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Ca^{2+}, Annexins, and GTP Modulate Exocytosis from Maize Root Cap Protoplasts

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Protoplasts isolated from root cap cells of maize were shown to secrete fucose-rich polysaccharides and were used in a patch–clamp study to monitor changes in whole-cell capacitance. Ca^{2+} was required for exocytosis, which was measured as an increase in cell capacitance during intracellular dialysis with Ca^{2+} buffers via the patch pipette. Exocytosis was stimulated significantly by small increases above normal resting [Ca^{2+}]. In the absence of Ca^{2+}, protoplasts decreased in size. In situ hybridization showed significant expression of the maize annexin p35 in root cap cells, differentiating vascular tissue, and elongating cells. Dialysis of protoplasts with maize annexins stimulated exocytosis at physiological [Ca^{2+}], and this could be blocked by dialysis with antibodies specific to maize annexins. Dialysis with millimolar concentrations of GTP strongly inhibited exocytosis, causing protoplasts to decrease in size. GTPγS and GDPβS both caused only a slight inhibition of exocytosis at physiological Ca^{2+}. Protoplasts were shown to internalize plasma membrane actively. The results are discussed in relation to the regulation of exocytosis in what is usually considered to be a constitutively secreting system; they provide direct evidence for a role of annexins in exocytosis in plant cells.

INTRODUCTION

The growth of plant cells depends on coordination between turgor-driven cell expansion and incorporation of cell wall and plasma membrane materials. The differentiation of plant cells typically requires the cell, plasma membrane, and wall to attain polarity, and the activities of differentiated plant cells frequently necessitate secretion of protein (e.g., from the aleurone cells of cereal grains) or polysaccharide (e.g., from the "slime"-secreting cells of the root cap) in the absence of concomitant growth. Exocytosis represents a key control point in all of these processes, because it determines when and where secretory vesicles bearing secretory products and building materials for cell wall and plasma membrane fuse with the existing plasma membrane. The balance between exocytosis and membrane retrieval must also be controlled to determine whether membrane growth accompanies secretion (Battey and Blackbourn, 1993; Kell and Glaser, 1993; Battey et al., 1996a).

Ca^{2+} is well known as a regulator of plant cell development (Hepler and Wayne, 1985), and we have suggested that one of the major mechanisms by which Ca^{2+} exerts its regulatory effect is by control over exocytosis (Battey and Blackbourn, 1993). The evidence for this is mainly circumstantial but is supported by direct measurement of exocytosis in protoplasts of cells from the barley aleurone layer (Zorec and Tester, 1992; Homann and Tester, 1997) and maize coleoptiles (Thiel et al., 1994). The apparent importance of Ca^{2+} in plant exocytosis raises the question of how the mechanisms controlling exocytosis compare in animals, yeast, and plants. The current belief, based almost exclusively on data from animals and yeast, is that all cells carry out "constitutive" exocytosis and that, in addition, some specialized animal cells (e.g., neurons) show "regulated" exocytosis. Regulated exocytosis is so described because it occurs in response to an external signal and involves coordinated exocytosis of a population of stored vesicles (Burgess and Kelly, 1987). Ca^{2+} is the critical mediator of this regulated response, and it can control exocytosis by indirect effects (e.g., Ca^{2+}-dependent phosphorylation) or directly through Ca^{2+} binding proteins that control vesicle docking and fusion (see Battey and Blackbourn, 1993; Burgoyne and Morgan, 1993). Candidate animal proteins for Ca^{2+}-dependent regulation of this type are synaptotagmin (Goda and Südhof, 1997) and annexins (Creutz, 1992; Raynal and Pollard, 1994; Donnelly and Moss, 1997). Circumstantial evidence has been obtained that suggests that plant annexins may regulate exocytosis, but direct evidence has been unavailable so far (see Clark and Roux, 1995).

The root cap consists of four distinct regions that are arranged as concentric layers in the overall conical-shaped cap (Rougier, 1981). At the center is the cap meristem. As cells are displaced outward, they become starch rich. They then lose their starch granules and become hypersecretory. Finally, in the outer layer, cell walls break down, the polysaccharide

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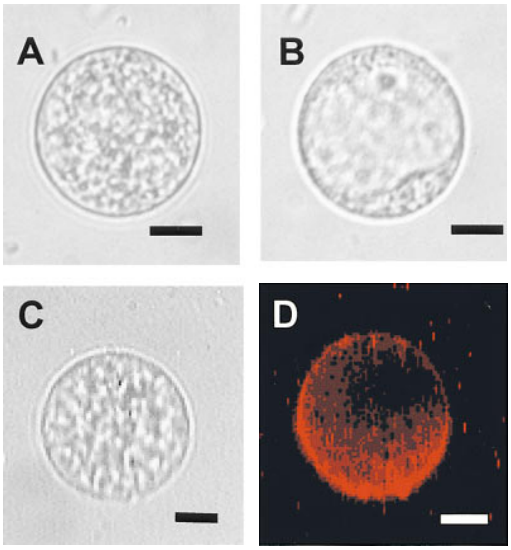

Figure 1. Protoplast Types Obtained from the Root Cap Region.

(A) Densely cytoplasmic protoplasts with granular appearance and no significant accumulation of starch grains.
(B) Highly vacuolate protoplasts.
(C) and **(D)** Bright-field and fluorescence microscopy of the same protoplast in the presence of rhodamine-labeled fucose-specific lectin. Bars in **(A)** to **(D)** = 10 μm.

slime is released, and cells are sloughed off. We wanted to measure exocytosis from the hypersecretory cells, because the secretion of polysaccharide from these cells reflects very active exocytosis that is strongly position dependent, indicating precise control.

Here, we show that Ca^{2+} stimulates exocytosis from root cap protoplasts and that annexins potentiate this stimulation at physiological Ca^{2+} concentrations; GTP can inhibit exocytosis, whereas GTPγS is relatively ineffective. Membrane retrieval is shown to take place at rapid rates in these protoplasts. These results indicate that exocytosis from root cap protoplasts cannot be categorized as regulated or constitutive but has some features in common with both pathways. We discuss the possibility that hypersecretory activity results from a positional signal mediated by Ca^{2+}.

RESULTS

Root Cap Secretory Protoplasts

Protoplasts from the hypersecretory layer were obtained by paring off the outer layers before gentle treatment with cell wall–degrading enzymes (see Methods). The resulting preparation contained predominantly two types of protoplasts differing in appearance (Figures 1A and 1B). Only the densely cytoplasmic protoplasts stained positively for fucose-specific lectin (Figures 1C and 1D). Positive staining occurred in ~70% of the protoplasts of this type after incubation for 4 hr in solution containing fucose-specific fluorescent lectin. These protoplasts were used for the electrophysiological measurements described below.

Measurements of Capacitance Changes Reflecting Exocytosis in Root Cap Protoplasts

Figure 2A shows that compensated whole-cell capacitance (C_m) was linearly proportional to cell surface area for populations of both secretory and vacuolate protoplasts. This correlation held for a range of values of access conductance (data not shown). Mean specific capacitance calculated from these data was 7.53 mF m^{-2} (±0.25; n = 11), which is

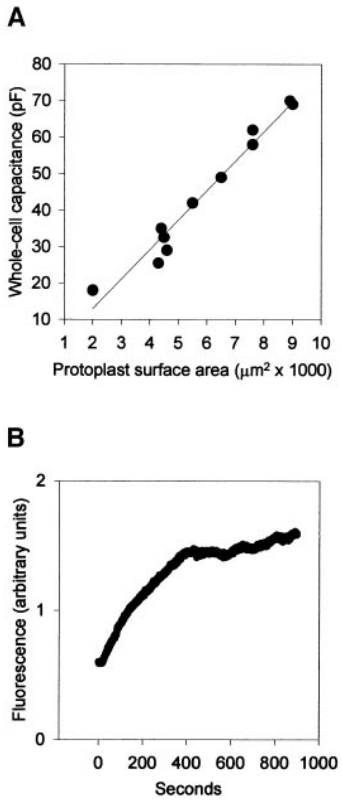

Figure 2. Capacitance Recordings from Different-Sized Protoplasts and Rate of Intracellular Dialysis during Whole-Cell Recording.

(A) Whole-cell C_m values were proportional to the cell surface monitored for a mixed population containing a range of sizes of nonvacuolate and vacuolate protoplasts.
(B) The increase in fluorescence of a secretory protoplast dialyzed via the patch pipette with intracellular recording solution containing 10-kD fluorescein isothiocyanate–dextran was determined. Zero time represents the beginning of whole-cell recording from the protoplast.

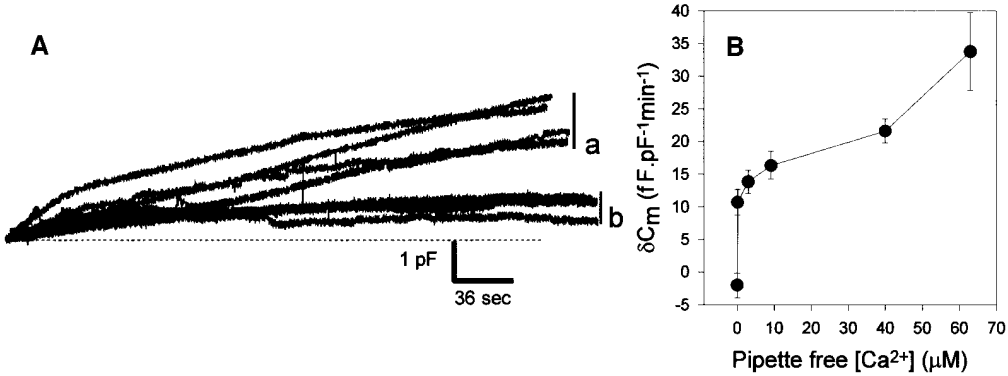

Figure 3. Changes in C_m in Protoplasts Dialyzed with Solutions Buffered to Varying [Ca^{2+}].

(A) Capacitance traces for individual protoplasts dialyzed with 0.2 μM (b) or 40 μM [Ca^{2+}] (a).
(B) [Ca^{2+}] dependence of the mean rate of capacitance increase for protoplasts dialyzed with EGTA-buffered intracellular solutions. Error bars show ±SE ($n > 4$).

close to values reported for other plant and animal cells (Pusch and Neher, 1988; Taylor and Brownlee, 1992; Zorec and Tester, 1992; Thiel et al., 1994). Because cells were dialyzed passively with experimental and control solutions from the patch pipette, it was necessary to determine whether the rate of perfusion was sufficient to allow equilibration of the intracellular contents with the patch pipette solution during the experiment. The diffusion of fluorescein isothiocyanate dextran (10 kD) into the cell from the patch pipette was monitored continuously by using fluorescence photometry. Intracellular fluorescence increased as dye diffused into the cell (Figure 2B), reaching a steady state value after a few minutes. Six replicate experiments gave a mean time to reach half-maximal fluorescence of 160 sec. Because C_m changes were monitored for at least 10 min during intracellular perfusion, this indicates that sufficient equilibration of intracellular contents occurred during the initial stages of an experiment.

Calcium Requirement for Exocytosis

Figure 3A shows representative traces of the change in C_m values of protoplasts with free Ca^{2+} set at 0.2 μM (lower four traces) and 40 μM (upper four traces) in the patch pipette. In most cases, the rate of change was approximately linear after the first 30 sec, allowing us to calculate mean rates at a range of free Ca^{2+} concentrations (Figure 3B). At Ca^{2+} levels close to zero, the protoplasts showed a net decrease in volume, indicating that under these conditions, plasma membrane retrieval exceeds its incorporation by exocytosis. [Ca^{2+}] at 0.2 μM was sufficient to sustain a steady rate of increase in C_m of ~10 fF^{-1} pF^{-1} min^{-1}. Elevation of Ca^{2+} to concentrations >1 μM caused a further, though less pronounced, stimulation of the rate of increase in C_m.

Annexins Potentiate Exocytosis at Elevated Levels of Ca^{2+}

To test their effect on exocytosis, we purified annexins p33 and p35 from maize coleoptiles (Figure 4A). These proteins represent ~70 to 80% of the total protein in this preparation: the annexin-like protein p68 and the non-annexin Ca^{2+}-dependent phospholipid binding protein p23 account for most of the remaining protein (see also Blackbourn et al., 1992; McClung et al., 1994). Previous work has shown that maize root tips contain a closely similar complement of the annexins p35 and p33 (Battey et al., 1996b). In situ hybridization revealed an accumulation of the annexin p35 transcript in the root cap region, in differentiating vascular tissue, and in the root elongation zone (Figure 4B). In the root cap region, accumulation of the transcript was observed in cells below the meristem and root cap initials in regions that labeled heavily for extracellular polysaccharide, as visualized using calcofluor white (Figure 4C). Sense control sections were blank (Figure 4D).

With pipette Ca^{2+} close to physiological levels (0.2 μM), annexins stimulated a C_m increase in a dose-dependent manner, with maximum enhancement occurring at ~20 μg cm^{-3} annexins (Figure 5A). At a pipette concentration of 18 μg cm^{-3} (equivalent to ~0.4 μM), the annexins stimulated the rate of C_m increase by up to threefold at a pipette [Ca^{2+}] of 0.2 and 0.45 μM (Figure 5B). With a pipette [Ca^{2+}] either significantly higher or lower than these values, no promotive effect of the annexins was observed (Figure 5B). The stimulation of exocytosis was specific to annexins because control pipette solutions containing boiled annexins (see Methods) did not enhance exocytosis (Figure 6). The annexin-induced stimulation did not occur when protoplasts were perfused with annexins that had been preincubated with anti-annexin antibodies (see Methods). The C_m increase was eliminated

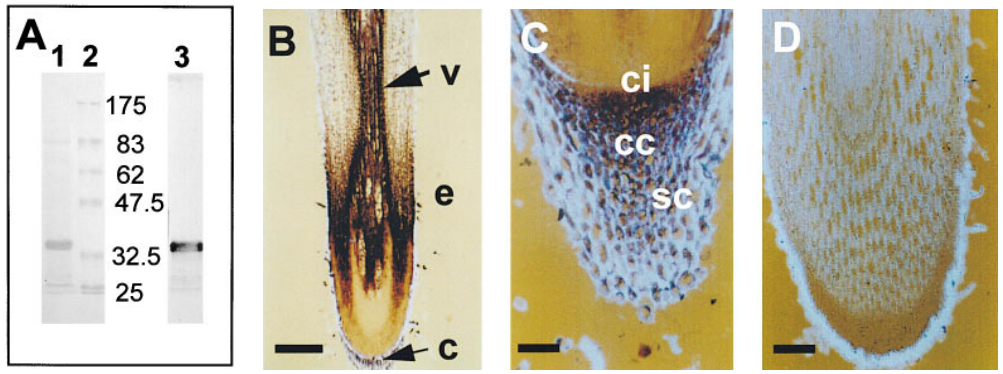

Figure 4. Annexin Purification and Localization of Annexin Gene Expression in Maize Roots.

(A) Characteristics of the preparation from maize coleoptiles used in electrophysiological experiments. Lane 1 contains annexins p33 and p35 after SDS-PAGE, blotting, and staining with Ponceau S; lane 2, molecular mass markers (in kilodaltons); and lane 3, cross-reaction with antiserum raised to annexins p35/p33 from maize. The preimmune control was blank.
(B) Digoxigenin-labeled antisense RNA in situ hybridization of annexin p35 in maize roots. Transcript accumulation is apparent in the differentiating vascular region (v), elongation zone (e), and root cap region (c). Bar = 600 μm.
(C) Within the root cap region, significant accumulation of p35 transcript is evident in the core cap cells (cc), below the cap initials (ci), and in the secretory cells (sc). Blue fluorescent staining shows calcofluor white staining of secreted polysaccharide. Bar = 100 μm.
(D) Sense RNA control section stained with calcofluor white. Bar = 100 μm.

by this treatment, whereas the preimmune serum was without effect. Perfusion with BSA significantly reduced the rate of C_m increase, suggesting a nonspecific inhibitory effect.

GTP Hydrolysis Inhibits Ca^{2+}-Activated Exocytosis

GTP at concentrations >0.5 mM completely inhibited the increase in C_m values with pipette [Ca^{2+}] buffered to 0.2 μM (Figure 7A). However, the net decrease in C_m values previously observed at very low [Ca^{2+}] (Figure 3) was also observed in the presence of GTP, suggesting that membrane retrieval is not inhibited by this nucleotide. GTPγS, a nonhydrolyzable analog of GTP, had only a small inhibitory effect on Ca^{2+}-stimulated exocytosis at the highest concentration used (2 mM); at lower concentrations, it was without effect (Figure 7A). The effects of GTPγS at varying [Ca^{2+}] were complex. GTPγS prevented the decrease in C_m values at a very low pipette [Ca^{2+}], was slightly inhibitory at a 0.2 μM [Ca^{2+}], and was without effect at a higher pipette [Ca^{2+}] (Figure 7B). Like GTPγS, GDPβS slightly inhibited the increase in C_m values at a 0.2 μM pipette [Ca^{2+}] (Figure 7C). The presence of annexins did not alleviate this inhibitory effect (Figure 7C).

Internalization of the Membrane Dye FM1-43 Indicates Rapid Membrane Retrieval in Root Cap Protoplasts

The fluorescent lipophilic probe FM1-43 (Smith and Betz, 1996) was used to monitor plasma membrane internalization in isolated protoplasts. Confocal laser scanning microscopy of dye fluorescence showed that the dye initially labeled the cell periphery (Figure 8A). Dye internalization was clearly visible as early as 5 min after incubation (Figure 8B) and continued to increase steadily over the following hour (Figures 8C to 8F). Saturation of internal dye fluorescence was not observed, indicating incomplete membrane turnover during this time period. Pretreatment of secretory protoplasts for 30 min with the inhibitor of Golgi vesicle transport brefeldin A (BFA) greatly reduced the internalization of FM1-43 (Figures 8G and 8H). Nonsecretory protoplasts showed a much lower degree of FM1-43 internalization compared with secretory protoplasts (Figure 8I).

DISCUSSION

The data presented here indicate that intracellular Ca^{2+} concentrations corresponding to those likely to occur in the cytoplasm of stimulated cells (~0.1 to 10 μM; e.g., Gilroy et al., 1993; Taylor et al., 1996) cause a sustained increase in the plasma membrane area of maize root cap protoplasts. Although an absolute Ca^{2+} requirement for exocytosis is evident from the decrease in C_m values at a very low [Ca^{2+}], the data also point to a regulatory role for Ca^{2+} because stimulation of exocytosis is apparent with increasing [Ca^{2+}] in the micromolar range.

The maize annexin preparation clearly stimulated Ca^{2+}-dependent exocytosis from root cap protoplasts, an effect that was dependent on the annexin concentration in the

patch pipette. Most interestingly, annexin stimulation of exocytosis occurred at physiological concentrations of free Ca^{2+}. This provides direct evidence for a role for annexins in plant exocytotic regulation and supports the idea that the high Ca^{2+} requirement for annexin binding and aggregation in vitro may reflect nonphysiological assay conditions (see Clark and Roux, 1995). The known capacity of some animal annexins to modulate the release of Ca^{2+} from intracellular stores (Diaz-Munos et al., 1990) and others to act as Ca^{2+} channels (Pollard et al., 1991) raises the possibility that plant annexins may act in a similar manner to stimulate exocytosis. However, the stimulation of exocytosis in pipette solutions buffered to submicromolar levels of free Ca^{2+} suggests that this is not the case in this study, and a direct effect on vesicle fusion is probably more likely. In situ hybridization indicated relatively high levels of expression of the annexin p35 in the root in cells undergoing secretion (root cap), expansion (elongation zone), and differentiation (vascular tissue). Biochemical experiments indicate that plant annexins cause Ca^{2+}-dependent aggregation of native secretory vesicles and artificial liposomes and are consistent with a role for these proteins in exocytosis (Blackbourn and Battey, 1993; Hoshino et al., 1995).

Although considerable evidence suggests that annexins act as Ca^{2+}-dependent regulators of exocytosis in animal cells (Creutz, 1992; Raynal and Pollard, 1994; Donnelly and Moss, 1997), their functional integration with the components of the vesicle docking and fusion pathway controlled by the *N*-ethylmaleimide–sensitive fusion protein is unclear (Wilson, 1995). On the other hand, there is now evidence for *N*-ethylmaleimide–sensitive fusion protein—independent secretory pathways. One of them is dependent on annexins (Fiedler et al., 1995; Ikonen et al., 1995; Harder and Simons, 1997). The fact that maize annexins are only ~35% identical to animal annexins (Battey et al., 1996b) suggests that caution is needed in extrapolating from animal to plant cells, but the results presented here provide good evidence that plant annexins can modulate Ca^{2+}-activated exocytosis.

GTP binding proteins are well-established regulators of the secretory pathway in yeast and mammals (Nuoffer and Balch, 1994). There is also evidence to suggest the similar

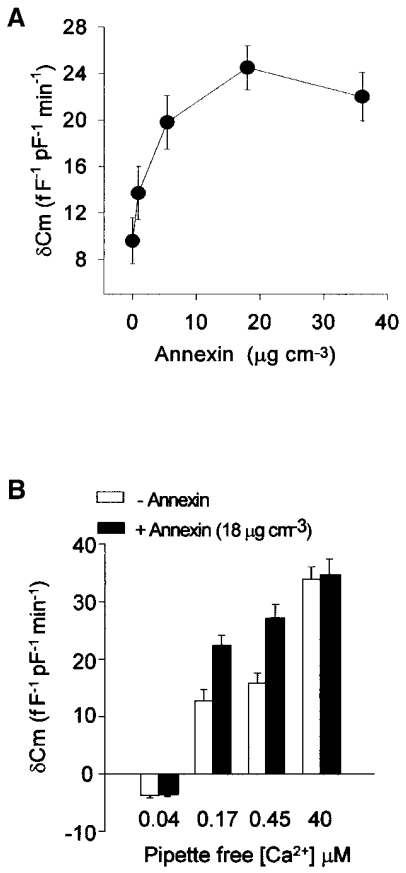

Figure 5. Potentiation of Exocytosis by Annexins.

(A) Increasing concentrations of maize annexins in the recording pipette caused elevated rates of C_m increase, saturating at ~20 μg cm^{-3} annexin. Pipette $[Ca^{2+}]$ was buffered to 0.2 μM.

(B) The presence of annexins in the patch pipette (+ Annexin, closed histograms) elevated the rate of capacitance increase at $[Ca^{2+}]$ in the submicromolar range. Annexins did not potentiate exocytosis at high $[Ca^{2+}]$, at which C_m increase was maximal, and they did not eliminate the capacitance decrease at very low $[Ca^{2+}]$.

Error bars show ±SE ($n > 4$).

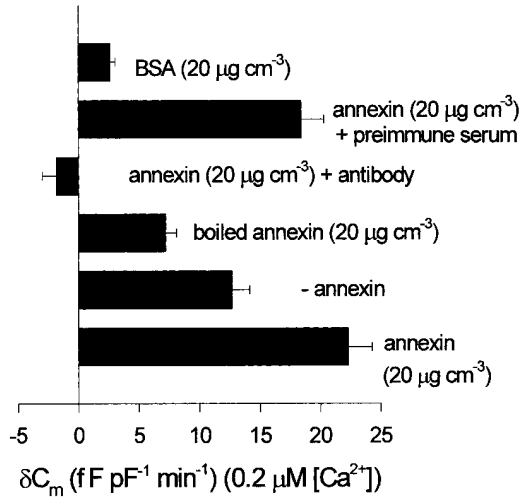

Figure 6. Annexin Antibodies Inhibit Exocytosis.

The addition of annexin antibodies to the pipette dialysis solution (annexin [20 μg cm^{-3}] + antibody) reduced capacitance increase to levels below those of controls without annexin (− annexin). Preimmune serum did not reduce the stimulation of exocytosis (annexin [20 μg cm^{-3}] + preimmune serum), although a nonspecific inhibitory effect occurred with BSA in the pipette (BSA [20 μg cm^{-3}]). Error bars show ±SE ($n > 4$).

involvement of small GTP binding proteins of the Rab family in the early stages of the plant secretory pathway (Verma et al., 1994; Staehelin and Moore, 1995). Recent experiments by Homann and Tester (1997) have indicated the presence of both Ca^{2+}-dependent and Ca^{2+}-independent control of exocytosis. Ca^{2+}-independent exocytosis was found to be insensitive to nonhydrolyzable GTP analogs, whereas the Ca^{2+}-dependent component was blocked strongly by GDPβS and weakly by GTPγS, suggesting the involvement of GTP binding proteins.

Our data indicate that GTP hydrolysis blocks exocytosis in root cap protoplasts. However, the absence of a stimulatory effect of GTPγS at physiological [Ca^{2+}] suggests that this block does not occur at intracellular levels of GTP. In mammalian cells, small GTP binding proteins usually are required for exocytosis so that GTPγS is inhibitory (Hess et al., 1993; Fischer von Mollard et al., 1994). In contrast, heterotrimeric G proteins can be activated by GTPγS (Lillie and Gomperts, 1993; Vitale et al., 1993). However, the effects of nonhydrolyzable analogs of GTP are complex, and their interpretation is difficult. Most pertinent to this study, GTPγS is not effective in all cell types (see Burgoyne and Handel, 1994); thus, the effects of the GTP analogs XTP and GppNHp need to be investigated in future work with root cap protoplasts.

The work described here raises some additional questions about the quantification of exocytosis. C_m measurements reflect the net opposing effects of exocytosis and membrane retrieval, and the latter is clearly shown to occur by the internalization of FM1-43. Moreover, the internalization could be visualized on the same time scale as the measured C_m changes. It would also be expected that for a differentiated nongrowing secretory cell such as the maize root cap cell, rates of exocytosis and membrane retrieval would be precisely matched under normal conditions. Thus, although it is likely that the observed capacitance changes in this and other studies (e.g., Zorec and Tester, 1992; Thiel et al., 1994; Homann and Tester, 1997) represent changes in exocytosis, future experiments need to address the quantification of membrane retrieval. This has been achieved recently in animal systems by simultaneous monitoring of C_m and FM1-43 fluorescence (Smith and Betz, 1996).

The data reported here for hypersecretory cells of the root cap represent an important step forward in our understanding of exocytotic regulation in plant cells. Constitutive exocytosis in yeast and mammals has no apparent requirement for Ca^{2+} but is controlled by small GTP binding proteins. In plants, this pathway has been assumed to be the rule (Griffing, 1991; Moore et al., 1991), although both Ca^{2+}-dependent exocytosis and Ca^{2+}-independent exocytosis have been demonstrated recently in barley aleurone cells (Homann and Tester, 1997). Our data, clearly indicating a Ca^{2+} requirement and inhibition by GTP with only a small inhibitory effect of GTPγS, suggest that exocytosis from root cap protoplasts is more similar to regulated exocytosis, in which Ca^{2+} is the dominant regulator.

However, regulated exocytosis in animals occurs from stored vesicles in response to external signals (Burgess and Kelly, 1987). The strongly position-dependent nature of hypersecretory activity in root cap cells, accompanying but distinct from "housekeeping" secretion of cell wall polysaccharides (Rougier, 1981; Roy and Vian, 1991), may imply

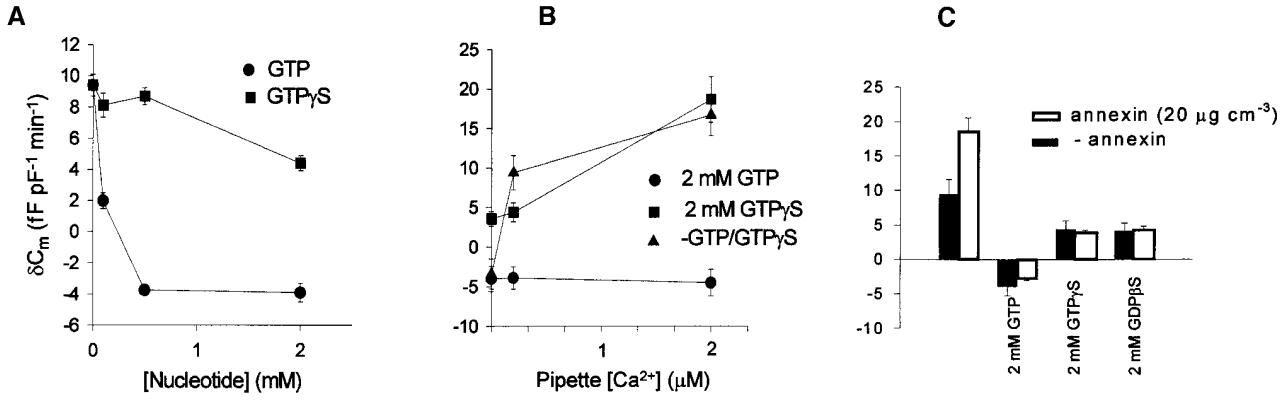

Figure 7. Effects of GTP and GTPγS on Exocytosis.

(A) Dialysis with GTP inhibited the C_m increase at submillimolar pipette concentrations, causing a net cell surface area decrease at concentrations >0.5 mM. GTPγS was inhibitory only at 2 mM. [Ca^{2+}] was buffered to 0.2 μM.
(B) GTPγS (2 mM) alleviated the capacitance decrease at low nanomolar [Ca^{2+}] and produced a slight inhibition of capacitance increase at submicromolar [Ca^{2+}] but was without effect at micromolar [Ca^{2+}]. The minus sign indicates no addition of GTP or GTPγS to the pipette dialysis solution.
(C) Both GTPγS and GDPβS inhibited the capacitance increase at 0.2 μM [Ca^{2+}]. The presence of annexins did not alleviate this inhibition. The minus sign indicates no addition of annexins to the pipette dialysis solution.
Error bars show ±SE ($n > 4$).

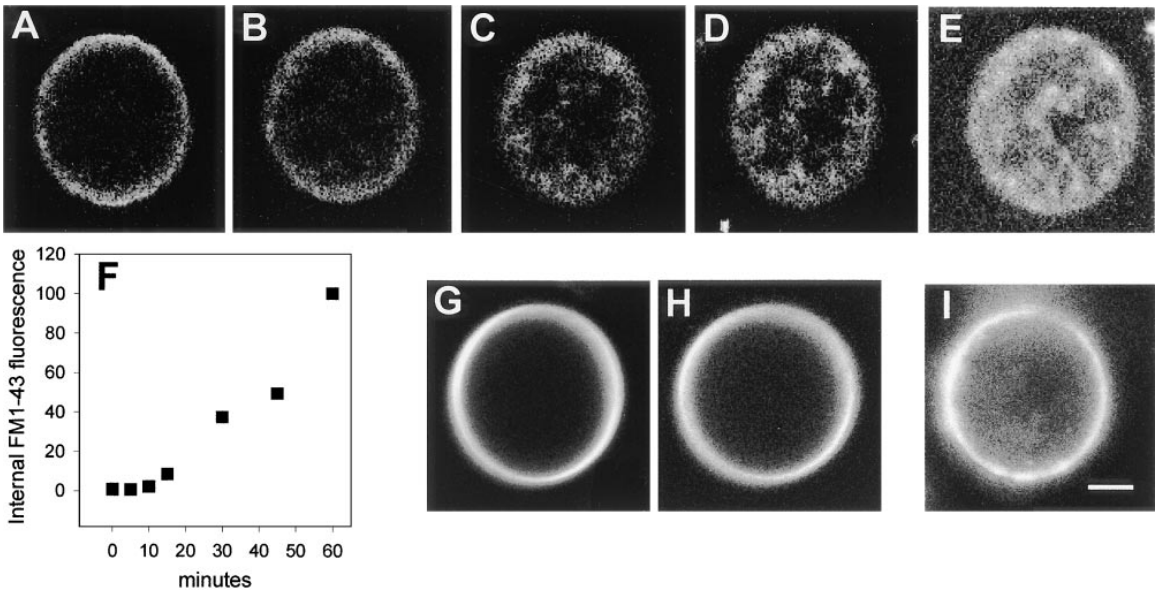

Figure 8. Secretory Protoplasts Show Significant Membrane Cycling.

(A) Confocal microscopy section of a protoplast incubated in the presence of FM1-43, showing initial fluorescence at the cell surface and reflecting dye incorporation into the plasma membrane after 2 min.
(B) Internalization of fluorescence detectable within 5 min.
(C) Fluorescence clearly visible in the cell interior after 15 min.
(D) Nonuniform intracellular fluorescence after 30 min.
(E) Localized regions of elevated intracellular fluorescence visible after 60 min.
(F) Mean relative increase (arbitrary fluorescence units) in intracellular fluorescence during a 60-min incubation period from five protoplasts of the type shown in **(A)** to **(E)**. Mean pixel intensity was measured for regions of interest, which included the whole-cell interior but excluded the fluorescence at the cell surface. Fluorescence increase did not show saturation during the 60-min recording period.
(G) and **(H)** Pretreatment of secretory protoplasts with 10 mg mL^{-1} BFA prevented FM1-43 internalization after 5 **(G)** and 60 **(H)** min.
(I) Nonsecretory protoplast of the type shown in Figure 1B, showing significantly lower internalization of FM1-43 after 60 min.
Bar in **(I)** = 10 μm for **(A)** to **(E)** and **(G)** to **(I)**.

control of differentiation and function by external positional information, as occurs in other root tissues (see Scheres et al., 1996). On the other hand, recent data indicate a Ca^{2+}-regulated exocytotic pathway in Chinese hamster ovary cells, previously regarded as a constitutively secreting cell type (Coorssen et al., 1996); therefore, it is also likely that the constitutive and regulated secretory pathways are present together in many different cell types. Our results on exocytosis from maize root cap protoplasts certainly suggest that these pathways are not clearly distinguishable in this cell type.

METHODS

Protoplast Preparation

Protoplasts were prepared from the root caps of 2-day-old maize seedlings (*Zea mays*). The root caps from ~70 seedlings were dissected and placed into a cell wall digestion buffer composed of Mes (10 mM), $MgCl_2$ (2 mM), $CaCl_2$ (2 mM), mannitol (300 mM), sucrose (10 mM), cellulase R10 Onozuka (1% [w/v]; Yakult Honsha Co., Tokyo, Japan), macerozyme R10 (0.5% [w/v]; Yakult Honsha Co.), and pectolyase Y23 (0.02% [w/v]; Kikkomann Corp., Tokyo, Japan), pH 5.5. Digestion was performed overnight at 15°C, after which the digest was filtered through a 60-μm nylon mesh, and the protoplasts were collected by centrifugation (500*g* for 5 min). The collected protoplasts were washed twice in the cell wall digestion buffer without the enzymes, after which the final pellet was resuspended in 2 cm^3 of the wash buffer and held on ice.

Lectin Staining of Fucose-Rich Polysaccharides

Protoplasts were incubated in cell wall digestion buffer without enzymes containing 1 mg cm^{-3} rhodamine-labeled fucose-specific lectin (UEA I-TRITC; Sigma) for up to 4 hr. Fluorescence (excitation 520 to 560 nm; emission >590 nm) and bright-field images were recorded with a cooled CCD camera (Digital Pixel, Brighton, UK) on an inverted microscope (Nikon, Tokyo, Japan).

Measurement of Plasma Membrane Capacitance

Changes in whole-cell capacitance (C_m) values of protoplasts prepared from secretory cells in the root cap of maize were monitored using standard whole-cell patch–clamp techniques by the software-driven phase-tracking technique of Fidler and Fernandez (1989) (see Battey et al., 1996a), using a modified Axopatch 200A amplifier and a TL-1 interface (Axon Instruments, Foster City, CA). Patch pipettes were fire polished to a resistance of 3 to 7 MΩ and coated in beeswax, giving mean access conductance (G_a) of 43 nS (SE = 2.27; n = 56). C_m was measured with the protoplast plasma membrane potential clamped at −60 mV. Mean recorded zero current potential was −47 mV (SE = 3.47; n = 56). For recordings, protoplast aliquots were diluted 20-fold with an external recording solution composed of K-glutamate (10 mM), $MgCl_2$ (5 mM), $CaCl_2$ (2 mM), Hepes-KOH (10 mM), and mannitol (350 mM), pH 7.2, and allowed to stand for 5 min to equilibrate to room temperature and to settle and adhere to the glass coverslip base of the recording chamber.

A range of microelectrode filling solutions covering [Ca^{2+}] between <1 nM and 40 μM was prepared from solutions containing K-glutamate (98 mM), $MgCl_2$ (2 mM), Hepes (10 mM), mannitol (300 mM), ± $CaCl_2$ (10 mM), Mg-ATP (2 mM), and EGTA (10 mM), pH 7.2. [Ca^{2+}] was varied by varying the Ca^{2+}/EGTA ratio. GTP, GTP analogs, and annexins were added to the intracellular solution immediately before recording. The osmolarities of the external and the pipette filling solutions were adjusted to within 2% of each other. Recordings were made against an agar bridge reference electrode containing the microelectrode filling solution without $CaCl_2$. In each experiment, an electrical seal >1 GΩ was formed between the plasma membrane and the fire-polished and coated microelectrode. Whole-cell recording configuration was achieved by suction. Whole-cell, fast, and slow capacitance transients were compensated, and the compensated response to a step voltage was checked at high gain before beginning the experiments. The initial membrane capacitance (C_i) was noted, and the diameter of the protoplast was measured using an eye-piece graticule. There was an average delay of ~20 sec between achieving whole-cell configuration and beginning the recording.

Throughout the course of an experiment, changes in C_m values were calibrated by switching in a decrease in whole-cell C_m compensation of 100 fF; similarly, a 500 MΩ dither resistor periodically was switched in to ensure that the capacitative element of the measured induced current was unaffected by changes in the current's resistive element. We used a software-driven phase-tracking technique (Joshi and Fernandez, 1988; Fidler and Fernandez, 1989; Battey et al., 1996a) to determine the appropriate phase angle at which to measure capacitative current. Phase tracking allows rapid and accurate determination of changes in the phase of the capacitative vector, thus allowing continual alignment of the current detector with the capacitance signal. This prevents errors in the capacitance measurements due to changes in series resistance. Each recording was sufficiently long to allow any change in C_m to reach a maximum within a minimum of 12 min. To account for the range of sizes of the protoplasts from which recordings were made, changes in C_m values with respect to time were normalized against C_i.

Annexin Isolation

Annexins were isolated from maize coleoptile tissue, as described by Blackbourn et al. (1991), except that after hydroxyapatite chromatography, the annexin preparation was dialyzed against 2 × 2-liter volumes of 5 mM Mes, pH 6.5, and concentrated to ~2 mg cm^{-3} in the same buffer by using Centriprep and Centricon microconcentrators (Amicon, Gloucester, UK), according to the manufacturer's instructions. Concentrated annexin protein was aliquoted, stored at −20°C, and used within 3 months of preparation. Protein gel blotting was performed according to Blackbourn et al. (1991), except that antiserum was used at a dilution of 1:10,000. In the patch–clamp dialysis experiments, antibodies to maize annexins (Blackbourn et al., 1991) were preincubated with annexin protein at a ratio of 2:1 for 30 min before dilution to a final annexin concentration of 20 μg cm^{-3}.

In Situ Hybridization

Tissue preparation and in situ hybridization using digoxigenin-labeled RNA probes were conducted according to Bradley et al. (1993). A clone containing a full-length cDNA encoding the maize annexin p35 (Battey et al., 1996b) was used to generate RNA probes. The cDNA was cut with BamHI to provide a template for T7 polymerase to generate the antisense RNA probe. Sense RNA, obtained using T3 polymerase and the p35 cDNA clone cut with KpnI, was used as a negative control.

Visualization of Membrane Retrieval

Protoplasts were incubated with 1 μM FM1-43 (Molecular Probes, Eugene, OR) in external recording solution (see above), and dye fluorescence (excitation 488 nm; emission 590 nm) was imaged using a confocal microscope (Molecular Dynamics, Sunnyvale, CA).

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Ca^{2+}, Annexins, and GTP Modulate Exocytosis from Maize Root Cap Protoplasts

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