Zn²⁺ depletion blocks endosome fusion

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Fusion among endosomes is an important step for transport and sorting of internalized macromolecules. Working in a cell-free system, we previously reported that endosome fusion requires cytosol and ATP, and is sensitive to *N*-ethylmaleimide. Fusion is regulated by monomeric and heterotrimeric GTP-binding proteins. We now report that fusion can proceed at very low Ca²⁺ concentrations, i.e. < 30 nM. Moreover, fusion is not affected when intravesicular Ca²⁺ is depleted by preincubation of vesicles with calcium ionophores (5 µM ionomycin or A23187) in the presence of calcium chelators (5 mM EGTA or 60 mM EDTA). The results indicate that fusion can proceed at extremely low concentrations of intravesicular and extravesicular Ca²⁺. However, BAPTA [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-

tetra-acetic acid], a relatively specific Ca²⁺ chelator, inhibits fusion. BAPTA binds other metals besides Ca²⁺. We present evidence that BAPTA inhibition is due not to Ca²⁺ chelation but to Zn²⁺ depletion. TPEN [*N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine], another metal-ion chelator with low affinity for Ca²⁺, also inhibited fusion. TPEN- and BAPTA-inhibited fusions were restored by addition of Zn²⁺. Zn²⁺-dependent fusion presents the same characteristics as control fusion. In intact cells, TPEN inhibited transport along the endocytic pathway. The results indicate that Zn²⁺ depletion blocks endosome fusion, suggesting that this ion is necessary for the function of one or more factors involved in the fusion process.

INTRODUCTION

Multiple membrane fusion events are required for the transport of macromolecules along the endocytic and secretory pathways. Reconstitution studies of different intracellular transport steps in cell-free systems and the analysis of transport-defective yeast mutants have shown that the process is complex and highly regulated [1,2]. However, the fusion machinery seems to share several components for most of the transport events analysed. Fusion among early endosomes is mediated by a specific version of this general mechanism. Endosome fusion requires *N*-ethylmaleimide (NEM)-sensitive factors, including NSF [3,4]. It is regulated by several GTP-binding proteins of the ARF [5], rab [6–9] and heterotrimeric [10] families. Energy in the form of ATP is also necessary.

Ca²⁺ is a major stimulus for several regulated exocytic processes. In intracellular trafficking, however, it is required only for transport from the endoplasmic reticulum to the Golgi apparatus [11]. Several other assays of transport *in vitro* can proceed at very low Ca²⁺ concentrations. Endosome fusion in particular does not require Ca²⁺ addition [12,13]. However, we have recently reported that Ca²⁺ concentrations in the micromolar range elicits fusion between endosomes [14]. Ca²⁺-dependent fusion is not affected by reagents known to block endosome fusion (e.g. NEM), suggesting that a different mechanism, probably involving annexins, is responsible for fusion in the presence of Ca²⁺.

A recent report showed that vesicle fusion during the reconstitution of the nuclear envelope *in vitro* is blocked by the Ca²⁺ chelator BAPTA [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid], suggesting that the process requires Ca²⁺ [15]. That report suggests that BAPTA inhibition may be caused by the specific property of this chelator of dispersing Ca²⁺ gradients very efficiently. When we tested this chelator in the endosome-fusion assay, we observed a remarkable inhibitory effect in conditions where Ca²⁺ was not added to the system

and only the NEM-sensitive fusion was functional. This would suggest that both Ca²⁺-dependent and NEM-sensitive mechanisms of endosome fusion require Ca²⁺. In the present paper we have searched for a Ca²⁺ requirement for endosome fusion and investigated the mechanism of the BAPTA inhibition of the fusion process. We concluded that Ca²⁺ is not an absolute requirement for fusion, and that the BAPTA effect is probably related to Zn²⁺ depletion and not to Ca²⁺ chelation.

EXPERIMENTAL

Reagents

TPEN [*N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine], BAPTA and BAPTA derivatives, and fura 2 were from Molecular Probes, OR, U.S.A. All other reagents were from Sigma Chemical Co. St. Louis, MO, U.S.A.

Assay for endosome fusion *in vitro*

Early endosomes were prepared by loading J774 E-clone macrophages with aggregated monoclonal anti-dinitrophenol (DNP) mouse IgG (via the Fc receptor) or with DNP-β-glucuronidase (via the macrophage mannose receptor) as previously described [12]. When EGTA-loaded endosomes were prepared, uptake was carried out in 10 mM EGTA/120 mM NaCl/8 mM phosphate buffer/20 mM Hepes/NaOH, pH 7.2. The antibody was used at 10 µg/ml (same concentration used for the standard uptake), whereas DNP-β-glucuronidase was internalized at 80 µg/ml (4-fold the concentration used for the standard assay). The mannose receptor requires Ca²⁺ for ligand binding, and the uptake was much less efficient in the presence of EGTA. However, under the conditions used, enough probe was internalized to generate a measurable signal in the assay of fusion *in vitro*. After uptake, cells were washed and homogenized in 250 mM sucrose/20 mM Hepes/KOH/0.5 mM EGTA

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; DNP, dinitrophenol; GTP[S], guanosine 5'-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; TPEN, *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine.

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(homogenization buffer) as previously described [12]. Postnuclear supernatants were stored in liquid nitrogen. To obtain endosome-enriched fractions, the postnuclear fractions were diluted 15-fold in homogenization buffer and centrifuged sequentially at 35000 *g* for 1 min and at 50000 *g* for 5 min. The second pellet was used for fusion reactions [16]. EDTA-washed endosomes were obtained by incubating the 35000 *g* supernatant in the presence of 60 mM EDTA and 5 μ M A23187 for 15 min at 25 °C. The fractions were diluted in 3 ml of homogenization buffer and endosomes isolated by centrifugation at 50000 *g* as described above. Fusion between two populations of endosomes containing either the antibody or the enzyme was performed as previously described [12]. The fusion buffer [250 mM sucrose/0.5 mM EGTA/20 mM Hepes/KOH (pH 7.0)/1 mM dithiothreitol/1.5 mM MgCl₂/50 mM KCl/1 mM ATP, containing 8 mM phosphocreatine, 31 units/ml creatine kinase and 0.25 mg/ml DNP-BSA] was supplemented with gel-filtered cytosol, guanosine 5'-[γ -thio]triphosphate (GTP[S]), ZnCl₂ or CaCl₂, as required.

Ca²⁺ and Zn²⁺ measurements

Free Ca²⁺ and free Zn²⁺ were measured by using the fura 2 fluorescence ratio (340 nm/380 nm excitation, 510 nm emission) [17,18]. To calculate free ion concentrations, the dissociation constant from fura 2 complexes used were 224 nM for Ca²⁺ and 3 nM for Zn²⁺ (Molecular Probes catalogue) [18]. Apparent dissociation constants for Zn²⁺-chelator complexes were calculated by iterative non-linear adjustment of curves similar to those shown in Figure 4(a) to the equation $[Zn] = K_d(Zn_T - [Zn]) / (Q_T - Zn_T + [Zn])$, where K_d = dissociation constant, $[Zn]$ = free Zn²⁺, Zn_T = ZnCl₂ added, Q_T = chelator added.

RESULTS

BAPTA inhibits endosome fusion

Endosome fusion *in vitro* is assayed as a routine in a buffer containing 0.5 mM EGTA. The free Ca²⁺ concentration in the assay estimated by fura II fluorescence is about 150 nM. Raising the EGTA concentration to 5.5 mM decreases free Ca²⁺ to < 30 nM. This chelator has no effect on fusion activity, suggesting that free Ca²⁺ is not necessary for fusion (Figure 1 and Table 1).

A recent report has shown that BAPTA, a fast Ca²⁺ chelator, is able to inhibit fusion among vesicles during the formation of the nuclear envelope *in vitro* [15]. When BAPTA was tested in our assay, we observed that this Ca²⁺ chelator inhibits fusion, with an IC₅₀ of 2.5 mM. Virtually total inhibition was observed with 5 mM BAPTA (Figure 1). As mentioned above, addition of 5 mM EGTA had no effect on endosome fusion. However, both chelators decrease the free Ca²⁺ concentration in the assay to the same extent (< 30 nM). A possible explanation for these observations is that a local Ca²⁺ gradient is required for fusion to occur. BAPTA would dissipate this gradient more efficiently than EGTA, because of its fast association with free Ca²⁺ (100 times faster than EGTA) [19]. An implication of this hypothesis is that a local release of Ca²⁺, probably coming from inside the vesicles, is required for the fusion process. We tried to deplete intravesicular Ca²⁺ to assess whether it affects endosome fusion. Addition of Ca²⁺ ionophores to endosomal fractions caused a measurable increase in extravesicular Ca²⁺ concentration, which was quenched by the addition of 5 mM chelator. As shown in Table 1, fusion was not affected when intravesicular Ca²⁺ was released by a 15 min preincubation at 25 °C in the presence of ionophores (ionomycin or A23187) and 5 mM EGTA, and

Figure 1 Effect of BAPTA and EGTA on endosome fusion

(a) Endosome fusion was assessed in the presence of 1.5 mg of cytosolic proteins/ml and increasing concentration of BAPTA alone (●), BAPTA plus 2.5 mM EGTA (▲), BAPTA plus 2.5 mM EGTA and 5 μ M ionomycin (○), EGTA alone (▼) or EGTA plus 5 μ M ionomycin (▽). Fusion was expressed as a percentage of the value observed in the absence of chelators and ionophore. (b) Fusion among endosomes loaded with 10 mM EGTA and tested in the assay in the absence of cytosol (Buffer), in the presence of 1.5 mg of cytosolic proteins/ml (Cytosol), in the presence of 0.2 mg of cytosolic proteins/ml and 40 μ M GTP[S] (Low Cy. + GTP[S]) and in the presence of 1.5 mg of cytosol/ml plus 5 mM EGTA (Cytosol + EGTA) or 5 mM BAPTA (Cytosol + BAPTA). Fusion was expressed as a percentage of the value observed with 1.5 mg of cytosol/ml. Vertical lines represent S.D. The results shown are representative of at least two experiments. Abbreviation: R.U., relative units.

Table 1 Effect of intravesicular and extravesicular Ca²⁺ chelation on endosome fusion

Control vesicles (left column) or vesicles washed with 60 mM EDTA in the presence of 5 μ M A23187 (see the Experimental section) (right column) were resuspended in fusion buffer supplemented with 1.5 mg of cytosolic proteins/ml in the absence or presence of calcium ionophores and/or EGTA. The samples were then incubated at 25 °C for 15 min and transferred at 37 °C for 45 min. Fusion was quantified and expressed as a percentage of the value obtained for control vesicles and no additions. Values are means $\pm$ S.D. of at least three experiments; —, not determined.

Conditions	Control vesicles	EDTA-washed vesicles
No addition	100 $\pm$ 7	66 $\pm$ 4
+ Ionomycin (5 μ M)	97 $\pm$ 1	—
+ A23187 (5 μ M)	104 $\pm$ 6	62 $\pm$ 1
EGTA (5mM)	102 $\pm$ 5	69 $\pm$ 6
+ Ionomycin (5 μ M)	104 $\pm$ 1	—
+ A23187 (5 μ M)	108 $\pm$ 9	63 $\pm$ 6

allowed to fuse in the presence of ionophores and EGTA. Besides, the inhibitory effect of BAPTA was not potentiated by the presence of ionomycin or ionomycin plus EGTA (Figure 1a). Moreover, washing endosomes with 60 mM EDTA for 15 min at 25 °C in the presence of A23187 caused only a modest (~ 35 %) decrease in fusion (Table 1). Addition of Ca²⁺ ionophore and EGTA to the fusion reaction produced no further loss of fusion activity (Table 1). After this treatment, free Ca²⁺ was undetectable in the assay and addition of Ca²⁺ ionophores did not cause any measurable release of Ca²⁺. To decrease the intra-endosomal Ca²⁺ content more directly, the probes (i.e. aggregate anti-DNP IgG and DNP- β -glucuronidase) were internalized for 5 min in a medium containing 10 mM EGTA. EGTA-loaded endosomes behave normally in the standard assay for fusion *in vitro* (Figure 1b). Taken together, our results indicate that intravesicular Ca²⁺

Figure 2 Inhibition of endosome fusion by TPEN, a chelator that binds several metal ions, but not Ca²⁺ and Mg²⁺

(a) Effect of increasing concentrations of TPEN on endosome fusion in the presence of 0.2 mg of cytosolic proteins/ml and 40 μ M GTP[S] (●) or in the presence of 20 μ M free Ca²⁺ (■). (b) Endosome fusion at increasing concentrations of cytosol in the presence (Δ) or absence (▲) of 0.5 mM TPEN. (c) Endosome fusion at increasing concentrations of cytosol in the presence of 40 μ M GTP[S] and 0.5 mM TPEN (Δ) or without TPEN (▲). Fusion was expressed as a percentage of the maximum value observed in each experiment. The results shown are representative of at least three experiments.

is not necessary for fusion and that a local Ca²⁺ increase is probably not an absolute requirement for endosome fusion.

Chelation of a metal ion different from Ca²⁺ inhibits fusion

BAPTA binds Ca²⁺ with high affinity. However, some other metal ions, including Zn²⁺, are chelated by BAPTA with even higher affinity. We wondered if BAPTA inhibition was due to the depletion of a metal different to Ca²⁺. TPEN is a good chelator for many metal ions, except Ca²⁺ and Mg²⁺. We tested the effect of this chelator in endosome fusion. Figure 2(a) shows that TPEN inhibits fusion with an IC₅₀ of 200 μ M. Both GTP[S]-stimulated fusion and control fusion were inhibited by 500 μ M TPEN (Figures 2b and 2c). TPEN did not decrease the free Ca²⁺ concentration in the system (free Ca²⁺ concentration in the fusion buffer was 140 nM in the presence of 0.5 mM TPEN), nor did it inhibit Ca²⁺-dependent fusion (Figure 2a). The results indicate that the chelation of a metal different from Ca²⁺ is required for fusion.

Addition of Zn²⁺ restores BAPTA- and TPEN-inhibited fusion

We tried to restore fusion by addition of increasing concentrations of several metals (Zn²⁺, Ni²⁺, Co²⁺, Fe²⁺, Cd²⁺, Cu²⁺, La²⁺) to TPEN-inhibited fusion mixtures. Only Zn²⁺ was able to restore fusion at low concentrations (Figure 3a). Zn²⁺

was inhibitory at higher concentrations. BAPTA-inhibited fusion was also restored by the addition of Zn²⁺ (Figure 3b). The concentrations of free Zn²⁺ ions (2–6 nM, estimated by fura 2 fluorescence) at the concentrations of ZnCl₂ necessary to recover TPEN and BAPTA inhibition were similar for both chelators. Zn²⁺-dependent fusion was regulated by GTP[S] and required ATP and cytosol. NEM and trypsin treatment inhibited fusion. Some of these results are shown in Figure 3(c). Hence the characteristics of Zn²⁺-dependent fusion are not those of Ca²⁺-dependent fusion. Indeed, Ca²⁺ also promotes fusion in the presence of TPEN, but fusion was not cytosol- or ATP-dependent, indicating that it triggers fusion by a different mechanism (Figure 3b). Our results indicate that TPEN and BAPTA inhibit a Ca²⁺-independent fusion process by depleting Zn²⁺.

Relationship between endosome-fusion inhibition and Zn²⁺ chelation

If Zn²⁺ is required for fusion, there should be a relationship between the inhibitory effect of different chelators and their ability to chelate Zn²⁺ ions. To test this hypothesis, the concentration of free Zn²⁺ was estimated by the fluorescence ratio of fura 2. In our system, EGTA was less efficient than BAPTA to

Figure 3 Recovery of TPEN- and BAPTA-inhibited endosome fusion by addition of ZnCl₂

(a) Endosome fusion (0.2 mg of cytosolic proteins/ml + 40 μ M GTP[S]) was assessed in the presence of 0.5 mM TPEN and increasing concentrations of ZnCl₂. (b) Endosome fusion (0.2 mg of cytosolic proteins/ml + 40 μ M GTP[S]) was assessed in the presence of 5 mM BAPTA and increasing concentrations of ZnCl₂ (●) or CaCl₂ (white symbols). Ca²⁺-dependent fusion was assessed under complete conditions (□), in the absence of ATP (Δ), or in the absence of cytosol and GTP[S] (▽). (c) Endosome fusion as shown in (a) was measured under complete conditions (●), in the absence of GTP[S] (○), or in the absence of ATP (Δ). Fusion was expressed as a percentage of the value observed in the absence of chelator and ZnCl₂. The results shown are representative of at least two experiments.

Figure 4 Relationship between affinity for Zn^{2+} and endosome-fusion-inhibitory potency for several chelators

(a) Free Zn^{2+} concentration estimated from fura 2 fluorescence ratio (excitation 340/380 nm, emission 510 nm) measured in fusion buffer supplemented with 4 μ M fura 2, 0.6 mM $ZnCl_2$, and increasing concentrations of TPEN (■), BAPTA (●) or EGTA (▼). (b) Plot of the IC_{50} for endosome fusion of several chelators versus their apparent dissociation constant (K_d) for Zn^{2+} complexes. K_d values were estimated from at least three experiments similar to that shown in (a). The BAPTA derivatives tested were 5,5'-dimethyl BAPTA (M-BAPTA), 5,5'-difluoro BAPTA (F-BAPTA), 5-nitro BAPTA (5N-BAPTA), and 5,5'-dibromo BAPTA (Br-BAPTA).

Figure 5 Inhibition of endocytic transport in intact cells by TPEN

(a) Cells were incubated at 4 $^{\circ}$ C for 1 h with ^{125}I -DNP-BSA-anti-DNP IgG complexes as described [21]. Unbound ligand was removed by washing. The cells were incubated at 37 $^{\circ}$ C for the period of time indicated in the presence of 0 μ M (●), 50 μ M (■) or 100 μ M (▲) TPEN. At each period of time, the trichloroacetic acid-soluble radioactivity was measured and expressed as a percentage of the total bound to the cells. (b) DNP- β -glucuronidase was internalized for 5 min at 37 $^{\circ}$ C and chased for an additional 15 min. Anti-DNP IgG, aggregated with a rabbit anti-mouse IgG antibody [22], was bound at 4 $^{\circ}$ C for 30 min. Excess anti-DNP IgG was washed away. Cells were incubated for different periods of time at 37 $^{\circ}$ C in the presence of 0 μ M (●), 50 μ M (■) or 100 μ M (▲) TPEN. Immune-complex formation was measured as described previously [22] and expressed as a percentage of the maximum value observed. Data are representative of at least two experiments done.

complex Zn^{2+} (Figure 4a). This may justify the low inhibitory effect of this chelator in endosome fusion. Several BAPTA derivatives were tested for fusion inhibition and Zn^{2+} chelation. The results are plotted in Figure 4(b). All BAPTA derivatives showed relatively high affinity for Zn^{2+} , and they presented an inhibitory effect in the endosome-fusion assay. The results indicate that there is a relationship between apparent K_d for Zn^{2+} complexes of the different chelators and their IC_{50} for endosome fusion inhibition.

TPEN inhibits endosomal transport in intact cells

TPEN is membrane-permeant. It has been used for inhibition of Zn^{2+} -dependent functions in intact cells [20]. We tested the effect

of the chelator in intact macrophages by two different transport-dependent assays: protein digestion and mixing of two ligands internalized sequentially. In the first assay, the arrival of a radioactive probe (aggregated antibody) to a proteolytic compartment is assessed by the formation of acid-soluble radioactivity [21]. In the second assay, the arrival of two sequentially internalized probes (DNP- β -glucuronidase and anti-DNP IgG) to the same endocytic compartment is assessed by the formation of immune complexes [22]. The results show that both assays are inhibited by TPEN (Figure 5). Inhibition was not complete and was saturated at TPEN concentrations about 50 μ M. No effect on cell viability (Trypan Blue staining) was observed with TPEN addition up to 150 μ M. Since some endopeptidases are Zn^{2+} -dependent enzymes, we tested the effect of TPEN on hydrolysis. Radioactive DNP-BSA was incubated with an endosomal fraction at pH 6 in the presence of 0.1% Triton X-100 and different concentrations of TPEN. The chelator did not inhibit hydrolysis up to 200 μ M (results not shown).

DISCUSSION

Intracellular trafficking of macromolecules among membrane-bound compartments requires several membrane-fusion steps. The fusion machinery seems to share several components for most of the transport events analysed [1,2]. Several transport steps require an NEM-sensitive factor (NSF) and NSF-associated proteins (SNAPs and SNAREs). SNAREs are involved not only in NSF binding to membranes but also in the recognition and docking of membrane-bound compartments. Several GTP-binding proteins are involved in the fusion processes. In addition, a series of other factors, such as protein kinases and protein phosphatases [23,24], motor proteins [25] etc., have been reported to be involved in intracellular transport. However, Ca^{2+} , an ion playing a central role in the regulation of the membrane-fusion process occurring during exocytosis, is not necessary for most of the transport steps reconstituted in cell-free systems. A remarkable exception is the transport from the endoplasmic reticulum to the Golgi that is blocked by EGTA, inhibition that is reversed by addition of Ca^{2+} [11]. We have reported that endocytic vesicles fuse in the presence of micromolar concentrations of Ca^{2+} [14]. However, the Ca^{2+} -dependent mechanism is not sensitive to NEM inhibition and does not require

ATP. A Ca²⁺-dependent mechanism is likely to be mediated by a different mechanism, based on annexin-like proteins. In agreement with other authors [13], we have not observed any requirement for Ca²⁺ for the NEM-sensitive mechanism of fusion. It was then surprising to observe that the Ca²⁺ chelator BAPTA inhibits endosome fusion. The effect of BAPTA could be due to the chelation of a metal ion different from Ca²⁺, or to the property of this compound to dissipate Ca²⁺ gradients very efficiently [19]. The requirement for a Ca²⁺ gradient has been proposed to justify specific inhibitory effects of BAPTA in several models, including fusion among vesicles during the reconstitution of the nuclear envelope [15]. However, we favour the hypothesis that BAPTA inhibits NEM-sensitive fusion among endosomes by depleting a metal ion different from Ca²⁺. A requirement for a Ca²⁺ gradient is unlikely, because the depletion of intra- and extra-vesicular Ca²⁺ does not affect endosome fusion. The BAPTA effect is reversed by Zn²⁺ addition, indicating that BAPTA inhibits by sequestering Zn²⁺ ions. This hypothesis is in agreement with the observation that TPEN, a chelator with low affinity for Ca²⁺ and Mg²⁺, also inhibits endosome fusion. Besides, there is a relationship between the affinity for Zn²⁺ of several chelators and their ability to inhibit endosome fusion. As mentioned above, we have observed that Ca²⁺ promotes fusion by an NEM-insensitive mechanism [14]. Zn²⁺ could reverse the effects of BAPTA and TPEN by a similar mechanism. However, Zn²⁺-dependent fusion presents the same requirement as the NEM-sensitive fusion. It is inhibited by NEM, requires cytosol and ATP, and is regulated by GTP[S].

At present, more than 300 proteins are known to bind Zn²⁺ [26]. Several Zn²⁺-proteins may be directly or indirectly involved in intracellular trafficking. Geranylgeranyltransferase, an enzyme necessary for the prenylation of rab proteins, binds Zn²⁺ [27]. Protein kinase C binds Zn²⁺ in its C1 cysteine-rich domain [28]. There is evidence indicating that protein kinase C and protein kinase C-like proteins may be involved in transport [29,30]. Which of these proteins is necessary for endosome fusion is still unknown.

In conclusion, our results indicate that a Zn²⁺-dependent factor is required for endosome fusion. They also suggest that BAPTA may affect several biological processes not because of its high affinity for Ca²⁺ or its ability to dissipate Ca²⁺ gradients, but because of its affinity for Zn²⁺ ions.

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