigaseal conditions. The merits of low resistance seals have been reviewed by Roberts and Almers (1992), and the results obtained for plant and fungal cells (Lew et al., 1990, 1992; Garrill et al., 1992, 1993; Levina et al., 1994) show that this method has advantages. For example, the protoplast isolation techniques used by others to obtain gigaseals from fungal cells (e.g. Gustin et al., 1988; Zhou et al., 1991) preclude the generation of any spatial distribution information. Furthermore, the process of forming gigaseals seems to involve major changes in the architecture of the membranes (Ruknudin et al., 1991) and the types of fungal protoplasts which give gigaseals appear very abnormal relative to normal cytoplasm-rich protoplasts (Hoch et al., 1992). Arguably, low resistance seals better reflect the normal properties of membranes.

With the use of the low resistance seals, we found that, in common with other tip-growing hyphae (Garrill et al., 1992, 1993; Zhou et al., 1991), both SP and SA channels in protoplasts derived specifically from the hyphal tips. Together with the observation of SA channels in another filamentous fungus, the basidiomycete *Uromyces* (Zhou et al., 1991), and in the ascomycetous yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Gustin et al., 1988; Zhou and Kung, 1992), these data show their generality among phylogenetically diverse fungi.

The SP channels are K^+ permeable, based on inhibition by TEA and the increase in current with elevated external K^+. K^+ movement through these channels is inward. We tentatively conclude that, like those of *Saprolegnia* (Garrill et al., 1993), they play a dispensable role in growth via turgor regulation because their inhibition with TEA causes only a temporary reduction in growth rate and reduced sensitivity to hypo-osmotic shock-induced bursting, presumably due to lower K^+ influx.

Similarly to *Saprolegnia*, the *Neurospora* hyphal tips also contain two SA channels of different amplitude. However, unlike *Saprolegnia*, in which the smaller amplitude channel was probably a Mg^{2+} channel (Garrill et al., 1992), both SA channels in *Neurospora* seem to be Ca^{2+} permeable channels because their amplitudes both increase with Ca^{2+} ion concentration.

Because *Neurospora* forms branches closer to the tips than *Saprolegnia*, we suspected that a greater incidence of branch initiation (and thus hypothetically channel-rich new tips) in the second and posterior protoplasts may have obscured a tip high gradient. However, detailed analysis of this factor showed that branch initiation occurred more frequently in the tip protoplasts in *Neurospora*, and should thus have enhanced the tip values. In contrast, in *Saprolegnia*, branch initiation is more likely to occur in the sub apical protoplasts, and thus reduce the slope of the gradient. Thus the previously shown slopes (Garrill et al., 1992; Levina et al., 1994) probably underestimate the real gradients.

Our lack of inhibition of hyphal growth with Gd^{3+} agrees with previous work on *Neurospora* (Takeuchi et al., 1988), where neither La^{3+} nor Gd^{3+} (40 μM) affected hyphal extension or the pattern of branching, but the rapid transient inhibition we report here may not have been observed. Furthermore, even 1 mM Gd^{3+} did not inhibit Ca^{2+} uptake in liquid cultures of *Neurospora* (Corzo and Sanders, 1992). Conversely, because normal extension of *Neurospora* hyphae requires external calcium (Schmid and Harold, 1988; Dicker and Turian, 1990; Reissig and Kinney, 1983; McGillviray and Gow, 1987), there may be Gd^{3+} insensitive Ca^{2+} permeable channels which are the major pathway of transporting Ca^{2+}, but whose amplitude might be smaller than the limits of resolution in our recordings. The presence of Ca^{2+} channels in the *Neurospora* plasma membrane was hypothesized based on the inhibition of Ca^{2+} uptake by La^{3+} (Corzo and Sanders, 1992) and the galvanotropism of hyphal tips (Lever et al., 1994). Whether these channels exist or participate in the regulation of apical growth, is unknown, but our voltage clamp data indicate that they are not important in the process.

A key feature of our argument that our voltage clamp data discounts a role for ion influx during tip growth is the relationship of the measured membrane potentials to those in the expanding apical dome. The most important morphogenic region is the most apical 5 μm of the tip where the cell surface is expanding, but the membrane potential measurements and voltage clamping were all made 12 μm or more behind the tip. Voltage clamping may not be complete at some distance away from the micropipette. However, in isolated hyphal segments, the error is only 10-15% over 300-400 μm (Gradmann et al., 1978). Therefore, as the tips grew to be no more than about 100 μm from the initial site of impalement, it is likely that the growing tip voltage was close to the clamped value.

It is possible that the results of the voltage clamp experiments were influenced by alteration of intracellular ion concentrations due to current injection. However, maximal clamping currents were about 20 nA. Over the duration of a voltage clamp treatment, this would not result in a significant electrical injection of either K^+ or Cl^- from the current-injecting barrel (less than 10 mM K^+ assuming an effective

hyphal length of 100 μm and a 60 second treatment, compared to 140 mM potassium intracellular concentration; Slayman, 1965).

If Ca^{2+} influx via the SA channels is not essential for tip growth, then what is their role? They may regulate turgor or be part of a signal transduction pathway in response to mechanical stress. The absence of a change in response of Gd^{3+} treated hyphae to hypotonic shock argues against a suggested role in turgor regulation in *Neurospora*. Morris and Horn (1991) have shown that mechanical stress likely to activate SA channels in animal cells has little influence on measureable whole cell current, suggesting that their effects may be very localized. Mechanical stress could activate SA channels, causing entry of Ca^{2+} through the channels and inducing subsequent transduction mechanisms such as activation of the proton pump (Lew, 1989) or other components involved in tip growth (Jackson and Heath, 1993).

In summary, *Neurospora* hyphae have SA channels. The SA channels may be ubiquitous among fungi and hyphal organisms, based on their reported presence in yeasts, *Neurospora*, *Uromyces* and *Saprolegnia*. While a physiological role for SA channels in growth regulation has been established in *Saprolegnia*, the lack of a tip-high gradient of the SA channels in *Neurospora*, and the inability to arrest growth by SA channel inhibition limits the potential role of these channels as universal growth regulators. However, in both *Saprolegnia* and *Neurospora*, a tip-high gradient of Ca^{2+} is associated with tip growth. It is reasonable to conclude that there is an intimate relationship between Ca^{2+} in the tip and the phenomenon of tip growth, but different means of maintaining the gradient seem to have evolved in different organisms.

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