CALCIUM ACCUMULATIONS WITHIN THE GROWING TIPS OF POLLEN TUBES

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Weisenseel et al. studied the electrical fields around pollen tubes and found a current entering the growing tip and almost the whole tube behind it (7). Moreover, their ion substitution experiments suggest that calcium is one component of this current (3). Because calcium ions form relatively tight chemical bonds to various cytoplasmic constituents, transcellular currents of this ion should establish relatively large cytoplasmic gradients and thus electrical fields. Such fields are hypothesized to move vesicles of new membrane and wall material to the growing tip (2). Here we report the first study of the ^{45}Ca distribution along growing pollen tubes (or, indeed, any tip-growing cell). It was executed via autoradiography of deep-frozen whole pollen. This method gives low resolution but is simple and reliable.

MATERIALS AND METHODS

Pollen suspensions were obtained by shaking anthers of *Lilium longiflorum* cv. Arai in a standard medium (of pH 5.2) containing 290 mM mannitol, 3.3 mM $CaCl_2$, 1.0 mM KOH, and 1.3 mM H_3BO_3. At room temperature, the pollen begins to germinate after 1–2 h. Germinated grains then grow at 6–8 μm/min.

Pollen was exposed to ^{45}Ca by transfer to a standard medium containing calcium with a specific activity of 2–20 mCi/mg. Suitably labeled pollen was then washed on a nylon net with non-radioactive medium. It was collected (and washed further) on a Nuclepore membrane (Nuclepore Corp., Pleasanton, Calif.) underlaid by a black Millipore filter (Millipore Corp., Bedford, Mass.) in turn supported by a sintered glass filter. To do this, fluid was pulled through the filters until the pollen was seen to be just stranded. Then we quickly picked up the Nuclepore, floated this hydrophobic membrane on fresh medium, and photographed it. (Pollen observed at this stage shows normal morphology, streaming, and growth.) Finally, the Nuclepore was peeled off the fluid and dropped into liquid nitrogen. The whole procedure, from the beginning of the wash to freezing, need take no more than 2 min. If the absolute ^{45}Ca concentrations in the pollen tubes were to be estimated, we attached ^{45}Ca standards to the frozen Nuclepore with some silicone grease. These standards were dummy pollen tubes made of 16–20 μm ID glass tubes filled with known concentrations of the isotope in aqueous solution. (Absorption by the 4 μm thick glass walls of these standards is estimated to have reduced the grain density produced by ^{45}Ca's 250 keV β particles by only 20%.) For autoradiography the frozen sample was then pressed against an emulsion as shown in Fig. 1. While this process crushes most of the 100 μm diameter pollen grains, it scarcely damages the 20 μm wide pollen tubes. It was done at −60°C in a

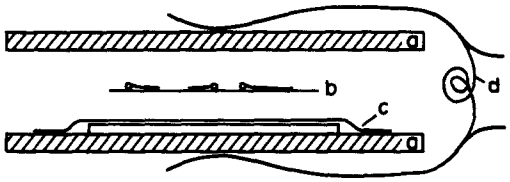

FIGURE 1 Sandwich for pressing frozen pollen against a nuclear emulsion. It is expanded to show its separate layers. (a), 25 × 30 mm glass slides. (b), Nuclepore membrane bearing pollen on its rough upper surface. This membrane is 10 μm thick, has 0.4 μm pores, and is quite flexible even at −160°C. (c), Kodak AR-10 stripping film draped over a cover slip waxed to a slide. (d), Bulldog clamp.

nitrogen-cooled bowl: Sorvall's no. FTS/LTC-2 (DuPont Instruments, Sorvall Operations, Newton, Conn.). The emulsion was then exposed at dry ice temperature (i.e. −78°C) for 2–30 days, separated from the sample, and developed. No pollen tissue ends up on the resultant autoradiograph.

We made photometric scans which indicated the relative grain densities along a 25 μm wide central strip of some tube images. This was done with a silicon photodiode put in the microscope's third tube and masked to receive the light from 25 μm wide spots of autoradiographs illuminated with a dark-field condensor. However, the images of some intensely labeled tube tips were necessarily over-exposed, so their photometric outputs underestimated their radioactivity. To reduce this error, their outputs were multiplied by a factor of 2–4 corresponding to their greater *widths*.

RESULTS

We have examined about 800 autoradiographic images of pollen ranging in length from 0.5 mm to 3 mm (thus 2–5 h old), grown in ^{45}Ca for periods ranging from 1 min to 5 h, and subsequently washed for periods ranging from 2 min to 1 h. About 500 of these images seemed reliable and are rather well represented by the dozen examples shown in Fig. 2.

At least 95% of the reliable images show obvious accumulations of silver grains at both ends: over the tube's growing tip and also over the pollen grain. Since the pollen tubes are of practically uniform diameter, an accumulation of silver over a tube's tip indicates a correspondingly high ^{45}Ca concentration within it. On the other hand, we calculate that the accumulations over the pollen grains can be accounted for by their greater width, without assuming any greater ^{45}Ca concentrations there. So we will focus this report on the more interesting tip accumulations.

In most cases, e.g., those illustrated in Fig. 2 A–C and G–J, the tip accumulation is moderate, but in cultures older than about 3 h some images show the remarkably intense, torchlike accumulations illustrated in Fig. 2 D–F and K–L. In 5-h old cultures, up to 25% of the population may be of this latter type. We very rarely observe an image which seems to be intermediate between the two types.

Most moderate tip accumulation images are almost isodiametric, thus 50–80 μm long, as illustrated in Fig. 2 A–C and G. A minority (illustrated by Fig. 2 I) are markedly elongate, usually 100–200 μm long, occasionally 300 or even 600–800 μm long. Such elongated tip accumulations were most frequent in one experiment in which the pollen was exposed to ^{45}Ca for periods between 3 and 30 min. Some intense accumulations, such as that shown in Fig. 2 E, have a simple pattern suggesting a single region of ^{45}Ca accumulation at the tip. Others, such as that shown in Fig. 2 F, have compound patterns suggesting two such regions, one at the tip and another just behind the first.

Fig. 3 shows some representative photometric scans of pollen grown in ^{45}Ca for several hours starting soon after wetting. In tubes with moderate tip accumulations the ^{45}Ca is two to four times more concentrated at the tip than in the bulk of the tube, while in tubes with intense accumulations it is about 200-fold greater.

^{45}Ca standards were included with two pollen samples which had been exposed to tracer for 3 h and washed for 3 min. If (as seems quite likely) the specific activity within these tubes had reached that in the exposure medium, then the standards indicated a concentration of about 0.5 mM calcium in the bulk of the tubes. On this same basis, the photometric scans of relative ^{45}Ca concentration along the tubes would indicate tip concentrations of about 1–2 mM calcium in pollen showing moderate tip accumulations and about 100 mM calcium in pollen showing intense accumulations.

As one would expect, the average concentration of ^{45}Ca in pollen is reduced when samples are exposed to tracer for shorter times or washed with nonradioactive media for longer times. Thus, to attain silver densities over tubes grown in ^{45}Ca for 1 min which were comparable to those grown in ^{45}Ca for several hours (and also washed for 3 min), we raised the product of the medium's specific activity and the emulsion's exposure time by about 20–80-fold. However, the *pattern* of ^{45}Ca concen-

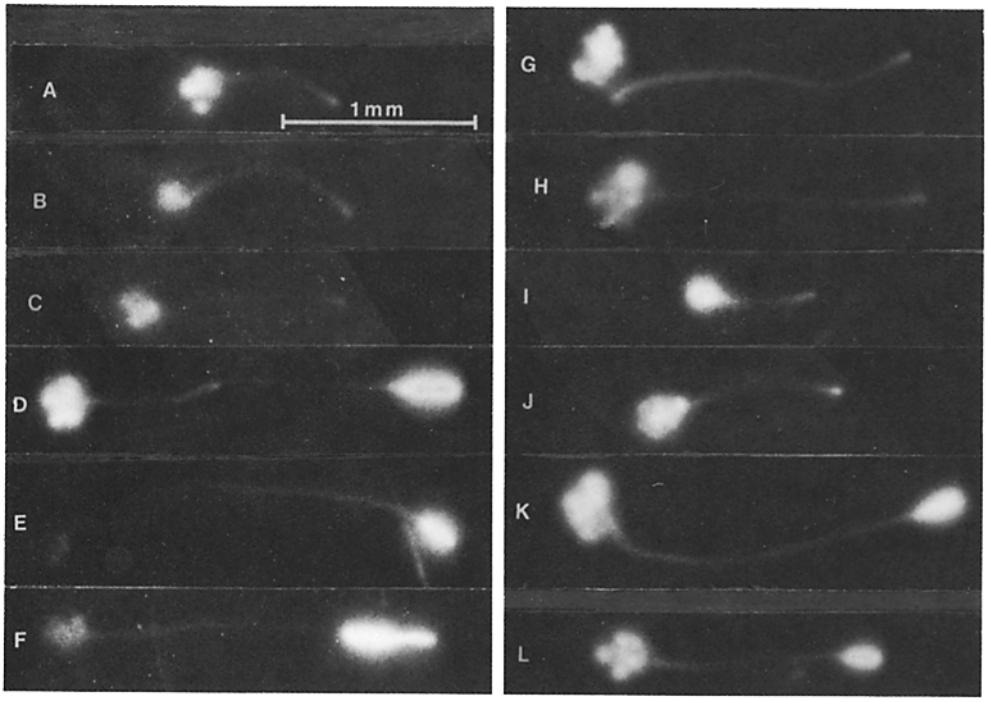

FIGURE 2 Autoradiographs of deep-frozen pollen grown in ^{45}Ca. Grains are to the left of each micrograph; growing tips to the right. Grains were crushed during autoradiography. Pollen tubes showing the usual moderate accumulations of ^{45}Ca at their growing tips are shown above in A–C and G–J; those with an intense accumulation, below. Pollen in the left-hand column were grown in radioactive media for 3–5 h starting soon after being wetted; pollen in the right hand column were grown in such media for only 1 min (G, H, K, L), or 3 min (I, J). Pollen A and D were washed for 1 h in nonradioactive media before being frozen; the rest, for 2–3 min. Photographed with Zeiss Epiplan optics, × 25. The apparent minima in silver density seen within images D and L are actually maxima produced by grain densities so very high as to reflect less light. Controls made with nonradioactive samples indicated that these images do not result from chemography or pressure artifacts.

tration along a tube showed little dependence upon exposure time or washing time. Similar ^{45}Ca gradients were seen whether the pollen tubes were grown in radioactive media for 1, 3, 10, 70, 120, or 300 min and then washed for 2, 3, 20, or 60 min. These gradients were likewise independent of age (i.e. time after wetting) in the investigated range of 2–5 h.

Finally, since exposures to ^{45}Ca as short as 1 min yielded typical moderate tip accumulations (as well as some intense ones), it might be inferred that calcium ions enter the pollen tubes most quickly at their growing tips. One complication that might cast doubt on this inference would be a redistribution of newly entered calcium by cytoplasmic streaming. In order to minimize this, we also pulse labeled some pollen tubes for 1 min and then washed them in ice-cold medium for 3 min. Ice-cold medium is seen to stop streaming immediately. Hence, the streaming should have had no more than 1 min to redistribute calcium in this experiment. Using Iwanami's value of 50 μm/min for the forward streaming rate near the tip (a figure we have roughly confirmed), one then calculates that calcium could have moved tipward by ≤ 50 μm here. Nevertheless, typical ^{45}Ca accumulations were seen even in these most briefly labeled tips.

DISCUSSION

Moderate Tip Accumulations after Long Periods of Growth in ^{45}Ca

Autoradiographs of most pollen tubes labeled for 2–5 h with ^{45}Ca show a two- to fourfold

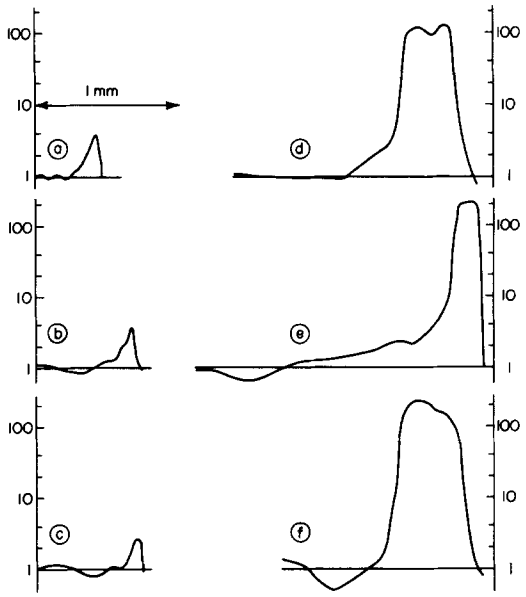

FIGURE 3 Photometric scans of autoradiographs shown in Fig. 2 A-F. Scan (a) is of the autoradiograph shown in Fig. 2 A; (b), of B, etc. Each extends back from the growing tip to include most of the tube but not the grain. Each is a semilog plot of relative silver density per unit length vs. distance along the tube. Those to the left include the usual moderate tip accumulations; those to the right are of the intense type.

accumulation of ^{45}Ca at the growing tip. Such accumulations persist even after an hour-long wash with nonradioactive medium. From Iwanami's careful observations of streaming rates in *L. auratum* (1) (in part confirmed by our own observations on *L. longiflorum*), we would estimate that the cytoplasm flows from one end of a 1-mm tube to the other in about 10 min. We would therefore infer that the *specific* activity is reasonably constant along the tube after these 2- to 6-h long experiments. Hence, the accumulations of ^{45}Ca seen at the growing tips should represent accumulations of total calcium there.

We would further infer that these tip accumulations of ^{45}Ca represent calcium concentrated in the tip's cytoplasm rather than the tip's wall. We infer this on the assumption that calcium's behavior in germinating lily pollen is sufficiently similar to that in a better studied system, germinating *Pelvetia* eggs. In this egg, 20% of the total calcium is in the cell wall, and this wall component shows an exchange half-time of only 5 min as compared to 2 h for the cytoplasm (4). If the corresponding figures for lily pollen are at all comparable, then the tracer distribution seen after 2–5 h of labeling followed by a 1-h wash would obviously represent a cytoplasmic rather than a wall gradient.

Due to scatter of β particles, the autoradiographic image of the pollen tube is wider than the actual tube; likewise, the length of the bright tip region must be longer than the actual region of calcium accumulation. The length and width of the bright region are usually of comparable dimensions, and since the actual tube is about 20 μm in width, we can conclude that the region of calcium accumulation is on the order of 20 μm in length.

This length corresponds with the 20–30-μm length of the optically clear "cap block" region that is seen at the growing tip of living pollen (see reference 1 for *L. auratum*, confirmed by our own observations for *L. longiflorum*). Electron microscopy of pollen rapidly fixed with osmium tetroxide shows that the cap block contains a relatively high concentration of 0.1–0.3 μm diameter vesicles and lacks the larger cytoplasmic inclusions (e.g., mitochondria) found in other regions of the tube (see Fig. 10 of reference 5). There is good evidence that these 0.1–0.3-μm vesicles fuse with the membrane of the growing tip, extending its length and depositing new wall (5, 6).

The apparent correspondence of the calcium accumulation with the region of such vesicle accumulation suggests that the extra calcium may be located within these vesicles. If this were the case, the transcellular calcium gradient would not produce a transcellular field. On the other hand, to the degree that the extra calcium was bound within the continuous phase of the tip's cytoplasm, electrophoretically significant fields should be produced there.

Pulse Labeling

While the tip accumulations of ^{45}Ca seen after 1 min of exposure to radioactive media resemble those obtained after hours of such exposure, they nevertheless require a different explanation. They cannot represent accumulations of net calcium in the tip cytoplasm, for only a small percent of the tip's calcium is exchanged after such brief exposure. They probably indicate that calcium ions enter the tube's growing tip fastest. Alternatively, they might indicate greater binding of calcium by the cell wall there.

Intense Accumulations

These extraordinary accumulations of ^{45}Ca are seen in the tips of some tubes exposed to

radioactive media for periods of 1 min up to several hours. At least, those seen after hours of exposure must represent accumulations of calcium, and accumulation in the cytoplasm rather than the wall, for they indicate an average concentration of at least 100 mM calcium in these tubes' tips. An assumption that all of this calcium lay in the wall, which occupies only 0.1 of a tube's volume, would imply the absurdly high concentration of at least 1000 mM there.

An interesting possibility is that these intense accumulations of cytoplasmic calcium are produced by the trains of current pulses which enter some pollen tube tips. (7). Both intense accumulations and pulsing first appear in a small fraction of the tubes when they get to be about 1 mm long.

SUMMARY

Pollen of *L. longiflorum* was grown in ^{45}Ca-labeled medium and washed with nonradioactive medium. Whole, labeled pollen was then frozen and autoradiographed at −78°C. The autoradiographs show striking accumulations of ^{45}Ca in the growing tips of the pollen tubes. This result is obtained when the pollen is labeled for times as short as 1 min, or as long as 5 h. In most cases, the tip concentration is about two to four times greater than that in the bulk of the pollen tube, and extends for a length of about 20 μm. In autoradiographs of tubes longer than 1 mm, a small fraction of cells show a distinctly larger ^{45}Ca accumulation, the tip containing more than 100 times that in the rest of the cell. The 1- to 5-h labeling experiments show that calcium is relatively concentrated within the cytoplasm of the growing tip. The 1- to 3-min labeling experiments suggest that calcium may enter the tip faster than it enters other regions. These patterns of calcium accumulation and flux may be related to the localized secretion of vesicles at the growing tip.

This work was supported by grant GB 35937 from the National Science Foundation. Dr. Weisenseel was supported by the Max Kade Foundation.

Received for publication 17 April 1975, and in revised form 21 July 1975.

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