Ion Regulation of Homotypic Vacuole Fusion in
*Saccharomyces cerevisiae*

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Biological membrane fusion employs divalent cations as protein cofactors or as signaling ligands. For example, Mg\(^{2+}\) is a cofactor for the N-ethylmaleimide-sensitive factor (NSF) ATPase, and the Ca\(^{2+}\) signal from neuronal membrane depolarization is required for synaptotagmin activation. Divalent cations also regulate liposome fusion, but the role of such ion interactions with lipid bilayers in Rab- and soluble NSF attachment protein receptor (SNARE)-dependent biological membrane fusion is less clear. Yeast vacuole fusion requires Mg\(^{2+}\) for Sec18p ATPase activity, and vacuole docking triggers an efflux of luminal Ca\(^{2+}\). We now report distinct reaction conditions where divalent or monovalent ions interchangeably regulate Rab- and SNARE-dependent vacuole fusion. In reactions with 5 mM Mg\(^{2+}\), other free divalent ions are not needed. Reactions containing low Mg\(^{2+}\) concentrations are strongly inhibited by the rapid Ca\(^{2+}\) chelator BAPTA. However, addition of the soluble SNARE Vam7p relieves BAPTA inhibition as effectively as Ca\(^{2+}\) or Mg\(^{2+}\), suggesting that Ca\(^{2+}\) does not perform a unique signaling function. When the need for Mg\(^{2+}\), ATP, and Sec18p for fusion is bypassed through the addition of Vam7p, vacuole fusion does not require any appreciable free divalent cations and can even be stimulated by their chelators. The similarity of these findings to those with liposomes, and the higher ion specificity of the regulation of proteins, suggests a working model in which ion interactions with bilayer lipids permit Rab- and SNARE-dependent membrane fusion.

Eukaryotic subcellular compartmentation requires selective membrane fusion. This fusion depends on specific lipids and conserved proteins, including SNAREs\(^1\) and their chaperones, Rab family GTPases, Rab effectors, and divalent cations (1). For example, membrane fusion at the synapse is triggered by an afferent wave of electrical depolarization, which opens a voltage-gated Ca\(^{2+}\) channel. The Ca\(^{2+}\) that enters binds to intracellular receptors, most notably the C2 domains of synaptotagmin, triggering a conformational change, which alters synaptotagmin interactions with SNAREs and with the apolar domain of the membrane bilayer (2). Although Ca\(^{2+}\) clearly regulates synaptic membrane fusion, in other membrane fusion events the Ca\(^{2+}\) channel, the trigger for Ca\(^{2+}\) flux, the Ca\(^{2+}\) receptor, and the modes of action of the receptors have received less study.

The vacuole (lysosome) of *Saccharomyces cerevisiae* is the major repository of cellular Ca\(^{2+}\) (3). Calcium is pumped into the vacuole by Pmc1p, an ATP-driven Ca\(^{2+}\) transporter (4), and by Vcx1p, a Ca\(^{2+}/H^+\) exchanger (5). Vacuole homotypic fusion occurs in three stages: ATP-dependent priming, docking, and finally bilayer fusion and content mixing (6). Docking is complex and requires Ypt7p (a Rab family GTPase), the HOPS (homotypic fusion and vacuole protein sorting)/Vps Class C complex (a Ypt7p effector that also binds to SNAREs), and the SNARE proteins Vam7p, Vam3p, Vti1p, and Nvr1p. The last stage of docking, the pairing of SNAREs in *trans*, triggers a dramatic release of luminal Ca\(^{2+}\) from the vacuole (7, 8). Experiments demonstrating the sensitivity of vacuole fusion to the Ca\(^{2+}\) chelator BAPTA, the relief of BAPTA sensitivity by added Ca\(^{2+}\), and the docking-dependent release of Ca\(^{2+}\) from the vacuole lumen (8, 9) have suggested that Ca\(^{2+}\) may signal successful docking and initiate the terminal stage of membrane fusion. Vacuole fusion requires the calcium-binding protein calmodulin (7), strengthening the connection between free Ca\(^{2+}\) and the downstream processes leading to vacuole fusion.

Several observations now lead us to re-examine the role of free Ca\(^{2+}\), and other ions, in vacuole fusion. Although the Ca\(^{2+}\) chelator BAPTA inhibits a late stage of vacuole fusion (10), BAPTA also inhibits the membrane association of two crucial peripheral membrane proteins, Vam7p and the HOPS/Vps class C complex, which do not have Ca\(^{2+}\) binding motifs. BAPTA, a tetravalent ion, may release these proteins from the membrane, and inhibit vacuole fusion, through its contribution to ionic strength. Vacuole fusion is commonly measured by an assay coupled to the activity of the zinc-metalloenzyme Pho8p, which is sensitive to divalent ion chelators (11). We now report that fusion assays with 5 mM Mg\(^{2+}\) show little sensitivity to BAPTA when they are adjusted to a constant ionic strength and are performed in the presence of the heavy metal ion chelator TPEN (to obviate the effect of Zn\(^{2+}\) chelation by BAPTA). At low Mg\(^{2+}\), the inhibition by BAPTA is not simply due to its ionic strength contribution, yet BAPTA inhibition can be bypassed by altering KCl, Mg\(^{2+}\), and Vam7p concentrations. Finally, under conditions that bypass the need for ATP or Sec18p, Ypt7p- and SNARE-dependent vacuole fusion requires moderate (125–250 mM) KCl but does not require appreciable free divalent cations; at certain KCl concentrations, fusion can even be stimulated by divalent ion chelators. We suggest a

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\(^{1}\) The abbreviations used are: SNARE, soluble NSF attachment protein receptor; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’-N’-tetraacetic acid; TPEN, N,N,N’,N’-tetrakis-2-pyridylmethyl)ethylenediamine; HOPS, homotypic fusion and vacuole protein sorting complex; PS, phosphatidyserine; PC, phosphatidylcholine; NSF, N-ethylmaleimide-sensitive factor.
working model in which Mg\(^{2+}\), Ca\(^{2+}\), and monovalent ions interact with the lipid bilayer to regulate vacuole fusion.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Modifications**—Vacuoles were purified from *S. cerevisiae* strains BJ3505 (MATα ura3–52 trpl1–3101 his3–200 lys2–801 gal2–1oc ade2–1oc can1–100 his3–11,15 leu2–3,112 trpl1–1 ura3–1), JGY149 (MATα ade2–1oc can1–100 cdc1–6 his3–11,15 leu2–3,112 trpl1–1 ura3–1), and JGY041 (MATα ade2–1oc can1–100 cdc1–3 his3–11,15 leu2–3,112 trpl1–1 ura3–1) (14) (generous gifts from Dr. Trisha Davis, University of Washington, Seattle, WA) bearing alleles of calmodulin that do not appreciably bind Ca\(^{2+}\). These strains were used to generate pep4Δ and pho8Δ derivatives for studies of vacuole fusion. Brieﬂy, the pep4::HIS3 allele and the pho8::TRP1 allele were PCR-ampliﬁed with ﬂanking chromosomal sequence from BJ3505 and DKY6281, respectively. These fragments were transformed into CRY1, JGY041, and JGY149 using the standard LiAc/ss-DNA/PEG transformation method (16), generating VSY3 (ΔCRY1 pep4::HIS3), VSY4 (ΔCRY1 pho8::TRP1), VSY5 (ΔJGY041 pep4::HIS3), VSY6 (ΔJGY041 pho8::TRP1), VSY7 (ΔJGY149 pep4::HIS3), and VSY8 (ΔJGY149 pho8::TRP1). Strain constructs were conﬁrmed by PCR analysis, and maintenance of the correct cDNA1 allele in each derivative was conﬁrmed by DNA sequencing.

**Viral Inoculation and in Vitro Fusion Assay Conditions**—Vacuoles were isolated as described previously (13). Vacuoles were purified from ade2–1oc strains VSY3, VSY4, VSY5, VSY6, VSY7, and VSY8 after growth in YPD with 0.002% (w/v) adenine hemisulfate (from a 2% stock dissolved in Me2SO).

In vitro fusion reactions contained 3 μg of pep4Δ vacuoles (from BJ3505) and 3 μg of pho8Δ vacuoles (from DKY6281). The following three fusion reaction conditions were used in this work: (a) high Mg\(^{2+}\) fusion reactions contained 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 1 mM MgCl\(_2\), 1 mM ATP (Amersham Biosciences), 1 mg/ml creatine kinase (Roche Applied Science), 29 mM creatine phosphate (Roche Applied Science), 10 μM coenzyme A (Sigma), and 330 mM puriﬁed Pho8p (IB2) (26); (b) low Mg\(^{2+}\) fusion reactions contained 10 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 0.5 mM MnCl\(_2\), 0.5 mM MgCl\(_2\), 0.5 mM ATP, 0.5 mg/ml creatine kinase, 14.5 mM creatine phosphate, 10 μM coenzyme A, and 330 mM puriﬁed IB2; and (c) bypass fusion, as previously described (21), can occur without ATP when recombinant Vam7p is supplied. Bypass reactions contained 10 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 10 μM coenzyme A, 2.8 μM recombinant Vam7p, and 330 mM puriﬁed IB2.

All reaction components except the vacuoles were mixed on ice. Vacuoles puriﬁed from pep4Δ and pho8Δ strains were premixed in equal amounts on ice and were added last to each reaction (6 μg of total per reaction). Reactions were incubated at 27 °C for 90 min unless otherwise noted.

**RESULTS**

We assay the fusion of yeast vacuoles that are puriﬁed from two strains, one that accumulates catalytically inactive pro-alkaline phosphatase due to the absence of vacuolar luminal proteases, and the other that has the normal proteases but is deleted for the PHO8 phosphatase gene (13). Upon vacuole fusion, the proteases gain access to the proPho8p and cleave it to the catalytically active form, Pho8p. This active form can be assayed colorimetrically and is a quantitative measurement of the extent of vacuole fusion. Pho8p has tightly bound Zn\(^{2+}\) and Cu\(^{2+}\), although only the Zn\(^{2+}\) is needed for phosphatase activity (11). The roles of divalent cations in fusion can be explored with chelators such as EDTA, BAPTA, and TPEN, each with their characteristic afﬁnities for divalent cations (Table I). BAPTA inhibits in vitro vacuole fusion (Fig. 1; compare squares to other symbols on the ordinate). Although added Ca\(^{2+}\) did not stimulate fusion, its addition relieved BAPTA inhibition (Fig. 1), suggesting that the BAPTA-mediated inhibition of vacuole fusion may reﬂect its calcium chelation properties. When BAPTA and calcium were equimolar, however, a modest amount of fusion inhibition remained. To explore the basis of this Ca\(^{2+}\)-independent inhibition, we sought BAPTA-sensitive aspects of vacuole fusion.

**BAPTA Releases Peripherally Bound Proteins from the Vacuolar Membrane**—BAPTA can affect biological systems in ways other than calcium chelation. For example, BAPTA has been shown to depolymerize microtubules (28) and to bind to proteins and phospholipids (29, 30). To study additional effects of BAPTA on the vacuole, vacuoles were incubated with 2.5 mM BAPTA and separated into pellet (P) and supernatant (S) fractions by centrifugation. As seen in Fig. 2, BAPTA promoted the release of Vam7p, a peripheral membrane SNARE, and HOPS (homotypic fusion and vacuole protein sorting/Vps-Class C), a multisubunit complex that includes Vps18p, Vps33p, and Vps39p, from the vacuole membrane. The retention of these proteins on the vacuole membrane is not thought to directly require Ca\(^{2+}\), because these proteins lack calcium-binding motifs, although Vps11p and Vps18p do contain Zn\(^{2+}\)-binding motifs. These data suggest that BAPTA may have multiple effects on in vitro fusion, including mechanisms that are distinct from Ca\(^{2+}\) chelation.

**BAPTA Contribution to Ionic Strength**—Because elevated ionic strength is often sufficient to release peripherally bound membrane proteins, BAPTA might inhibit fusion through its contribution to ionic strength. Ionic strength is proportional to the square of the net charge borne by each ionized species in solution, according to the formula, $I = \frac{1}{2} \sum_{i} M C_{i}^{2}$, where $r$ is the ionic strength, $M$ is the molarity of each ion, and $C_{i}$ is its net charge. Thus, $K_{i}$ BAPTA would contribute 10-times as much to the ionic strength as equimolar KCl. BAPTA might also chelate other metal ions such as Zn\(^{2+}\), which might contribute to fusion or to the catalytic activity of matured Pho8p, and BAPTA might alter the pH of an insufficiently buffered reaction. To test whether part of the BAPTA inhibition derives from its contribution to ionic strength, we made compensatory adjustments to the KCl concentration of the reactions. To control for insufficient buffering capacity, reactions were performed in 50 mM PIPES-KOH instead of the standard 20 mM. To prevent BAPTA from inhibiting through binding Zn\(^{2+}\), the membrane-
permeant heavy-metal chelator TPEN (31) was added; all fusion reactions containing 0.1 mM TPEN included 0.1 mM ZnCl₂ in the final Pho8p assay solution.

Vacuole fusion is inhibited by high [KCl] (Fig. 3, squares). The inhibitory effect of 3 mM BAPTA (circles) is only seen as the reaction KCl concentration rises over 100 mM and approaches bars 5 KCl tested (25 mM), even 9 mM BAPTA had little effect on concentrations of CaCl₂ (Standard high Mg²⁺ HOPS.

FIG. 1. BAPTA inhibition of vacuole fusion is reversed by Ca²⁺.

High Mg²⁺ fusion reactions (see “Experimental Procedures”) were incubated at 27 °C for 90 min without chelator (squares) or with 1 mM (triangles), 3 mM (circles), or 5 mM (diamonds) BAPTA and the indicated concentrations of CaCl₂ (abscissa).

FIG. 2. BAPTA alters the vacuole association of Vam7p and HOPS. Standard high Mg²⁺ fusion reactions without inhibitor or with 5 mM BAPTA were incubated for 90 min at 27 °C, then fractionated into membrane pellets and supernatants by centrifugation (13,000 × g, 15 min, 4 °C). Membranes were resuspended in 30 µl of PS buffer with protease inhibitors (1 µM leupeptin, 5 µM pepstatin, and 0.1 µM Pefabloc-SC). Equivalent portions of the pellets and supernatants were mixed with SDS-loading buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Membranes were probed with antibodies to Vam7p, the HOPS subunits Vps33p, Vps39p and Vps18p, and the GTPases Ypt7p and Rho1p.

Although vacuoles will fuse in the presence of BAPTA upon lowering the KCl concentration, the high vacuole luminal Ca²⁺ levels and the docking-triggered Ca²⁺ flux may lead to brief spurts of Ca²⁺ at the vacuole surface, which could conceivably act even faster than chelation by BAPTA. We therefore tested vacuoles lacking the known Ca²⁺ ATPase, Pmc1p, and the Ca²⁺/H⁺ exchanger, Vcx1p, for their fusion in the presence of BAPTA. These vacuoles accumulate far less luminal Ca²⁺ (5) yet fuse normally (10). We considered that these vacuoles might be more sensitive to BAPTA under fusion conditions due to the lack of luminal calcium and that simple adjustment of [KCl] would not bypass a sensitivity to BAPTA. However, this was not the case (Fig. 5).

Factors other than ionic strength can also make modest contributions to the BAPTA inhibition of fusion. Even when the ionic strength is kept constant through adjustment of the KCl concentration, fusion inhibition due to BAPTA (Fig. 4, bars 1 versus 2) is somewhat relieved by the inclusion of ZnCl₂ in the Pho8p assay buffer (bars 3 and 4). BAPTA solutions may also contribute to adverse pH changes, because increasing the buffer from 20 to 50 mM makes a modest contribution to BAPTA resistance (bars 5 and 6). Finally, in the presence of TPEN, with its extraordinary affinity for zinc, it becomes clear that a small part of the BAPTA inhibition of our assay signal was due to zinc chelation (bars 7 and 8). In sum, salt, pH, and zinc chelation each contribute to the inhibition by BAPTA.
Fig. 5. Reduced luminal Ca\(^{2+}\) does not confer BAPTA sensitivity. High Mg\(^{2+}\) fusion reactions using vacuoles from BJ3505 and DKY6281 (PMC1 VCX1) or their pmc1 vcx1 deletion derivatives (BJ3505 vcx1::URA3 pmc1::TRP1) and (DKY6281 vcx1::URA3 pmc1::TRP1) (10) were performed with the indicated concentrations of added CaCl\(_2\) (A), BAPTA (B), or equimolar BAPTA-CaCl\(_2\) (C). The KCl concentration of each reaction was adjusted to compensate for the ionic strength of the divalent ion/chelator addition. All reactions contained 0.1 mM TPEN, and 0.1 mM ZnCl\(_2\) was present in the Pho8p assay solution.

To ensure that the fusion seen in the presence of BAPTA occurs by the well studied pathway that requires Rab and Rho GTPases, regulatory lipids, SNAREs, and HOPS, we added inhibitory ligands that target these fusion catalysts to reactions with 50 mM PIPES-KOH, pH 6.8, 0.1 mM TPEN, 65 mM KCl, and 6 mM BAPTA. Vacuole fusion in the presence of BAPTA was sensitive to each of the tested inhibitors (Fig. 6) and therefore proceeds via the authentic, physiological pathway. As a second control, we directly determined the effective concentration and the calcium-chelating properties of our BAPTA stock under fusion assay conditions. We exploited the fact that the extinction coefficient of BAPTA at 254 nm changes as it binds calcium (32) to show that our BAPTA stock is ~90% active (Fig. 7A). In addition, the dissociation constant (K\(_d\)) of BAPTA for Ca\(^{2+}\) under our standard vacuole fusion conditions is 126 nM (Fig. 7B), in accord with the published K\(_d\) of 107 nM (32). Thus, BAPTA remains an effective calcium chelator under our fusion reaction conditions.

**BAPTA and High Salt Show Similar Inhibition of Fusion**

Although BAPTA inhibits vacuole fusion through its contribution to ionic strength, we have previously reported (10, 19) that BAPTA is a reversible late-acting fusion inhibitor. We therefore tested whether elevated salt can also act as a reversible late-acting fusion inhibitor. Fusion inhibition by 6 mM BAPTA (Fig. 8B, lane 2) was relieved by 6 mM CaCl\(_2\) (lane 3). This is consistent with the BAPTA inhibition being largely due to ionic strength; because BAPTA has a charge of ~4 and the stable BAPTA-Ca\(^{2+}\) complex has a net charge of ~2, the ionic strength of an equimolar mixture of Ca\(^{2+}\) and BAPTA is far lower than the sum of their separate ionic strengths. Fusion in the presence of BAPTA-Ca\(^{2+}\) remained completely insensitive to antibodies (Fig. 8B, compare lane 3 to lanes 4–6) that inhibited the standard reaction (Fig. 8A). However, when the BAPTA inhibition was reversed by the addition of Ca\(^{2+}\) at 35 min, the ensuing fusion (Fig. 8B, lane 8) was fully resistant to the priming inhibitor α-Sec18p (lane 9) while remaining sensitive to α-Ypt7p or α-Vam3p (lanes 10 and 11). In a standard incubation without inhibitors, the reaction becomes resistant to each of these antibodies by 35 min (9, 19). Thus vacuole priming can occur in the presence of BAPTA, but essential Ypt7p and Vam3p SNARE functions are blocked.

Fusion reactions inhibited by 250 mM KCl behave similarly in this type of experiment (Fig. 8C). Inhibition by 250 mM KCl can be reversed by diluting the reaction 2-fold to a final concentration of 125 mM KCl prior to warming the reaction to 27 °C (Fig. 8C; compare lane 2, diluted to lower the salt, to lane 6, diluted but maintained at 250 mM KCl). Reversal of salt inhibition through dilution at the start left the reaction still sensitive to each antibody (lanes 3–5). When reactions were incubated at 27 °C with 250 mM KCl, mixed with inhibitors at 30 min, then diluted to lower the salt concentration at 35 min, the pattern of fusion resembled calcium-mediated BAPTA reversal. During incubation with 250 mM KCl, the reaction had acquired resistance to α-Sec18p (compare lanes 8 and 9) but remained largely sensitive to α-Ypt7p (lane 10) or α-Vam3p (lane 11). Thus the BAPTA inhibition of fusion closely resembles inhibition caused by elevated ionic strength. We note that these results differ from our earlier studies (10, 19), which had placed the action of BAPTA after the acquisition of resistance.
to α-Ypt7p or α-Vam3p. These earlier studies may not have allowed the inhibitory antibodies sufficient time to act before reversing the BAPTA block.

**BAPTA Effects on Fusion under Other Salt Conditions**—Earlier studies of BAPTA inhibition of vacuole fusion (7, 10) had employed closely related reaction conditions that differed in the concentrations of buffer and divalent cations. Under these conditions (10 mM PIPES-KOH, pH 6.8, 0.5 mM Mg/ATP, 0.5 mM MnCl₂, 125 mM KCl, 200 mM sorbitol, 10 μM coenzyme A, and 330 mM IB2), hereafter referred to as low Mg²⁺ conditions, vacuole fusion proceeded (Fig. 9A, filled squares) as in high Mg²⁺ reaction conditions. As reported (7), fusion is blocked by BAPTA, especially at KCl concentrations >100 mM (filled circles). This inhibition is not relieved by the addition of ZnCl₂ to the Pho8p assay (Fig. 9A, open symbols). Fusion at high or low Mg²⁺ remained sensitive to α-Vam3p (data not shown).

To resolve the effects of BAPTA on vacuole fusion from any effects on the availability of Zn²⁺ for Pho8p activity, BAPTA was added to low Mg²⁺ condition reactions, which also contained 100 μM TPEN, and Zn²⁺ was restored during the Pho8p assay. BAPTA caused a dose-dependent inhibition of fusion activity at higher KCl concentrations (Fig. 9B), while having less effect at salt concentrations of 100 mM KCl or less. Only the addition of 6 mM BAPTA to the reaction caused a significant inhibition of fusion at 100 mM KCl (filled squares). These ionic...
strength effects of BAPTA on vacuole fusion are similar to the results obtained under high Mg²⁺ conditions (Fig. 3) and suggest a similar inhibitory mechanism between these two reaction conditions.

If the inhibitory effects of BAPTA on fusion resulted from the chelation of free Ca²⁺, the addition of CaCl₂ should completely restore fusion when the free [Ca²⁺] reaches a level that is required for fusion. Alternatively, fusion might be restored by reducing the amount of total free [BAPTA] available. To distinguish between these possibilities, CaCl₂ was titrated into fusion reactions containing 6 mM BAPTA, low Mg²⁺, and no TPEN. The Pho8p assay solution did not contain ZnCl₂, E, all reactions contained 0.1 mM TPEN and were assayed for Pho8p activity with 0.14 mM ZnCl₂ in the Pho8p assay solution. Fusion reactions contained: filled squares, no BAPTA; filled circles, 1 mM BAPTA; filled triangles, 3 mM BAPTA; filled diamonds, 5 mM BAPTA; filled squares, 6 mM BAPTA. C, all reactions contained 0.1 mM TPEN and were assayed for Pho8p activity with 0.14 mM ZnCl₂ in the Pho8p assay solution. BAPTA and CaCl₂ solutions were premixed before addition to the fusion reaction. Free BAPTA and Ca²⁺ concentrations were estimated with WEBMAXC STANDARD (65). Fusion reactions contained the following total concentrations of BAPTA and CaCl₂: open squares, 0 mM BAPTA/0 mM CaCl₂; filled squares, 6 mM BAPTA/0 mM CaCl₂; filled triangles, 6 mM BAPTA/6 mM CaCl₂; D, all fusion reactions were as in C, except without TPEN. The Pho8p assay solution did not contain ZnCl₂. E, all reactions contained 0.1 mM TPEN and were assayed for Pho8p activity with 0.14 mM ZnCl₂ in the Pho8p assay solution. Fusion reactions contained: filled squares, no BAPTA; filled circles, 6 mM BAPTA; open triangles, 6 mM BAPTA and 2.8 μM rVam7p; open diamonds, 6 mM BAPTA and 6 mM MgCl₂.

certain conditions of limited free magnesium, low ionic strength, and (see below, Fig. 9E) in the absence of free Vam7p.

It was possible that the effect of CaCl₂-dependent relief of BAPTA inhibition seen in Fig. 9C was specific to the inclusion of TPEN in the assay, or ZnCl₂ in the Pho8p assay. Therefore, we repeated this assay in the absence of TPEN and ZnCl₂ (Fig. 9D) and found a similar profile of fusion under these conditions (compare panels D and C). In addition, fusion showed a similar salt profile and total yield in the presence or absence of TPEN (compare Fig. 9A and 9E, filled squares) and yet remained sensitive to BAPTA (filled circles).

Other reaction components were tested for their ability to reverse the BAPTA-mediated inhibition. There was a dramatic reversal of BAPTA inhibition by the addition of 5.5 mM Mg²⁺ (Fig. 9E, open diamonds), consistent with our fusion studies under high Mg²⁺ reaction conditions (Figs. 1–8). BAPTA inhibition is also reversed by added recombinant Vam7p (open triangles), in accord with earlier studies (8). The fusion supported by Vam7p in the presence of BAPTA is sensitive to o-Vam3p and o-Ypt7p antibodies (data not shown) and is thus authentic Rab- and SNARE-dependent fusion. Although Vam7p addition can stimulate a Ca²⁺ efflux of several micromolar (8, 33), this would have little impact on the free Ca²⁺ concentrations in the presence of 6 mM BAPTA and is thus unlikely to be responsible for the Vam7p-dependent reversal of
Bivalent Cations and Yeast Vacuole Fusion

Calcium Binding to Calmodulin Is Not Required for Vacuole Fusion—It has been suggested that free calcium might activate the essential calcium-binding protein calmodulin to trigger vacuole fusion (7). In light of our current findings, we re-evaluated the requirement for Ca<sup>2+</sup> binding to calmodulin. We assayed the fusion of vacuoles bearing mutant calmodulins with severely lowered ability to bind calcium. Either pho8Δ or pep4Δ were introduced into <i>S. cerevisiae</i> harboring either wild-type calmodulin (CMD1) or either of two calmodulin alleles, cmd1–3 or cmd1–6. The proteins encoded by the cmd1–3 and cmd1–6 alleles bind calcium with dissociation constants of at least 300 μM, and possibly higher (15). In comparison, the wild-type CMD1 protein can bind Ca<sup>2+</sup> with a <i>K<sub>d</sub></i> of ~3 μM (15, 34). Vacuoles purified from the cmd1–3 and cmd1–6 genetic backgrounds fuse just as well as those purified from the CMD1 background, even when assayed under low Mg<sup>2+</sup> conditions (Fig. 10). Although it is formally possible that the particular calmodulin mutants we have employed are in a “locked-on” signaling mode with regard to membrane fusion, this seems unlikely because cmd1–6 strains cannot survive a challenge by α-factor mating pheromone, and this pheromone operates through a signaling cascade that requires the locked-on, Ca<sup>2+</sup>-bound conformation of calmodulin (14). The fusion of these vacuoles remained sensitive to known fusion inhibitors and thus represents authentic Ypt7p-, SNARE-, and HOPS complex-dependent homotypic vacuole fusion. The reason for the discrepancy between this study and a previous report that vacuoles from the cmd1–3 background would not fuse (7) is unclear.

Divalent Ions and Chelators Alter Vacuole Fusion at Different Ionic Strengths—These data suggest that divalent ions, although important in fusion, are not required for a specific signaling pathway. To further explore the requirements for ionic strength or divalent ions, we exploited the fact that vacuoles will fuse in the absence of ATP when provided with sufficient Vam7p (21), a condition that we have termed “bypass fusion,” because it bypasses the need for Sec18p/ATP-dependent priming. This permits a decoupling of any requirement for Mg<sup>2+</sup> ions for fusion from the role of Mg<sup>2+</sup> as a cofactor for ATP hydrolysis by Sec18p. During incubation without ATP and with added Vam7p, vacuoles fuse at elevated KCl concentrations (Fig. 11, filled squares). The addition of 6 mM MgCl<sub>2</sub> generates a distinct shift in the salt requirement for fusion (Fig. 11, filled circles). The increased fusion afforded by Mg<sup>2+</sup> at lower salt is not solely explained by the ionic strength contribution of MgCl<sub>2</sub>, because fusion at 118 mM KCl (which has the same ionic strength as 100 mM KCl plus 6 mM MgCl<sub>2</sub>) is not stimulated to the same extent. The addition of 6 mM CaCl<sub>2</sub> supported fusion in a manner that was indistinguishable from the addition of MgCl<sub>2</sub> (data not shown), suggesting that these ions behave in a similar manner and that they do not promote fusion by a unique Mg<sup>2+</sup>- or Ca<sup>2+</sup>-dependent signaling mechanism. When divalent cationic salts were omitted entirely and a mixture of divalent cation chelators was added (0.1 mM TPEN and 1 mM each of EDTA and BAPTA), the salt profile of fusion was significantly altered (Fig. 11, open squares). Remarkably, this chelator mixture actually stimulates fusion at 100 mM KCl. The divalent ions already associated with the vacuole may be inhibitory or the chelators themselves might alter the physical properties of the lipid bilayer to promote fusion. To determine which chelators stimulate under these narrowly defined conditions, we added the chelators singly or in combination. Bypass fusion with 100 mM KCl requires either divalent ions (Fig. 11) or chelators (Fig. 11 and Table II). The addition of low concentrations (0.1–1 mM) of divalent ion chelators in various combinations promotes fusion, with maximal fusion occurring in the presence of all three chelators (TPEN, EDTA, and BAPTA), a condition where any free divalent ions would be at subnanomolar concentrations. Although divalent ions can directly associate with lipid bilayers to promote fusion (35–37), these data

![Fig. 10. Vacuole fusion does not require Ca<sup>2+</sup>-bound calmodulin. Low Mg<sup>2+</sup> reactions ("Experimental Procedures") were on ice or at 27 °C for 90 min. Antibody inhibitors were added from the start of the reaction. Top, fusion of vacuoles isolated from VSY3 and VSY4 (CMD1, see "Experimental Procedures"). Middle, fusion of vacuoles isolated from VSY5 and VSY8 (cmd1–3). Bottom, fusion of vacuoles isolated from VSY7 and VSY8 (cmd1–6).](http://www.jbc.org/content/16760/13/24999/F1.large.jpg)
suggest that free divalent cations are not required for association with signaling proteins during vacuole membrane fusion.

**DISCUSSION**

Divalent cations serve as cofactors and signaling ligands for membrane fusion proteins such as NSF and synaptotagmin. Divalent ions can also drive fusion of protein-free liposomes (35–37). It has been unclear how each of these two ion effects relate to physiological membrane fusion. When serving as a protein cofactor, Ca$^{2+}$ and Mg$^{2+}$ are usually not interchangeable and cannot be replaced by monovalent salt. Our current studies are consistent with a working model in which divalent ions modulate the capacity of lipids to rearrange during bilayer fusion.

We find that vacuole fusion can occur despite the chelation of Ca$^{2+}$ by BAPTA if sufficient Mg$^{2+}$ or Vam7p are present. This suggests that free Ca$^{2+}$ may not have a unique role in vacuole fusion. Although calmodulin can serve as a Ca$^{2+}$ sensor for other biological processes, Ca$^{2+}$ binding by calmodulin is not required for its function during vacuole fusion (Fig. 10). Furthermore, Rab- and SNARE-dependent vacuole fusion can occur in a reaction with sub-nanomolar concentrations of free divalent ions (Fig. 11 and Table II). Fusion can even be stimulated by divalent ion chelators under certain salt and reaction conditions. Finally, vacuoles lacking the major Ca$^{2+}$ uptake systems, Pmc1p and Vcx1p, fuse normally in the presence of up to 6 mM BAPTA (Fig. 5), providing further evidence that a calcium signal is not a unique prerequisite for vacuole fusion, at least *in vitro*. Although luminal vacuolar Ca$^{2+}$ is released after trans-pairing of SNAREs (7, 8), and our results do not exclude the possibility that this docking-dependent efflux of vacuolar calcium might trigger fusion under specific intracellular conditions of limited free Mg$^{2+}$ or Vam7p, they broaden our thinking about possible fusion mechanisms to include the direct fusogenic effects of Ca$^{2+}$, Mg$^{2+}$, and monovalent ions on lipid bilayer rearrangements.

In addition to the roles of proteins in catalyzing fusion, the necessary rearrangements of bilayer lipids during fusion are substantial and have received extensive study and consideration. For example, DAG is fusogenic in membrane model systems due to its induction of negative membrane curvature (38) and is required for vacuole fusion as well (33). Ca$^{2+}$ and Mg$^{2+}$ can directly stimulate the aggregation and fusion of liposomes composed of phosphatidylycerine (PS) or PS-phosphatidic acid (35, 36). Monovalent salts can also promote or, at higher concentrations, inhibit liposome aggregation and fusion (39), as we now report for vacuole fusion. There has been little prior evaluation whether the Rab- and SNARE-dependent fusion of biological membranes may also be governed by the same interactions of ions with membrane lipids as seen in these liposome studies. Four regulatory lipids (ergosterol, phosphatidylglycerol, phosphatidylethanolamine, and diacylglycerol) function together with the vacuolar Rab, Rab effectors, and SNAREs to assemble a vacuole membrane microdomain for Rab- and SNARE-dependent vacuole fusion (40). Similarly, an enzymatically controlled and spatially distinct interconversion of phosphatic acid and lysophosphatidic acid has been suggested to control Golgi membrane fusion by altering lipid bilayer shape and physical properties (41, 42).

Despite these advances, the relationship between proteins such as SNAREs and specific regulatory lipids at the last stages of membrane fusion remains unclear. Although Mg$^{2+}$ is required for NSF to couple ATP hydrolysis to SNARE complex disassembly (43, 44), the NSF ATPase is not required for single rounds of *in vitro* membrane fusion (1, 21). During synaptic vesicle exocytosis, a wave of membrane depolarization triggers Ca$^{2+}$ influx, then calcium-bound synaptotagmin undergoes a conformational change that alters its interactions with phosphoinositides (45) and SNARE complexes (46–48) as well as mediating synaptotagmin oligomerization (49, 50). These steps link Ca$^{2+}$ signaling to regulated neuronal membrane fusion, as elegantly reconstituted in a defined model reaction (51), but it remains unclear whether Ca$^{2+}$ is directly involved in regulating the fusion of other membranes, such as the vacuole, which lack synaptotagmin. One model of bilayer mixing during vacuole fusion posits a radially expanding, proteinaceous “fusion pore” of apposed, oligomerized vacuolar ATPase V0 sectors (52–54). Alternatively, there is evidence for a “hemifusion” transition state in membrane fusion consisting of a predominantly lipidic “neck” (55–57). The driving forces to establish or resolve either of these states may include bilayer strain from trans-pairing of SNAREs (58–61), although ionic conditions alone can drive PC-PA liposomes into the hemifusion state (35). In support of this, *in vitro* studies of plant vacuole fusion suggest that Mg$^{2+}$ can drive rapid lipid mixing while maintaining separate luminal contents (62). These studies offer a glimpse into the role Mg$^{2+}$ and Ca$^{2+}$ can play in directly altering the arrangement of lipid bilayers during membrane fusion. How, then, do these concepts, founded largely on model
studies with liposomes, relate to Rab- and SNARE-dependent fusion systems such as the vacuole?

We suggest a working model for the regulation of vacuole fusion by interactions of ions with the bilayer. Although Mg\(^{2+}\) is required for Sec18p ATPase activity, we suggest that Mg\(^{2+}\) and Ca\(^{2+}\) also bind directly to bilayer lipids, thereby regulating the lipid rearrangements, from separate apposed bilayers to hemifusion and on to subsequent pore formation, which are the essence of membrane fusion. This is in accord with recent findings that ligands to regulatory lipids block bilayers to hemifusion and on to subsequent pore formation, especially if the resting ionic state of the cell suffices. In either case, ions may have specific, protein-dependent functions.

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Ion Regulation of Homotypic Vacuole Fusion in *Saccharomyces cerevisiae*

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