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Ca^{2+}-independent and Ca^{2+}/GTP-binding protein-controlled exocytosis in a plant cell

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ABSTRACT **Exocytosis allows the release of secretory products and the delivery of new membrane material to the plasma membrane. So far, little is known about the underlying molecular mechanism and its control in plant cells. We have used the whole-cell patch-clamp technique to monitor changes in membrane capacitance to study exocytosis in barley aleurone protoplasts. To investigate the involvement of Ca^{2+} and GTP-binding proteins in exocytosis, protoplasts were dialyzed with very low (<2 nM) and high (1 μM) free Ca^{2+} and nonhydrolyzable guanine nucleotides guanosine 5′-[γ-thio]triphosphate (GTP[γS]) or guanosine 5′-[β-thio]-diphosphate (GDP[βS]). With less than 2 nM cytoplasmic free Ca^{2+}, the membrane capacitance increased significantly over 20 min. This increase was not altered by GTP[γS] or GDP[βS]. In contrast, dialyzing protoplasts with 1 μM free Ca^{2+} resulted in a large increase in membrane capacitance that was slightly reduced by GTP[γS] and strongly inhibited by GDP[βS]. We conclude that two exocytotic pathways exist in barley aleurone protoplasts: one that is Ca^{2+}-independent and whose regulation is currently not known and another that is stimulated by Ca^{2+} and modulated by GTP-binding proteins. We suggest that Ca^{2+}-independent exocytosis may be involved in cell expansion in developing protoplasts. Ca^{2+}-stimulated exocytosis may play a role in gibberellic acid-stimulated α-amylase secretion in barley aleurone and, more generally, may be involved in membrane resealing in response to cell damage.**

Exocytosis is a vital process in plant growth and development. During exocytosis, vesicles fuse with the plasma membrane that results in the delivery of membrane material and membrane-associated proteins to the plasma membrane and/or the release of secretory products such as cell wall precursors and extracellular proteins. This process must be highly controlled, but so far little is known about the underlying molecular mechanisms in plant cells (1–3).

In animal cells, cytosolic Ca^{2+} activity, GTP-binding proteins, and protein phosphorylation/dephosphorylation are known to be involved in the regulation of secretion (4, 5). Ca^{2+} also appears to be important in controlling secretion in plant cells (1, 6), and a rise in cytosolic Ca^{2+} activity has been shown to stimulate exocytosis in barley aleurone (7) and maize coleoptile protoplasts (8). Ca^{2+}-independent exocytosis, which is also common in animal cells (9), has not yet been demonstrated in plants. To our knowledge, there has also been no direct evidence published for the involvement of other regulators of exocytosis in plant cells, such as GTP-binding proteins. However, homologues of animal and yeast GTP-binding proteins that are involved in the process of secretion have been identified in plants (3, 10).

To investigate the mechanisms involved in the regulation of exocytosis in plant cells, we have studied the role of Ca^{2+} and GTP-binding proteins in exocytosis in protoplasts from barley aleurone layers. Exocytosis is clearly essential in the central function of these cells, namely, the secretion of hydrolytic enzymes upon germination. Aleurone cells should therefore contain all the components to allow exocytosis. In addition to providing a model system for studying secretion, they also proved to be an excellent system for the analysis of vacuole development in plants (11). Vacuolation occurs whether or not cells are secreting and is associated with an increase in cell diameter and, thus, insertion of new plasma membrane material via exocytosis (12, 13). As all cells studied in the current work had started to vacuolate (see *Results*), they were likely to be in the process of exocytosis.

By using the patch-clamp technique, we have monitored changes in plasma membrane area, resulting from endo- and exocytotic events, by measuring the membrane capacitance (C_m) (14, 15). This technique also allows us to control the cytosolic composition by dialyzing the cell via the patch pipette. To analyze the role of Ca^{2+} and GTP-binding proteins in exocytosis, we have dialyzed the cytosol of protoplasts with solutions of low and high Ca^{2+} activity in the absence or presence of nonhydrolyzable guanine nucleotides, guanosine 5′-[γ-thio]triphosphate (GTP[γS]) and guanosine 5′-[β-thio]diphosphate (GDP[βS]). These nucleotides have been widely used in animal cells to investigate the involvement of GTP-binding proteins in exocytosis (for example, see refs. 9, 16, and 17). Our results demonstrate a Ca^{2+}-independent exocytotic pathway in barley aleurone in addition to the previously described Ca^{2+}-stimulated exocytosis. We found that Ca^{2+}-independent exocytosis was not affected by GTP[γS] or GDP[βS], but stimulation of exocytosis by Ca^{2+} was slightly inhibited by GTP[γS] and completely abolished in the presence of GDP[βS]. These results are consistent with the involvement of GTP-binding proteins in a Ca^{2+}-stimulated exocytotic pathway in barley aleurone cells.

MATERIALS AND METHODS

Isolation of Aleurone Protoplasts. De-embryonated grains of barley (*Hordeum vulgare* L. cv. Himalaya) were cut in half longitudinally, surface-sterilized by incubation in NaOCl (0.5% available chlorine) for 20 min at room temperature, then rinsed five times in sterile distilled water, and imbibed for 24 h in the dark at 30°C in sterile conditions. About 30 imbibed "half-seeds" were placed in a Petri dish and incubated for 15–17 h in the dark at 30°C in 3.5 ml of filter-sterilized (0.2-μm pore size, Millipore) enzyme solution, containing 4.5% cellulase Onozuka RS (Yakult Honsha, Tokyo), 0.05% pectolyase Y-23 (Seishin, Tokyo), and 2% (wt/vol) bovine serum albumin (fraction 5), dissolved in culture medium, which consisted of 100 mM glucose, 10 mM L-arginine, 20 mM $CaCl_2$, 10 mM Mes·HCl (pH 5.5), 58 mM sucrose, 1.1 mM NaH_2PO_4, 25 mM

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Abbreviations: C_m, membrane capacitance; GA_3, gibberellic acid; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; mOsmol, milliosmole(s); BAPTA, bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate.

KNO_3, 1 mM $(NH_4)_2SO_4$, and 1 mM $MgSO_4$ (after ref. 18), and then adjusted to 830 ± 10 milliosmoles (mOsmol)/kg with sorbitol (approximately 450 mM). After incubation, the enzyme solution was replaced by culture medium and the Petri dish was tapped several times to release protoplasts sticking to the half-seeds. Protoplasts were purified by centrifuging at $50 \times g$ for 5 min on a Nycodenz (Nycomed, Oslo) step gradient, 70:50:0% (wt/vol) in culture medium, collected immediately after centrifuging from the 50:0% interface and stored in culture medium at room temperature.

Capacitance Measurements. Standard patch-clamp whole-cell recordings were used to measure C_m (14). Capacitance was measured with a two-phase lock-in amplifier (SWAM IIC; Henigman, Ljubljana, Slovenia). Computer-aided data processing (19–21) allowed on-line recording of C_m, parallel combination of seal resistance and membrane conductance, and access conductance with software provided by J. Dempster (University of Strathclyde, Glasgow, U.K.).

Patch pipettes with a tip resistance of 3–5 MΩ were prepared from glass capillary tubing (Kimax 51, Kimax Products, Vineland, NJ) by using a DMZ Universal Puller (Zeitz Instrumente, Augsburg, Germany) leading to an average access conductance of 137 ± 5 nS ($n = 67$). The shank of the pipette was coated with beeswax to reduce stray capacitance. Protoplasts were clamped at −50 to −70 mV; no effects of the holding voltage on changes in C_m were observed. Protoplast diameter was measured with an eye-piece micrometer. Data are presented as the mean ± SEM.

Solutions. Protoplasts were bathed in a solution of 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, and10 mM Mes·NaOH (pH 5.6). The pipette solution for measurements with low cytosolic Ca^{2+} contained 30 mM potassium gluconate, 10 mM KCl, 5 mM $MgCl_2$, 20 mM K_4-BAPTA [where BAPTA is bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate], 4 mM K_2ATP, and 10 mM Mes·KOH (pH 7.2). In measurements where BAPTA was replaced by 8 mM EGTA or 20 mM free acid 5,5′-dibromo-BAPTA, the amount of potassium gluconate was increased to keep the K^+ concentration constant. The pipette solution for measurements with 1 μM cytosolic free Ca^{2+} contained 100 mM potassium gluconate, 10 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 6 mM K_4-BAPTA, 4 mM K_2ATP, and 10 mM Mes·KOH (pH 7.2). Ca^{2+} activity was calculated with the computer program GEOCHEM-PC. $Li_4GTP[\gamma S]$ and GDP[βS] were added to the pipette solution on the day of use. Nucleotides were from Sigma or from Boehringer, with no differences in responses noticed between chemicals from the two sources. 5,5′-Dibromo-BAPTA was obtained from Fluka. All other chemicals were from Sigma. The osmolarities of all bath and pipette solutions were adjusted to 830 ± 10 mOsmol/kg with sorbitol and were always within

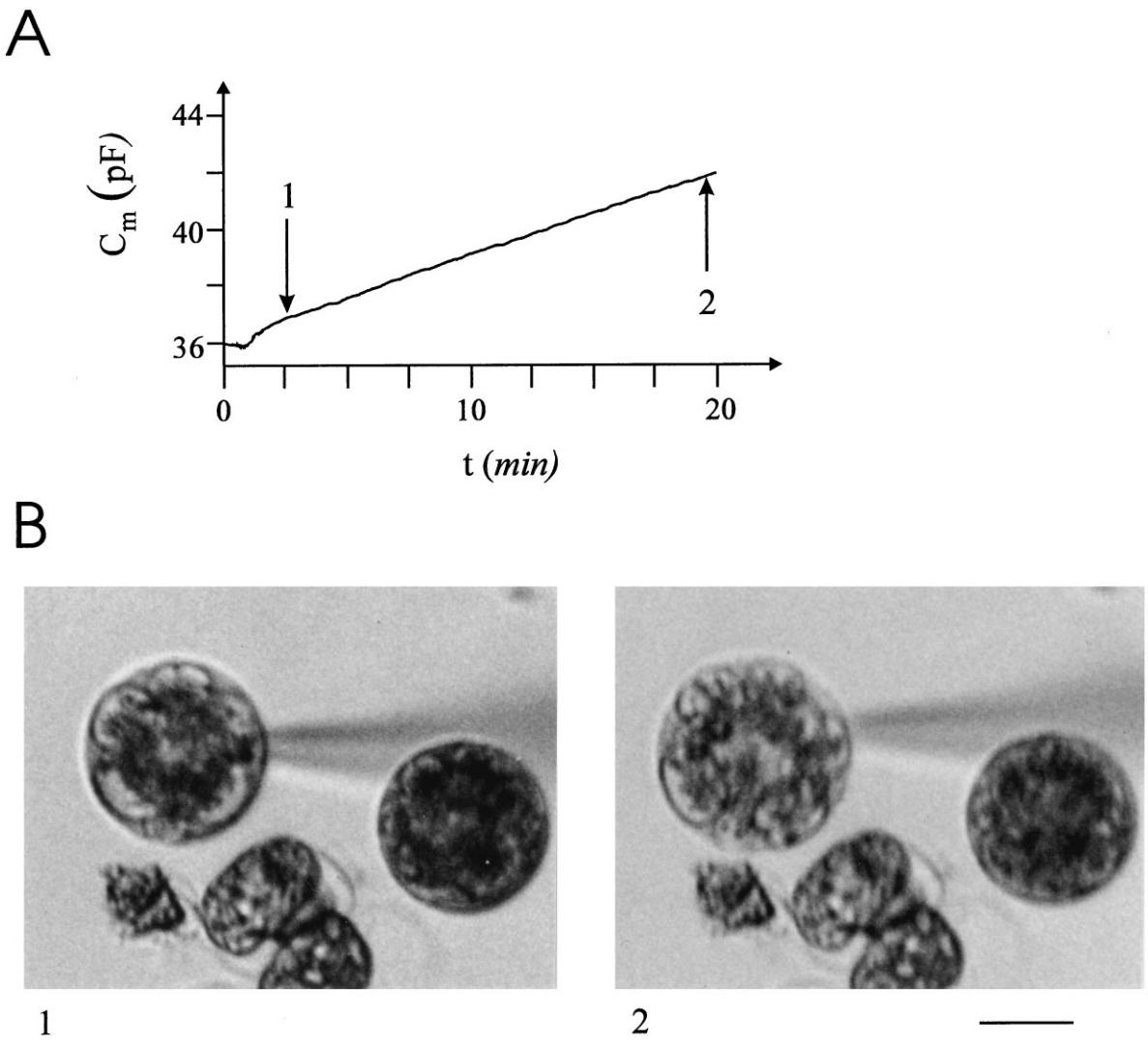

FIG. 1. Parallel increase in C_m and cell surface area. (*A*) Change in C_m after cytosolic dialysis with 1 μM free Ca^{2+}. Arrows indicate time at which photographs in *B* were taken. (*B*) Aleurone protoplast in stage II (13) photographed during the capacitance measurement at 3 min (*1*) and 20 min (*2*) after dialysis with 1 μM free Ca^{2+}. Bar = 20 μm.

1% of each other. The osmotic pressure of solutions was measured with a vapor pressure osmometer (model 5100, Wescor, Logan, UT). All solutions were filtered (0.22-μm pore size, Millipore) before use. Measurements were carried out at room temperature (20–27°C) with continuous bath perfusion (three to five bath volumes per min).

RESULTS

General Features of Exocytosis. The development of aleurone protoplasts has been divided into four stages, based on the degree of vacuolation (13, 22). All measurements presented were carried out on protoplasts that had just started to vacuolate (developmental stage II; Fig. 1). The average diameter of the protoplasts studied was 35.0 ± 0.4 μm (n = 67) with an average initial capacitance (C_m upon formation of a whole-cell recording) of 30.1 ± 0.7 pF, giving a specific capacitance of 7.6 ± 0.04 mFm^{-2}. Fig. 1*A* shows the increase in capacitance of an aleurone protoplast dialyzed with a pipette solution containing 1 μM free Ca^{2+}. Capacitance increased by 6 pF over 20 min, concomitant with a visible increase in diameter from 39 to 42 μm (Fig. 1*B*). There was no change in specific capacitance during these experiments, demonstrating that the rise in C_m was due to an increase in cell surface area as a result of exocytosis and not as a result of stretching of membranes (see also ref. 7).

Ca^{2+}-Independent and Ca^{2+}-Stimulated Exocytosis. After dialyzing the protoplasts with 1 μM free Ca^{2+} for 20 min, we found an average relative increase in C_m of 14.7 ± 2.3% (n = 8) (Fig. 2). This corresponds to a rate of increase of 4.0 ± 0.8 fF/s, similar to that in previous measurements on barley aleurone (7).

Exocytosis was not prevented at very low cytosolic Ca^{2+} activities, which is in contrast to earlier measurements by Zorec and Tester (7). Protoplasts dialyzed with a solution containing 8 mM EGTA and no added Ca^{2+} ("0 Ca^{2+}" conditions) showed an average increase of 5.4 ± 2.3% (n = 4; Fig. 2). Under these conditions, free Ca^{2+} was calculated to be less than 2 nM, even if as much as 100 μM Ca^{2+} was added as a contaminant of other chemicals (such as sorbitol). The observed increase was not the result of an osmotic effect as the osmolarities of the bath and pipette solutions were adjusted to within 1% of each other (see *Materials and Methods*). To test whether this apparently Ca^{2+}-independent increase in C_m was the result of a local rise in cytosolic Ca^{2+} due to a Ca^{2+} influx down the large gradient across the plasma membrane and insufficient intracellular buffering, we increased cytosolic Ca^{2+} buffering and reduced the external Ca^{2+} concentration. Replacement of EGTA with the Ca^{2+} buffers BAPTA or dibromo-BAPTA, which are known to be more efficient than EGTA in dissipating local Ca^{2+} gradients (23, 24) did not prevent exocytosis. Within 20 min of dialyzing the protoplast with 20 mM BAPTA or dibromo-BAPTA, C_m increased by 3.6 ± 0.6% (n = 10) and 5.8 ± 3% (n = 6), respectively (Fig. 2). There was no significant difference between the three Ca^{2+} buffers tested ($P > 0.5$, Student's t test). Changing external Ca^{2+} from 10 mM to submillimolar activities also had no effect on the increase in C_m (Fig. 3), nor did addition of up to 10 mM La^{3+}, a potential blocker of Ca^{2+} channel-mediated Ca^{2+} influx (25, 26) (data not shown). Our results therefore demonstrate that the exocytosis measured under 0 Ca^{2+} conditions is not the result of a stimulation by a localized increase in cytosolic Ca^{2+}. This suggests the existence of an exocytotic pathway in barley aleurone cells that does not require Ca^{2+} (now referred to as Ca^{2+}-independent exocytosis), in addition to the Ca^{2+}-stimulated exocytosis described above.

Involvement of GTP-Binding Proteins in Ca^{2+}-Independent and Ca^{2+}-Stimulated Exocytosis. To study the role of GTP-binding proteins on Ca^{2+}-independent and Ca^{2+}-stimulated exocytosis, we dialyzed the cytosol of protoplasts with nonhydrolyzable analogues of guanine nucleotides (GTP[γS] and GDP[βS]) with low or high (1 μM) Ca^{2+}. With low cytosolic Ca^{2+} (maintained with 20 mM BAPTA), the increase in C_m was small (3.6 ± 0.6%, n = 10) and was not affected by addition of 100 μM GTP[γS] or 500 μM GDP[βS]. The average increase in C_m after 20 min was 4.3 ± 1.5% (n = 6) and 6.0 ± 1.8% (n = 9), respectively (Fig. 4 *A* and *C*). These are not significantly different from the control ($P > 0.2$, Student's t test) and indicates that GTP-binding proteins are not involved in the modulation of Ca^{2+}-independent exocytosis. With 1 μM free cytosolic Ca^{2+}, exocytosis was greatly increased (14.7 ± 2.3%, n = 8), an effect that was reduced by 36% by addition of GTP[γS] (average increase in C_m of 9.4 ± 1.6%, n = 6; P = 0.08, Student's t test) (Fig. 4 *B* and *C*), suggesting that GTP-binding proteins may play a role in Ca^{2+}-stimulated exocytosis. This was strongly supported by the effect of GDP[βS] on Ca^{2+}-stimulated exocytosis. Dialyzing the proto-

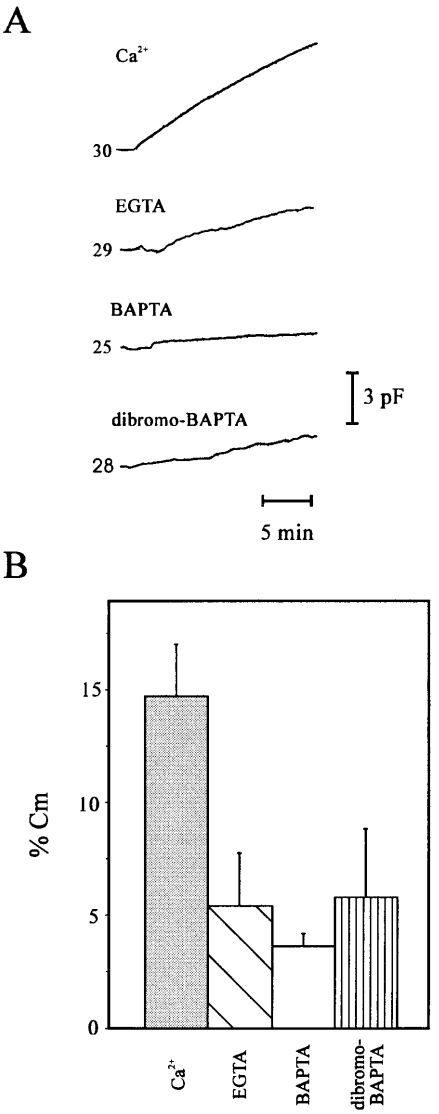

FIG. 2. Effect of different Ca^{2+} buffers on Ca^{2+}-independent exocytosis compared with Ca^{2+}-stimulated exocytosis. (*A*) Example traces illustrating changes in C_m for 20 min after the start of cytosolic dialysis with 1 μM free Ca^{2+} (top trace), or with no added Ca^{2+} and addition of Ca^{2+} buffers as indicated. Numbers at start of traces indicate resting capacitance (in pF) measured upon formation of the whole-cell configuration. (*B*) Average relative changes in C_m (% C_m) determined 20 min after the start of cytosolic dialysis with 1 μM free Ca^{2+} (n = 8), 8 mM EGTA (n = 4), 20 mM BAPTA (n = 10), or 20 mM dibromo-BAPTA (n = 7). Bars represent SEM.

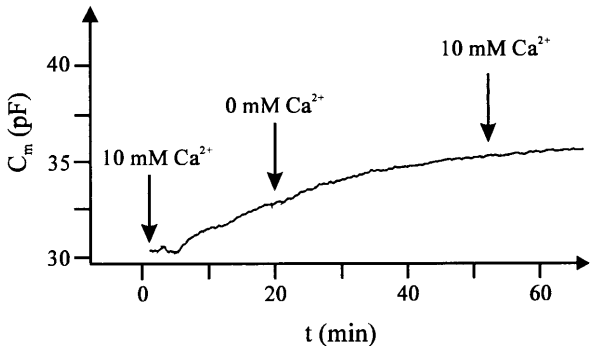

FIG. 3. Effect of external Ca^{2+} on Ca^{2+}-independent exocytosis. Change in C_m after the start of cytosolic dialysis with 8 mM EGTA and no added Ca^{2+}. Measurements started in standard bath solution (BS) containing 10 mM Ca^{2+}, and arrows indicate change to 0 mM Ca^{2+} (standard BS with 0.5 mM EGTA and no added Ca^{2+}) at t = 21 min and back to 10 mM Ca^{2+} (standard BS) at t = 52 min. Note different time scale to that used in other figures.

plast with 1 μM Ca^{2+} and 500 μM GDP[βS] eliminated the stimulatory effect Ca^{2+} (Fig. 4 *B* and *C*). Under these conditions, the average increase in C_m was only 3.7 ± 1.9% (n = 6), which is not significantly different from nonstimulated cells (P = 0.9, Student's t test). This clearly demonstrates that GTP-binding proteins are involved in the Ca^{2+}-stimulated exocytotic pathway.

DISCUSSION

We have studied the modulation of exocytosis by Ca^{2+} and nonhydrolyzable guanine nucleotides in barley aleurone cells *in vivo*, by measuring the C_m with the patch-clamp technique. Changes in C_m represent a balance between rates of exo- and endocytosis and, unless single fusion events can be resolved, it is not possible to distinguish between an increase in membrane area resulting from an increase in exocytosis or a decrease in endocytosis. However, in studies where the two mechanisms have been separated, an increase in C_m could be explained by an increased rate of exocytosis (27) and only after prolonged stimulation did exo- and endocytosis overlap (28). Therefore, in our discussion of the experiments, we interpret effects on C_m to be only due to effects on exocytosis.

Two Exocytotic Pathways in Barley Aleurone Cells. Our results show the existence of a Ca^{2+}-independent exocytotic pathway in barley aleurone in addition to a previously described stimulation of exocytosis by an increase in cytosolic free Ca^{2+} (7). Protoplasts that had been dialyzed with a solution containing less than 2 nM free Ca^{2+} still showed an increase in C_m (Fig. 2). In contrast, Zorec and Tester (7) found a decrease in C_m after dialyzing the protoplasts with low cytosolic Ca^{2+} activity (<30 nM). The decrease was thought to be due to a reduction in the basal rate of exocytosis that normally balances membrane retrieval by endocytosis. A similar decrease in C_m was also detected in maize coleoptile protoplasts (8). One explanation for these findings might be that, in contrast to our measurements, there was no continuous perfusion of the bath during the earlier experiments. This could have resulted in evaporation increasing the osmolarity of the external solution, causing an efflux of water and subsequent endocytosis. In the current work, we found that a change in the osmolarity of the external solution of only 4% resulted in a change in C_m (data not presented). Another possible reason for the different findings might be that the protoplasts studied by Zorec and Tester (7) were mainly in stage I (M.T., unpublished observations) and bathed in a solution of higher osmolarity (1100 mOsmol/kg), which might have blocked vacuolation and cell expansion. In the absence of GA_3, vacuolation in aleurone is inhibited by increasing osmolarity (12, 22),

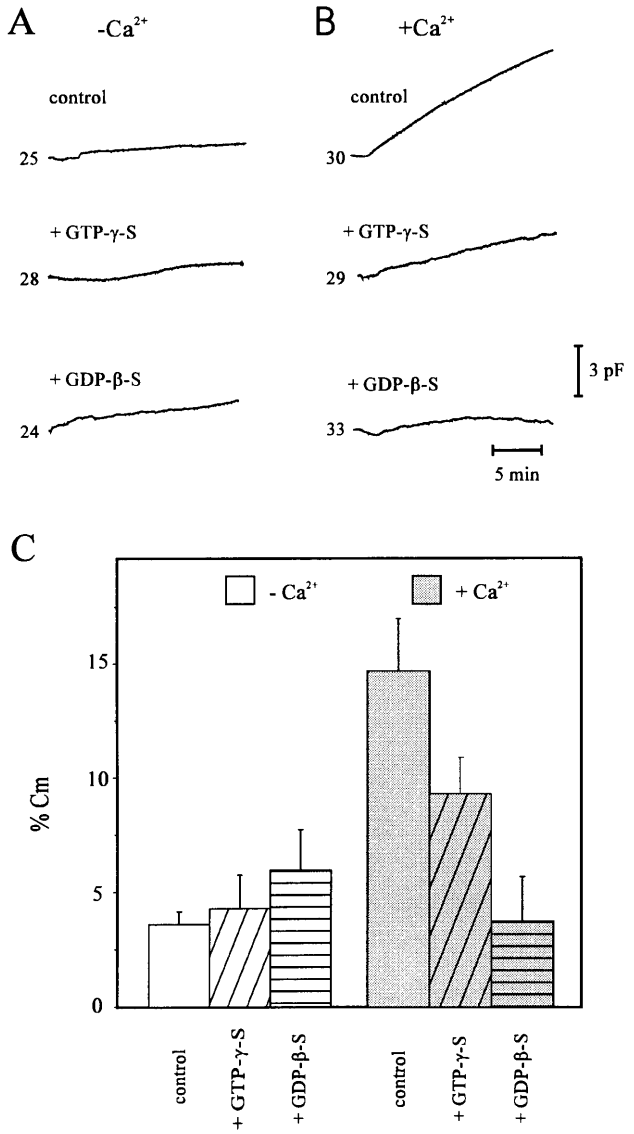

FIG. 4. Effect of GTP[γS] and GDP[βS] on Ca^{2+}-independent and Ca^{2+}-stimulated exocytosis. (*A*) Change in C_m for 20 min after the start of cytosolic dialysis with 20 mM BAPTA and no added Ca^{2+} (top trace), and with addition of 100 μM GTP[γS] (middle trace), or 500 μM GDP[βS] (bottom trace). Numbers at start of traces indicate resting capacitance (in pF) measured upon formation of the whole-cell configuration. Increase in C_m for the cells shown was 4, 3, and 7%. (*B*) Change in C_m for 20 min after the start of cytosolic dialysis as in *A* but with 1 μM free Ca^{2+}. Increase in C_m for the cells shown was 16, 10, and 1%. (*C*) Relative change in C_m (% C_m) determined 20 min after the start of cytosolic dialysis with 20 mM BAPTA and no added Ca^{2+} (n = 10) and with addition of 100 μM GTP[γS] (n = 6) or 500 μM GDP[βS] (n = 9) (*Left*) and 1 μM Ca^{2+} (n = 8) and addition of 100 μM GTP[γS] (n = 6) or 500 μM GDP[βS] (n = 6) (*Right*). Bars represent SEM.

and no development beyond stage II occurs, even after 5 days, when the osmolarity was as high as 990 mOsmol/kg (11). In contrast, the data presented in the present study are derived from protoplasts in stage II (which have already started to vacuolate) and were bathed in a solution of only 820 mOsmol/kg.

Ca^{2+}-independent exocytosis has also been demonstrated in animal cells, with high rates of exocytosis observed even with cytosolic Ca^{2+} activities as low as 1 nM (9). This is similar to our findings with barley aleurone cells. In Chinese hamster ovary cells, the Ca^{2+}-independent exocytosis was completely abolished by GTP[γS], indicating that GTP-binding proteins are involved in the control of Ca^{2+}-independent exocytosis (9).

This does not seem to be the case for barley aleurone cells as neither GTP[γS] nor GDP[βS] had any effect on Ca^{2+}-independent exocytosis. Phosphorylation also does not appear to play a role in the modulation of Ca^{2+}-independent exocytosis, as dialyzing the protoplasts without added ATP in 0 Ca^{2+} conditions did not change the observed increase in C_m (average relative increase in C_m was 5.1 $\pm$ 2.0%, $n = 12$).

Physiological Relevance of the Two Exocytotic Pathways. We found that two exocytotic pathways exist in barley aleurone cells, one that is Ca^{2+}-independent and whose regulation is currently unknown and another that is stimulated by Ca^{2+}. Because development of barley aleurone protoplasts does occur in the absence of external Ca^{2+} (13), it seems likely that Ca^{2+}-independent exocytosis is the exocytotic process underlying the cell expansion during protoplast development and Ca^{2+}-stimulated exocytosis is involved in a different exocytotic process. Exocytosis in animal cells is often divided into two distinct pathways: constitutive and regulated exocytosis. Constitutive exocytosis is thought to be independent of cytosolic free Ca^{2+}, whereas regulated exocytosis is stimulated by an increase in cytosolic free Ca^{2+} (9). One could therefore propose that the observed Ca^{2+}-independent exocytosis is part of a constitutive exocytotic pathway and the Ca^{2+}-stimulated exocytosis belongs to a regulated exocytotic pathway. However, the distinction between constitutive and regulated pathways might not be appropriate for plant cells (1) and has become less rigid in animal cells (for example, see refs. 29 and 30).

A recent study on Chinese hamster ovary cells demonstrates that these cells are not only capable of exocytosis that is independent of cytosolic Ca^{2+} activity but also of Ca^{2+}-stimulated exocytosis (30). This is similar to our findings in barley aleurone. Coorssen *et al.* (30) suggest that all eukaryotic cells are capable of rapid Ca^{2+}-stimulated exocytosis as a mechanism for the repair of plasma membrane damage, as resealing of cell membrane requires Ca^{2+}-stimulated exocytosis (31, 32). Thus, Ca^{2+}-stimulated exocytosis in barley aleurone protoplasts might be involved in the mechanism of resealing disrupted plasma membrane.

On the other hand, barley aleurone cells are potentially highly secretory cells. Zorec and Tester (7) therefore suggested that the observed Ca^{2+} stimulation of exocytosis in barley aleurone is likely to be physiologically relevant to the stimulation of secretion of hydrolases by the plant hormone GA_3, as cytosolic Ca^{2+} activity has been shown to increase in the presence of GA_3. However, the protoplasts investigated by Zorec and Tester (7) and the protoplasts used in the present study have not been treated with GA_3. Thus, it seems unlikely that the measured exocytotic events correspond to secretion of α-amylase, because no more than 10% of non-GA_3-treated protoplasts secrete α-amylase (33). Nevertheless, studies on animal cells suggest that resealing may be the evolutionary ancestor of other membrane additions, such as Ca^{2+}-triggered secretion, because resealing plasma membrane involves mechanisms that are similar to neurotransmitter release (31, 32). Therefore, the Ca^{2+}-stimulated exocytotic pathway in barley aleurone cells may not only be involved in GA_3-stimulated α-amylase secretion but, more generally, also play a role in membrane resealing in response to cell damage.

Involvement of GTP-Binding Proteins in Ca^{2+}-Stimulated Exocytosis. Several mono- and heteromeric GTP-binding proteins have been isolated from a range of plants (2, 10, 34), including barley aleurone (35). In animal cells, both mono- and heteromeric GTP-binding proteins are involved in exocytosis (36–38). Most of the monomeric GTP-binding proteins that have been isolated from plants are more similar to members of the YPT/rab family, which are involved in membrane trafficking, than to other monomeric GTP-binding proteins. This suggests that in plant cells, as in animal cells, GTP-binding proteins could be involved in exocytosis. To investigate the role of GTP-binding proteins in Ca^{2+}-stimulated exocytosis in barley aleurone, we dialyzed protoplasts with nonhydrolyzable guanine nucleotides, GTP[γS] and GDP[βS]. We found that 100 μM GTP[γS] slightly inhibits and 500 μM GDP[βS] completely inhibits Ca^{2+}-stimulated exocytosis. Our results therefore indicate that GTP-binding proteins are involved in Ca^{2+}-stimulated exocytosis in barley aleurone cells.

In most animal cells, the effects of GTP[γS] and Ca^{2+} on secretion were found be additive, with GDP[βS] having the opposite or no effect at all (4). Nevertheless, GTP and its analogues have been reported to inhibit exocytosis in some cells (4). The release of neurotransmitters is inhibited by both GTP[γS] and GDP[βS] (16), suggesting the involvement of monomeric GTP-binding proteins whose function requires a cycling between GTP- and GDP-bound forms. Arrest of the cycle in either form by GTP[γS] or GDP[βS] results in an inhibition of exocytosis. However, the inhibitory effect of GDP[βS] on Ca^{2+}-stimulated exocytosis in barley aleurone protoplasts was much stronger than the inhibition by GTP[γS]. This may indicate the involvement of at least two different GTP-binding proteins. One of these, when activated by GTP[γS] inhibits Ca^{2+}-stimulated exocytosis; and another inhibits Ca^{2+}-stimulated exocytosis when blocked by GDP[βS].

Conclusion. Our findings demonstrate the existence of two exocytotic pathways in barley aleurone cells. One is Ca^{2+}-independent and may be involved in the delivery of membrane material during cell expansion in developing protoplasts. The other is Ca^{2+}-stimulated and modulated by GTP-binding proteins; this may be involved in the resealing of disrupted plasma membrane and may also be involved in GA_3-stimulated α-amylase secretion.

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