J. Membrane Biol. 123, 261–268 (1991)
002226319100124E

The Journal of
Membrane Biology

Characterization of Constitutive Exocytosis in the Yeast *Saccharomyces cerevisiae*

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Summary. Constitutive exocytosis was investigated in the yeast *Saccharomyces cerevisiae* using temperature-sensitive mutant (*sec*) strains which do not allow vesicle fusion to the plasma membrane at the restrictive temperature. Secretory vesicles were accumulated in the cell at the restrictive temperature and then protein synthesis was blocked with cycloheximide. Upon returning the cells to the permissive temperature the contents of the accumulated vesicles were secreted. This allowed the study of constitutive exocytosis independent of the processes responsible for vesicular biosynthesis. Neither the kinetics nor magnitude of exocytosis were affected by removal of external Ca^{2+} or perturbations of cytosolic Ca^{2+}. This suggests that in those systems where calcium is required for exocytosis it is a regulatory molecule and not part of the mechanism of membrane fusion. Release occurred over a very broad range of pH and in media with different ionic compositions, suggesting that ionic and potential gradients across the plasma membrane play no role in exocytosis in yeast. High osmolarity inhibited the rate, but not the extent, of release. A novel inhibitory effect of azide was detected which occurred only at low pH. Vanadate also inhibited release in a pH-independent manner. Secretion occurred at the same rate in cells with or without accumulated vesicles. This infers a rate-limiting step following vesicle accumulation, perhaps a limiting number of release sites on the plasma membrane.

Key Words exocytosis · secretion · yeast · calcium

Introduction

Exocytosis, the fusion of secretory vesicles with a cell's plasma membrane, occurs in both a constitutive and a regulated manner (Kelly, 1985). Over the past 20 years it has become generally accepted that an essential step in regulated exocytosis is calcium entry into a cell (Katz, 1969; Llinás & Heuser, 1977) and, more significantly, a calcium increase immediately under the plasma membrane adjacent to the release sites (Simon & Llinás, 1985). It is not clear whether this reflects a ubiquitous use of calcium in the signal transduction apparati of specialized secretory cells or a fundamental role for calcium in the fusion process itself.

Many model systems have been developed to study regulated exocytosis (Knight & Baker, 1982; Mundy & Strittmatter, 1985; Zimmerberg, Sardet & Epel, 1985; Barrowman, Crockcroft & Gomperts, 1986; Vilmart-Seuwen et al., 1986). The fact that many vesicles accumulate before a stimulus triggers release means that one can study the effects of perturbing the system on the release of vesicle contents without considering indirect effects on earlier steps in vesicle formation. This is not the case in constitutive exocytosis, where vesicles fuse with the plasma membrane soon after they are formed in the golgi, and do not accumulate in large numbers. Thus analogous studies of constitutive exocytosis have not been performed.

This problem can be circumvented in yeast by using the temperature sensitive "*sec*" mutants isolated by Novick and Sheckman (Novick & Scheckman, 1979; Novick, Field & Scheckman, 1980). These mutant yeast strains secrete constitutively at 25°C but stop secreting when shifted to 37°C. Some of these mutants accumulate secretory proteins in post-golgi secretory vesicles when shifted to 37°C. A few of these strains have reversible phenotypes: when the temperature is shifted back down to 25°C, many of the accumulated vesicles are released (Novick et al., 1980). By looking at the secretion upon shift-down in these strains it is possible to study exocytosis in the constitutive pathway separately from processes leading to vesicle production.

In this paper evidence is presented that constitutive exocytosis in yeast is insensitive to changes in cytosolic calcium levels and is unaffected by changes in the voltage and ionic gradients across the plasma membrane. Release was slowed in media of elevated osmolarity and inhibited by vanadate and azide.

Over the past decade a number of systems have been established for studying the in vitro transport of vesicles through the secretory pathway. The util-

ity of an in vitro system is constrained by its ability to mimic the *in situ* physiology. A study of constitutive exocytosis in intact cells is required for establishing the benchmarks by which in vitro systems must be evaluated.

Materials and Methods

The yeast strains HMSF-1 (*sec*1), HMSF-136 (*sec*6), and HMSF-143 (*sec*9) were obtained from the Yeast Genetic Stock Center, University of California, Berkeley, CA 94720. Chemicals were from Sigma, and Yeast Extract and Peptone were from Difco.

For all experiments except those in Fig. 1, cells were grown in YPD (1% Yeast Extract, 2% Peptone, 2% glucose) to log phase (about 2×10^7 cells/ml), then centrifuged for 5 min at 3000 $\times$ *g* and resuspended at 2×10^7 cells/ml in $YP^{0.1}D$ (1% Yeast Extract, 2% Peptone, 0.1% glucose) to induce invertase synthesis. They were shifted to 37°C immediately upon resuspending and incubated with aeration for 70 min to accumulate invertase in secretory vesicles. The cells were pelleted, resuspended in the various test media at a density of 2×10^8 cells/ml, and incubated at 25°C for the indicated times. 200 μl samples were taken into 1 ml of ice-cold 10 mM NaN_3 to halt secretion, and the external invertase activity was determined as described (Goldstein & Lampen, 1975). Normalized shift-down release was calculated by subtracting the basal signal present before shift-down (usually 5–10% of maximal signal) and dividing by the maximal signal. YPD and $YP^{0.1}D$ lacking phosphate were prepared as described (Bisson & Thorner, 1982).

Protein synthesis was measured by the incorporation of ^{35}S-methionine into TCA precipitable material.

Uptake of ^{45}Ca was measured essentially as described (Borbolla & Pena, 1980): *sec*1 cells were grown in YPD at 25°C to 3×10^7 cells/ml, centrifuged and resuspended in 40 mM Na · PIPES at pH 6.0, 100 mM glucose, 90 μM $CaCl_2$ at 3×10^8 cells/ml. A23187 was added from a 2 mM stock in ethanol to a final concentration of 20 μM (an equivalent amount of ethanol was added to control samples). After a 1-min incubation at 25°C to restart cellular metabolism, 1/100 volume of 1 mM, 2 mCi/ml $^{45}CaCl_2$ was added. Triplicate 100 μl samples were removed at the indicated times, deposited on 0.45 μm nitrocellulose filters (Millipore) and washed twice with 10 ml of ice-cold 10 mM $CaCl_2$. Subsequent washes removed <5% of the cell-associated ^{45}Ca, showing that this had entered the cell and was not merely bound to the cell wall. The filters were dried, immersed in scintillation fluid, and counted.

Sodium orthovanadate (Na_3VO_4) was added just before each experiment from a 100 mM stock made fresh from solid.

Results

For all the studies reported in this paper, the mutant strains *sec*1, *sec*6, and *sec*9 were used. All accumulate secretory vesicles at 37°C and release a fraction of them (30–50%) when shifted back to 25°C in the presence of protein synthesis inhibitors (Novick et al., 1980). Release was measured by assaying the appearance of invertase at the cell surface. The secretory protein invertase is synthesized in yeast in a glucose-regulated manner: transcription of the invertase gene is repressed by high glucose levels, and derepressed when cells are grown in media containing low glucose (Dodyk & Rothstein, 1964; Carlson et al., 1983). Upon derepression, invertase synthesis begins within 10 min, the enzyme traverses the secretory pathway with a half-time of approximately 5 min, and is released into the space between the plasma membrane and the cell wall, where it remains trapped and its activity can be easily measured (Novick, Ferro & Scheckman, 1981).

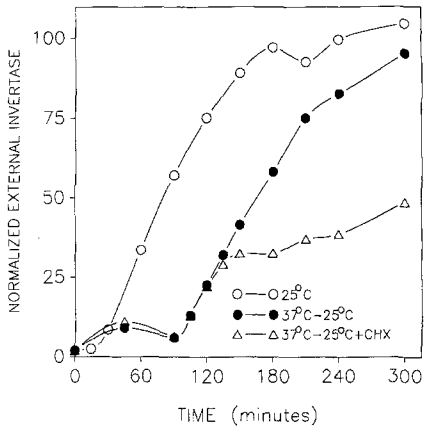

Fig. 1. Kinetics of shift-down invertase release. *sec*9 cells were grown in YPD to 2×10^7 cells/ml, harvested, and resuspended in $YP^{0.1}D$ to induce invertase synthesis. Cells were incubated continuously at 25°C (open circles) or at 37°C for 90 min followed by a further 210 minutes at 25°C in the absence (filled circles) or in the presence (open triangles) of 0.1 mg/ml cycloheximide. Samples were taken at the indicated times and assayed for external invertase activity. Parallel samples were taken and assayed for cell number by measuring the $O.D._{260}$ of the culture. Values for external invertase were adjusted for cell number by dividing by the relevant $O.D._{260}$, and the normalized external invertase/cell is plotted in arbitrary units.

Cells were grown in YPD medium (containing 2% glucose) at 25°C to a density of 2×10^7/ml, then transferred to $YP^{0.1}D$ medium (containing 0.1% glucose) and incubated at 37°C for 70 min. This led to an accumulation of invertase in secretory vesicles within the cell, and very low basal levels of invertase outside the plasma membrane (Fig. 1, 37°C, 90 min). We measured invertase release upon shift-down to 25°C in the presence of >0.1 mg/ml cycloheximide. This concentration of cycloheximide was sufficient to block 98% of cellular protein synthesis (*data not shown*). As expected, no release was observed if the 37°C preincubation was omitted (*data not shown*), confirming that our signal came solely from the fusion of accumulated vesicles.

The rate of invertase release after shift-down (Fig. 1) was very similar to that observed in cells

induced to secrete invertase at 25°C; no initial burst of release was seen despite the large number of vesicles backed up at 37°C.

All the treatments (below) which affected release had similar effects in all three mutant strains, indicating that the effects seen are due to fundamental aspects of yeast exocytosis rather than the result of a particular mutant lesion.

Effect of Calcium Perturbations on Exocytosis

To determine whether extracellular calcium was required for exocytosis in yeast, invertase release was measured in 50 mM Tris-HCl (pH 7.4), 100 mM glucose, and 0.2 mg/ml cycloheximide supplemented with either 1 mM $CaCl_2$ or 50 mM EGTA (estimated $Ca^{2+} < 10^{-11}$ M). As shown in Fig. 2*A*, neither the rate nor the extent of invertase release was affected by removal of external calcium (open circles versus open triangles). Thus, calcium entry from outside is not involved in exocytosis in yeast. Similar experiments were performed in YPD, with identical results.

To study the role of cytosolic calcium in exocytosis we made use of the ionophore A23187. Control experiments (Fig. 2*B*) demonstrated that addition of 20 μM A23187 accelerated ^{45}Ca influx into yeast cells, showing that the ionophore penetrated the cell wall and partitioned into the plasma membrane. A23187 (at 20 μM) also caused an 80% inhibition of protein synthesis (*data not shown*), an effect observed previously in mammalian cells (Bottenstein & de Vellis, 1976; Tartakoff & Vassali, 1977) providing further evidence that the ionophore gained access to the cell. However, the ionophore in combination with either 1 mM $CaCl_2$ (Fig. 2*A*, filled circles) or 50 mM EGTA (filled triangles) had no effect on the rate or extent of invertase release for 2 hr after shift-down (Fig. 2*A*). Thus, exocytosis is insensitive to perturbations of cytosolic calcium levels.

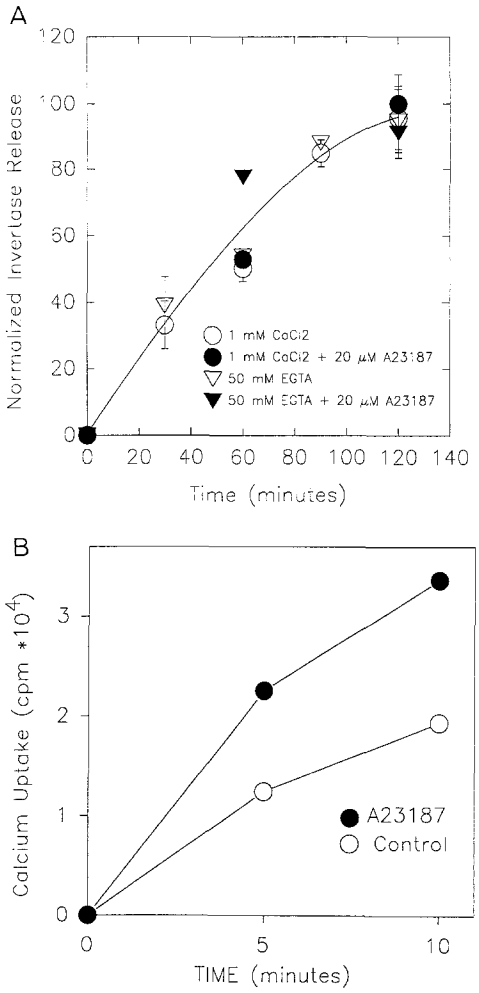

Fig. 2. Effect of Ca^{2+} levels on exocytosis. (*A*) *sec*1 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 50 mM Tris · HCl at pH 7.5, 100 mM glucose, 0.2 mg/ml cycloheximide, and either 1 mM $CaCl_2$ (circles) or 50 mM EGTA (triangles). A23187 was added to 20 μM to half the cultures (filled symbols). Cells were incubated at 25°C with aeration, and invertase release was determined at the indicated times. Means + SE from triplicate samples are shown. (*B*) *sec*1 cells were grown to 3×10^7 cells/ml, harvested, and resuspended in 50 mM NaPIPES at pH 6.0, 100 mM glucose, 0.1 mM $^{45}CaCl_2$, with (filled circles) or without (open circles) 20 μM A23187. ^{45}Ca uptake was measured as described in Materials and Methods. Means + SD from triplicate samples are shown (standard deviations were less than 3% of the mean values, so the error bars are not visible).

Effect of Ionic Composition on Exocytosis

Yeast maintains a membrane potential of 50–100 mV (cytosol negative, depending on medium pH (De La Pena et al., 1982; Borst-Pawels, 1981)) generated mainly by a H^+ ATPase in the plasma membrane (Serrano, 1984). Cytosolic levels of Na^+ and K^+ (measured by NMR (Ogino, den Hollander & Shulman, 1983)) are similar to those in mammalian cells (130–170 mM K^+, 2.5 mM Na^+). To investigate the role of ionic and voltage gradients across the plasma membrane, invertase release was measured in various media (Fig. 3 and the Table).

Release was remarkably insensitive to external pH (Fig. 3) over a very broad range (pH 2–10, assayed using a variety of buffers). Incubation in a medium mimicking the ionic composition of the cytosol (Table, line 2) in order to reduce the membrane potential also had no effect. Addition of diva-

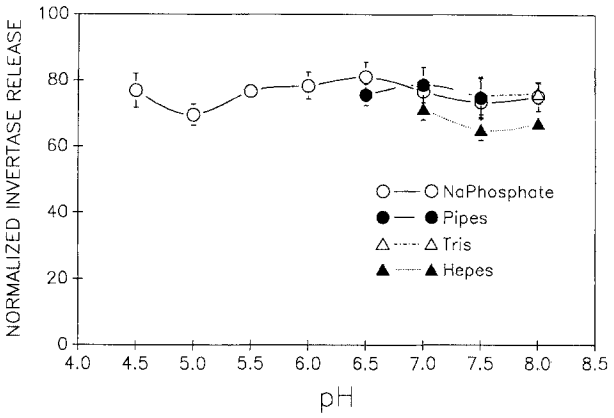

Fig. 3. Effect of external pH on exocytosis. *sec*1 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 100 mM Tris (open triangles), NaHEPES (filled triangles), Na phosphate (open circles), or 50 mM NaPIPES (filled circles), 10 mM glucose, 1 mg/ml cycloheximide, buffered to the indicated pH. The cells were incubated for 1 hr at 25°C and assayed for external invertase. Means ± SE from sextuplicate samples are shown.

Table. Effect of ionic composition on exocytosis

Na^+ (mM)	K^+ (mM)	Cl^- (mM)	Glutamate (mM)	Mg^{2+} (mM)	Ca^{2+} (mM)	Release
5	120	130	—	2.5	[a]	100 ± 5
5	120	10	120	2.5	[a]	102 ± 5
120	5	130	—	2.5	[a]	109 ± 4
20	120	120	—	—[b]	[b]	109 ± 6
—	—	200	—	100	[a]	98 ± 14
—	—	200	—	—	100	75 ± 4

[a] No calcium added.
[b] 10 mM EDTA added.
*sec*6 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 20 mM K · PO_4 (lines 1–4) or Tris · HCl (lines 5,6) at pH 7.5, 10 mM glucose, 0.2 mg/ml cycloheximide, and the indicated ions. Cells were incubated for 1 hr at 25°C and assayed for external invertase. Means ± SE from quintuplicate samples are shown.

lent cations to 100 mM did not markedly affect release (lines 5 and 6), neither did complete removal of divalents (line 4). Exocytosis occurred at the same rate in media containing different monovalent anions or cations (lines 1–4). Thus we found no evidence for a role of ionic gradients across the plasma membrane in exocytosis.

Effect of Osmolarity on Exocytosis

The experiments shown in Fig. 4*A* demonstrate that raising the osmolarity of the medium above 1 Osm inhibited exocytosis in yeast. This was not dependent on the osmoticant used; sorbitol, KCl, and PEG400 had similar effects (*data not shown*). Figure 4*B* shows that high osmolarity affected the rate, but not the extent, of exocytosis (*compare* 1.6 or 2 M sorbitol with 0.8 M sorbitol).

Yeast, because of their cell wall, are able to withstand media with osmolarities below isotonicity. Lowering the external osmolarity to under 100 mOsm usually caused a small reduction in both the rate and the extent of release (Fig. 4*B*, *compare* 0 with 0.8 M sorbitol).

Inhibitors of Exocytosis in Yeast

Studies in several systems have identified an ATP requirement for exocytosis (Knight & Baker, 1982; Vilmart-Seuwen et al., 1986). Novick et al. (1981) have shown that removal of glucose and addition of azide to the medium completely inhibited shift-down invertase release in the reversible *sec* mutants, consistent with an energy requirement for exocytosis in yeast. In agreement with these results, we found that release decreased as glucose was lowered, and was completely inhibited in medium with no glucose and added azide (*data not shown*). Surprisingly, however, addition of azide to cells in glucose-containing media also inhibited release, in a pH-dependent manner (Fig. 5). Under these conditions, the cells derive most of their energy from glycolysis (Fraenkel, 1982), and the inhibition of respiration by azide would not be expected to significantly affect intracellular ATP pools. Indeed, the proton ionophore CCCP which uncouples respiration from ATP synthesis (Reid & Schatz, 1982) had no inhibitory effect at any pH and did not significantly enhance the pH-dependent inhibition by azide (Fig. 5).

Vanadate, an inhibitor of the yeast H^+ ATPase (Borst-Pauwels & Peters, 1981), produced a dose-dependent inhibition of invertase release (Fig. 6*A*). This inhibition was more extreme in cells grown in phosphate-depleted medium (Fig. 6*B*) to induce the phosphate transport system in the plasma membrane (Borst-Pawels, 1981), suggesting that the vanadate entered the cell via this transporter, as was observed in *Neurospora* (Bowman, 1983). Inhibition by vanadate was insensitive to pH (Fig. 6*C*, filled circles), even in the presence of CCCP (filled triangles), suggesting that it was not due to acidification of the cytosol following inhibition of the H^+ pump.

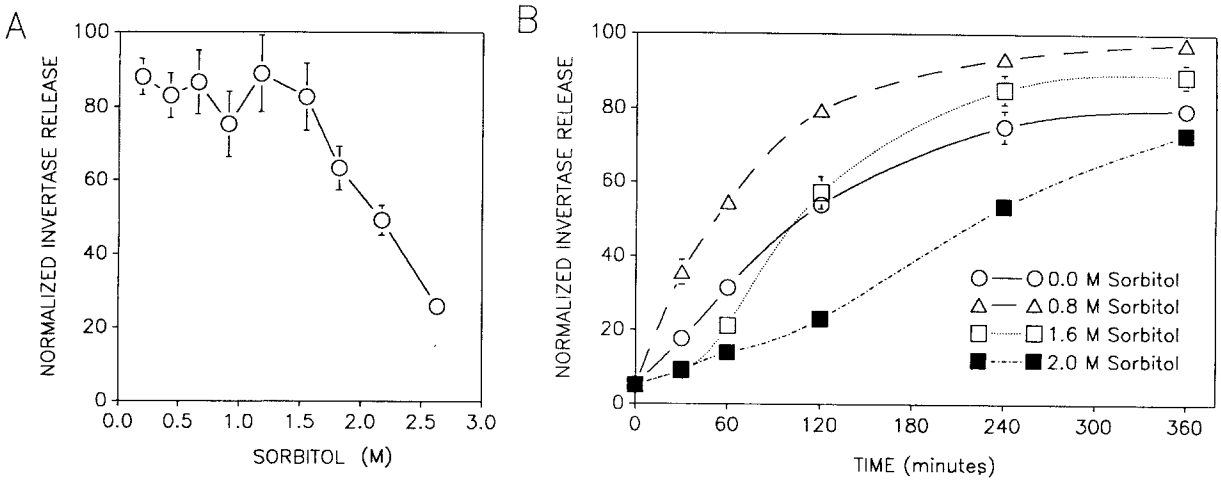

Fig. 4. Effect of osmolarity on exocytosis. (*A*) *sec*1 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 10 mM glucose, 0.2 mg/ml cycloheximide, and the indicated concentrations of sorbitol. The cells were incubated for 1 hr at 25°C and assayed for external invertase. Means ± SE from sextuplicate samples are shown. (*B*) *sec*1 cells were treated as in *A* except that the incubations were carred out for different periods of time as indicated. Means ± SE from sextuplicate samples are shown.

Discussion

By using reversible temperature-sensitive mutants of yeast, several aspects of constitutive exocytosis were investigated. After accumulating vesicles at the nonpermissive temperature there was no initial burst of invertase release upon shifting back to the permissive temperature. The rate of release was constant and paralleled release by unshifted cells. This suggests that there are a limited number of release sites in the plasma membrane and that these are fully occupied by vesicles during normal growth. An alternative interpretation would be that the mutant products reacquired function gradually upon shift-down, but this seems unlikely as release commenced rapidly and with similar kinetics in all three mutant strains.

In yeast cells, the constitutive fusion of secretory vesicles is localized to the bud portion of the plasma membrane (Tkacz & Lampen, 1973; Field & Scheckman, 1980). An attractive hypothesis to explain this would be that calcium channels are restricted to this region, and the resulting local elevation in calcium levels is responsible for release. However, removal of extracellular calcium (thus preventing calcium entry) had no effect on exocytosis.

A definitive study on the relationship between calcium and exocytosis in yeast would require the development of techniques to measure the cytosolic free calcium concentration in this system. Disruption of intracellular calcium has been proposed to disrupt vesicular traffic in yeast (Schmitt, Puzicha & Gallwitz, 1988). This was based on the phenotype of a *ts* mutant (*ypt1*) which slows secretion at the restrictive temperature but can be rescued by very high (100 mM) concentrations of calcium in the

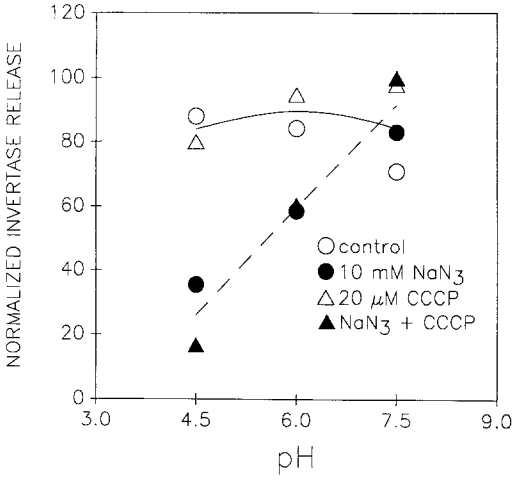

Fig. 5. Effect of azide on exocytosis. *sec*6 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 50 mM K · PO_4 at pH 4.5, pH 6.0, or pH 7.5, 100 mM glucose, 0.2 mg/ml cycloheximide (open circles), supplemented with 10 mM NaN_3 (filled symbols) or 20 μM CCCP (triangles). The cells were incubated for 1 hr at 25°C and assayed for external invertase. Means from duplicate samples are shown.

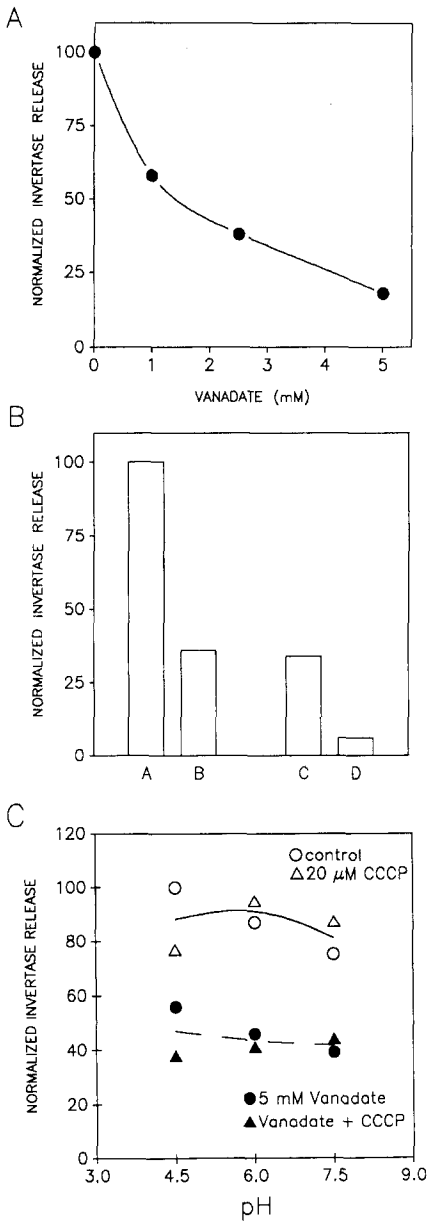

Fig. 6. Effect of vanadate on exocytosis. (*A*) *sec*6 cells were grown in YPD without phosphate and induced to accumulate invertase in vesicles at 37°C in $YP^{0.1}D$ without phosphate. Cells were harvested, resuspended in 50 mM PIPES.K at pH 7.2, 10 mM glucose, 0.2 mg/ml cycloheximide, with various levels of sodium orthovanadate, incubated for 90 min at 25°C, and assayed for external invertase. Means from triplicate samples are shown. (*B*) *sec*6 cells were grown and induced to accumulate invertase in media containing (*A*, *B*) or lacking (*C*, *D*) phosphate. Cells were resuspended in 50 mM K · PO_4 at pH 4.5, 100 mM glucose, and 0.2 mg/ml cycloheximide, with (*B*, *D*) or without (*A*, *C*) 5 mM vanadate, incubated for 60 min at 25°C, and assayed for external invertase. Means from duplicate samples are shown. (*C*) *sec*1 cells were induced to accumulate invertase in vesicles as described in Materials and Methods, harvested, and resuspended in 50 mM K · PO_4 at pH 4.5, pH 6.0, or pH 7.5, 100 mM glucose, 0.2 mg/ml cycloheximide (open circles), supplemented with 5 mM vanadate (filled symbols) or 20 μM CCCP (triangles). The cells were incubated for 1 hr at 25°C and assayed for external invertase. Means from triplicate samples are shown.

growth medium (Schmitt et al., 1988). However, our results with the ionophore A23187 suggest that constitutive exocytosis is insensitive to perturbations in cytosolic calcium levels. This suggests that the secretory defect observed in the *ypt1* mutant may not be a direct result of disregulated calcium or, alternatively, that an earlier step in the secretory pathway is affected by the disruption of calcium levels.

The rate of vesicle release was identical in different ionic media, indicating that ionic or potential gradients do not play a role in exocytosis. This is consistent with the finding in many systems that permeabilizing the plasma membrane does not destroy competence for exocytosis (Knight & Baker, 1982). Release was also unaffected by variations of external pH between pH 2 and 10, or by the protonophore CCCP in media of pH 4.5 to 7.5. While CCCP, which uncouples mitochondria and therefore eliminates the mitochondrial contribution to intracellular ATP pools, did not affect release, azide did inhibit release. Furthermore, inhibition by azide was pH dependent, occurring only at low pH. The basis for this remains unclear.

Fusion of phospholipid vesicles with bilayers is driven by the osmotic gradient across the vesicle membrane (Finkelstein et al., 1986). Exocytosis in several cell types is inhibited by placing the cells in hyperosmotic media (Finkelstein, Zimmerberg & Cohen, 1986; Holz, 1986). A similar inhibition has been observed in permeabilized cells (Knight & Baker, 1982) and isolated sea urchin oocyte cortices (Zimmerberg et al., 1985), suggesting that the osmotic gradient across the vesicle membrane, rather than the plasma membrane, is important for vesicle fusion. The rate (but not the extent) of constitutive exocytosis in yeast was decreased in media of high osmolarity, showing that this effect is not restricted to regulated systems, or to cells with dense-core secretory vesicles. It remains unclear whether this inhibition reflects a role for the vesicular osmotic gradient in fusion or an indirect effect of dehydration (e.g. alterations in vesicle size, cytosol viscosity, or concentrations of cytosolic proteins).

Vanadate had an inhibitory effect on exocytosis. The observation that this effect was more extreme in cells with induced phosphate transport systems suggests that the vanadate has to enter the cell in order to inhibit release. The intracellular site of inhibition does not, however, seem to be the proton pump as the inhibition was insensitive to pH changes even in the presence of CCCP. Recently, a family of P-type (vanadate sensitive) ATPases homologous to the proton pump have been identified in yeast (Rudolph et al., 1989). Mutations in one of these, PMR1 (which has been proposed to be a calcium pump based on sequence comparisons), result

in aberrant secretion (Rudolph et al., 1989), suggesting that some of these ATPases may play a role in maintaining competence for secretion. It is attractive to speculate that the vanadate sensitivity observed here reflects a role for one of these pumps in exocytosis.

These results constitute an initial characterization of constitutive exocytosis in yeast. A comparison of results obtained studying constitutive exocytosis with those obtained from the investigation of various regulated systems should allow one to distinguish between requirements for the transduction of the stimulus signal and general requirements for vesicle fusion.

In many regulated secretory systems, a transient rise in the cytosolic calcium level (Kao & Schneider, 1986; Sage & Rink, 1987) or fall (Shoback et al., 1983; Nemeth, Wallace & Scarpa, 1986) precedes or accompanies exocytosis. Removal of extracellular calcium in some systems prevents exocytosis (Katz & Miledi, 1967; Llinás & Nicholson, 1975), and studies in several permeabilized cell systems have shown that raising the calcium level is sufficient to trigger exocytosis (Knight & Baker, 1982). In neurons the release of transmitter is quantitatively dependent on the time course and magnitude of presynaptic calcium entry (Llinás & Heuser, 1977; Llinás et al., 1981, 1982). All these observations argue for an intimate relationship between calcium and vesicle fusion. This has led to the belief that calcium is required, either to crosslink phospholipids, or help dehydrate phospholipids, as part of the membrane fusion mechanism (Rand & Parsegian, 1986; Papahadjopoulos et al., 1990). Recently, it has been observed that, in certain instances, release can be triggered without macroscopic elevations of calcium, both in vivo (Rink, Sanchez & Hallam, 1983) and in vitro (Fernandez, Neher & Gomperts, 1984; Vallar, Biden & Wollheim, 1987). In yeast, a disruption of cellular calcium severe enough to kill protein synthesis has no effect on the kinetics or magnitude of exocytosis. In these systems calcium cannot be functioning as a fundamental component of the fusion mechanism. This suggests that, in those systems where it is required, calcium is a regulatory molecule, a provocateur and not the causatory agent. The latency between calcium entry and transmitter release is under 180 μsec. This delay is so brief that in regulated systems vesicles must be docked to the release sites and primed for exocytosis. Only binding of calcium in the presynaptic active zones immediately adjacent to the vesicles (Simon & Llinás, 1985) is required to trigger the fusion apparatus.

The requirements for constitutive exocytosis described in this paper form the benchmarks by which an in vitro reconstitution of exocytosis must be judged. Most in vitro systems for vesicular fusion require a calcium concentration higher than the resting cellular level. Unfortunately, calcium can be used to fuse many membrane systems—even pure liposomes—in a nonphysiological manner. The observation that the kinetics of exocytosis in yeast are unperturbed by changes of cellular calcium indicates that an in vitro reconstitution of constitutive exocytosis must be equally insensitive to calcium ion levels.

The strength of yeast as a model system lies mainly in the ability to genetically manipulate this organism, which allows the detection and isolation of mutations that disrupt particular functions, such as those used in this report. Hopefully, some of these mutants will identify components of the biochemical machinery which underlies exocytosis. Recently, Salminen and Novick (1987) have described a *ras*-like GTP-binding protein encoded by the sec4 locus, which promises to be such a component. Development of a cell-free system which reconstitutes yeast exocytosis would help to identify mutations of interest and permit a biochemical analysis of the role played by their gene products.

We would like to thank Dr. Peter R. MacLeish for his constant interest, encouragement, and support; without his enthusiasm this work would not have been possible. D.J.L. was supported by the Lucille P. Markey Charitable Trust. S.M.S. is grateful for the support of a Irma T. Hirschl-Monique Weill-Caulier Career Scientist Award.

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Received 8 February 1991