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# In Vitro Fusion of Endocytic Vesicles: Effects of Reagents That Alter Endosomal pH

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**Abstract** Ricin, a plant toxin that binds to galactose-terminated glycoproteins and glycolipids on the cell surface, is internalized into endosomes before reaching the cytosol where it exerts its toxic activity. Fusion of early endosomes containing ricin or transferrin was demonstrated by using postnuclear supernatant fractions from K-562 cells. For both ligands, fusion depended on time, temperature, and ATP and was blocked by preincubation with N-ethylmaleimide. Some reagents that increase endosomal pH, the ionophores monensin and nigericin and the weak base chloroquine, stimulated the rate of fusion. However, bafilomycin $A_1$, a specific inhibitor of vacuolar $H^+$-ATPases, did not alter the rate of fusion. Moreover, it reduced or eliminated stimulation caused by monensin, nigericin, or chloroquine. Thus, the increased rate of fusion did not correlate with the higher lumenal pH of the endosome. The results suggest instead that fusion was stimulated by reagents that promoted accumulation of cations within the vesicles. 

**Key words:** ricin, transferrin, monensin, bafilomycin A1, chloroquine

## INTRODUCTION

To study endosome fusion, several in vitro assays have been developed with probes introduced into endosomes either by receptor-mediated endocytosis or by fluid phase endocytosis [Braell, 1987; Woodman and Warren, 1988; Diaz et al., 1988; Gorvel et al., 1991]. Fusion requires ATP, temperatures above 20°C, and cytosolic protein(s) that is inactivated by N-ethylmaleimide (NEM). Early endosomes and coated vesicles [Woodman and Warren, 1991] have the greatest activity in these in vitro fusion assays [Gruenberg and Howell, 1989]. To examine the fusion properties of early endosomes containing ricin, we adapted an assay that employs complementary vesicle populations containing proteins labeled by avidin or biotin [Braell, 1992].

Ricin, a highly toxic, 60 KDa protein from castor beans, is a disulfide-linked heterodimer in which the A chain has enzymatic activity and the B chain is a lectin. For its lethal action, the A subunit must reach the cytosol, where it cleaves a specific, essential adenine residue from ribosomal RNA, thereby blocking protein synthesis [Endo and Tsurugi, 1987]. The B subunit mediates entry of ricin into the cell by binding to terminal galactose residues of diverse glycoproteins and glycolipids on the cell surface. Internalization of ricin appears to occur both via clathrin-coated pits as well as from smooth areas on the cell surface [Sandvig and van Deurs, 1994; van Deurs et al., 1990]. Ricin affinity for galactosyl moieties is less at pH 5 than at pH 7 [Frenoy, 1986], but only a small fraction of ricin dissociates from its glycoprotein and glycolipid receptors at the pH of early endosomes [van Deurs et al., 1988].

After internalization, ricin is initially delivered to the early endosome, a dynamic compartment responsible for sorting and routing ligands to their various destinations. A substantial fraction of the internalized toxin recycles unaltered to the plasma membrane [van Deurs et al., 1990; McIntosh et al., 1990; Magnussen et al., 1993].

Abbreviations used: $A_{enz}$, avidin-β-galactosidase; B-ricin, biotinyl ricin; B-Tf, biotinyl transferrin; CHO, Chinese hamster ovary; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FCCP, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; FITC, fluorescein isothiocyanate; GTP-γ-S, guanosine 5'-(3-thiotriphosphate); HEPES, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PNS, postnuclear supernatant; TGN, trans Golgi network.

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Another fraction (presumably ricin that dissociates from its membrane-bound receptors) is routed via the late endosome to lysosomes. Finally, a small amount of ricin (5 to 15%) reaches the TGN and the Golgi region after 60 min [van Deurs et al., 1988]. Although Beaumelle et al. [1993] have recently detected ATP-dependent translocation of ricin A chain out of isolated endosomes, several studies indicate that passage of ricin to the trans Golgi network (TGN) or the Golgi complex [Sandvig et al., 1986; Youle and Colombatti, 1987; Yoshida et al., 1990] or to the endoplasmic reticulum [Wales et al., 1992] is required for toxicity. With a goal of better understanding the early intracellular trafficking events, we sought to demonstrate and characterize the fusion properties of early endosomes that contained ricin. In addition, we examined the effects on fusion of agents known to alter endosomal pH.

## METHODS

### Materials

The human erythroleukemia cell line K562 was obtained from American Type Culture Collection (Rockville, MD). Avidin-β-galactosidase, biotin amidocaproate-N-hydroxysuccinimide ester, human holo transferrin, biotin-labeled insulin, biotin-labeled ricin (Toxin $RCA_{60}$), FITC-labeled ricin (Toxin $RCA_{60}$), goat anti-human transferrin (fractionated antiserum), rabbit anti-*Ricinus communis* lectin fractionated antiserum, valinomycin, nigericin, chloroquine, monensin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), Sepharose 6B, and dialyzed fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Bafilomycin $A_1$ was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Protease inhibitors were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Microtiter well strips were Titertek FB High Binding strips from ICN/Flow (no longer available), or radius edge FB strips from Labsystems (Marlboro, MA). The source of growth media has been reported [Wellner et al., 1994]. All other reagents were obtained from Sigma.

A commercial preparation (Sigma) of biotinyl ricin (2.2 mol biotin/mol ricin) was used for experiments with CHO cells. Its cytotoxicity was 60% of that of the native toxin as determined by cell proliferation assays (Wellner et al., 1994). Both ricin and transferrin were biotinylated as described by Braell [1992] using a 10-fold molar excess of biotin amidocaproate-N-hydroxysuccinimide ester. Incorporation was 5.4 mol biotin per mol diferric transferrin and 2.2 or 2.6 mole biotin per mol ricin. The biotinyl ricin preparations retained over 60% of their original toxicity, and vesicles containing them have same fusion activity as those prepared with the commercial biotinyl ricin that lacked a spacer between biotin and the toxin.

FCCP, valinomycin, and bafilomycin $A_1$ were stored as concentrated solutions in DMSO and diluted into homogenization buffer before adding them to the fusion assay. Monensin and nigericin were stored as concentrated solutions in ethanol. The final concentrations of solvent in the assay were 0.2% or less, and neither 0.2% DMSO nor 0.2% ethanol altered fusion activity. A protease inhibitor mix (1 mg/ml chymostatin, 1 mg/ml pepstatin A, 2 mg/mlL E64, 1 mg/ml antipain) was stored at −20°C in DMSO and diluted 100-fold into the appropriate medium [Woodman et al., 1992].

### Cell Culture and Preparation of Cytosol and Vesicle Fractions

Cells were grown at 37°C in a humidified chamber containing 5% $CO_2$ in air. Nonadherent K562 cells and CHO cell monolayers were grown in α-minimal essential medium containing 7.5% FBS, 2 mM glutamine, and antibiotics (penicillin, 200 U/ml; streptomycin sulfate, 200 μg/ml). Vero cell monolayers were grown in Eagle's Minimal Essential Medium containing 5.0% FBS plus antibiotics. Cell viability was determined by exclusion of trypan blue. Before preparation of cytosol or postnuclear supernatant (PNS) fractions, the medium was changed to Dulbecco's modified Eagle's medium supplemented with nonessential amino acids and 7% dialyzed FBS when cells were half confluent monolayers (CHO, Vero) or when there were 5–7 × $10^5$ cells/ml (K562). The cells were then incubated overnight (14–16 h) [Braell, 1987].

To prepare PNS fractions containing avidin-β-galactosidase ($A_{enz}$PNS), a suspension (8 × $10^7$ cells/ml) of washed K562 cells in uptake buffer (0.15 M NaCl; 25 mM HEPES, pH 7.4; 1 mg/ml glucose; 1 mg/ml bovine serum albumin) containing 0.5 mg/ml avidin-β-galactosidase, was incubated for 1 h at 18–20°C, as reported by Braell [1987, 1992]. To prepare PNS fractions containing biotinyl ricin (B-ricin), biotinyl trans-

ferrin (B-Tf), or both biotinyl proteins (BR + BTf), the biotinyl proteins (each 100 nM) were incubated with K562 cells in the same way for 5 min at 37°C. Cells were preincubated for 30 min at 37°C in serum-free, HEPES-buffered Dulbecco's modified Eagle's medium before all internalizations of transferrin. The incubations were stopped by dilution into cold phosphate-buffered saline (PBS) (containing 0.1 M galactose after uptake of B-ricin), and the cells were collected, washed five times with the same buffer, and suspended in homogenization buffer (75 mM KCl, 25 mM NaCl, 85 mM sucrose, 20 mM HEPES, pH 7.4, 20 μM ethylene glycol-bis[β-aminoethyl ether] N, N, N′, N′-tetraacetic acid). For CHO or Vero cells, uptake was carried out in 10 cm cell culture dishes. Medium was removed from the cells and replaced with prewarmed uptake medium (3 ml per plate) containing 16 nM B-ricin (1 μg/ml) and incubated for 5 min (or as indicated) at 37°C. The incubation was stopped by removing the medium, and the cells were washed four times by incubating 5 min with cold 0.1 M galactose in PBS, 3 ml/plate, then once with homogenization buffer. The plates were inverted, drained for 90 sec, then scraped with a rubber policeman to remove the cells (about 1.2 ml of cell suspension, $60 \times 10^6$ cells per six plates) [Woodman and Warren, 1989]. In all cases, washed cells (about 60 million cells per ml) were broken with a stainless steel homogenizer until about 25% (for PNS fractions) or 5% (for cytosol) of the cells remained viable. The homogenates were centrifuged at 4°C for 5 min at $800 \times g$, and the PNS fractions were frozen and stored in liquid nitrogen in aliquots suitable for one set of assays.

Sepharose 6B-excluded fractions were obtained by gel filtration of 200 μl of the PNS fraction (about 10 mg/ml protein) on columns (0.5 × 7 cm) of Sepharose 6B. The turbid, excluded fractions (200 μl, about 3 mg/ml) were collected and assayed immediately. To prepare cytosolic protein, the PNS fraction from K562 cells was centrifuged 1 h at $100,000 \times g$ at 4°C. The supernatant fraction was dialyzed against homogenization buffer [Braell, 1987] and then stored in liquid nitrogen.

### Vesicle Fusion Assay

The fusion assay was carried out essentially as reported by Braell [1992]. Unless otherwise indicated, assays (25 μl) contained cytosolic protein (45 μg), 8 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 1 mM ATP (neutralized with NaOH), 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml of biotinyl-insulin, and the two vesicle fractions, $A_{enz}$PNS and B-ricin PNS (each 25 μg protein) in homogenization buffer. For experiments performed in the absence of ATP, the reagents phosphocreatine, creatine phosphokinase, and ATP were replaced by 5 mM glucose and 50 units/ml hexokinase. Reactions were carried out at 37°C for various times and were stopped by placing the reaction mixtures in ice. The vesicles were lysed by adding 5 μl of detergent solution (10% Triton X-100, 1% SDS, and 50 μg/ml biotinyl-insulin). After 15 min, the assays were mixed with 225 μl of dilution buffer (0.05% Triton X-100, 0.30 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mg/ml heparin). The six-fold increase in NaCl concentration compared to the original procedure [Braell, 1992] reduced the otherwise high background fluorescence that was observed when the concentration of vesicle protein was low ($<50$ μg total vesicle protein), particularly in the absence of ATP and/or added cytosolic protein. After centrifugation (2 min, $10,000 \times g$), 200 μl aliquots of the supernatant fractions were transferred to microtiter wells coated with anti-ricin antibody (or anti-transferrin antibody where appropriate) and incubated for 3 h at 37°C to permit binding of ricin-containing complexes. The wells were prepared, blocked, washed, and assayed for bound β-galactosidase by using 4-methylumbelliferyl-β-galactoside, which is converted to a fluorescent product, as described by Braell [1987, 1992]. Fluorescence emitted at 450 nm was measured on a Perkin Elmer 650–40 fluorescence spectrometer with excitation at 366 nm. The assay was designed such that the fusion complex (ricin-biotin.avidin-β-galactosidase) can form only upon fusion of sealed vesicles, and only β-galactosidase in this complex should bind to the antibody-coated wells [Braell, 1987]. To assay PNS containing cointernalized proteins (BR + BTf), all assay volumes were increased four-fold. After adding 900 μl of dilution buffer and centrifugation, two 200 μl aliquots were transferred to microtiter wells coated with antibody specific for ricin or transferrin.

### Other Assays

The total B-ricin content of PNS preparations was estimated by a modification of the fusion assay. Cytosolic protein, $A_{enz}$ PNS, and biotin-insulin were omitted and replaced by 5 μg of

soluble avidin-β-galactosidase and 0.2% Triton X-100. Exposed B-ricin probe was measured in the same manner except that detergent was omitted. Latency (total minus exposed) of the biotinyl ricin was 75% to 85% in various PNS preparations.

## RESULTS

### Optimization of Fusion Activity of B-Ricin Vesicles

For all studies reported here, a postnuclear supernatant fraction containing avidin β-galactosidase ($A_{enz}$ PNS) was prepared from K562 cells according to the methods reported by Braell [1987, 1992]. To optimize the fusion activity of vesicle preparations loaded with biotinyl ricin (B-ricin PNS), CHO cells were incubated with B-ricin for various times at 37°C, PNS fractions were prepared and tested for B-ricin content (Fig. 1A) and for fusion activity (Fig. 1B). Significant B-ricin was detected in the PNS fractions from cells incubated with B-ricin on ice, but these fractions had no fusion activity. After 5 min incubation at 37°C, the total B-ricin content increased, and fusion activity was observed. Increasing the time of internalization to 15 or 60 min (Fig. 1B) or preincubating the cells for 60 min on ice with B-ricin before the 37°C internalization (data not shown) resulted in no further increase in total B-ricin content or in fusion activity. Experiments with K562 cells produced similar results.

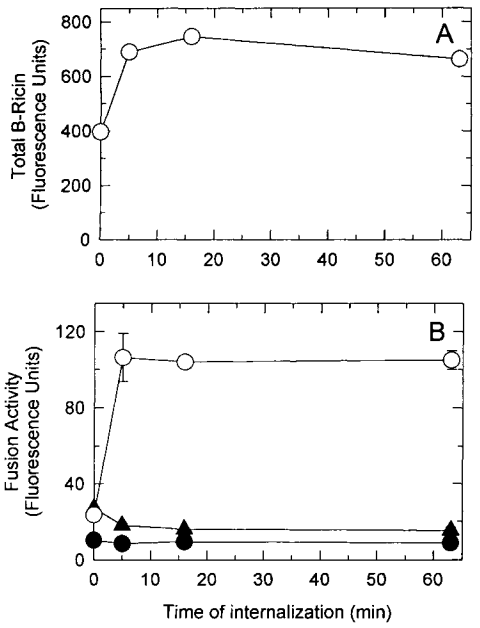


**Fig. 1.** Dependence of B-ricin content and fusion activity of PNS fractions on time of B-ricin uptake. CHO cells were allowed to internalize B-ricin, as described in Methods, using prewarmed uptake medium containing 1 μg/ml B-ricin and incubation for 0 to 60 min at 37°C. Results shown for 0 min at 37°C were from cells incubated 10 min on ice with B-ricin. PNS fractions containing B-ricin (102 μg protein) were assayed in a total volume of 50 μl for **(A)** total B-ricin content and **(B)** fusion activity with $A_{enz}$ PNS (65 μg protein) after 40 min incubation in the presence of ATP at 37°C (○) or at 4°C (●), or in the absence of ATP at 37°C (▲).

CHO cells were allowed to internalize B-ricin at 37°C for 5 min, then were chilled, washed, and reincubated for various times at 37°C without B-ricin. Both total B-ricin content and fusion activity dropped rapidly during the chase ($t_{1/2}$ = 5 min, total B-ricin, Fig. 2A; $t_{1/2}$ = 10 min, fusion, Fig. 2B). K562 cells that internalized FITC-ricin for 5 min retained only 25% of the original ligand after a 15 min chase (data not shown). Although we have not excluded degradation of ricin, these results are consistent with the rapid and extensive recycling of intact ricin to the cell surface observed by others [van Deurs et al., 1988; McIntosh et al., 1990; Magnusson et al., 1993].

Fusion activity of PNS fractions from three different cell lines, CHO, Vero, and K562, that had internalized B-ricin and/or B-transferrin were compared directly (Fig. 3). Similar levels of fusion activity were observed in PNS fractions from K562 cells incubated with either B-ricin (Fig. 3A) or B-transferrin (Fig. 3B) or when both ligands were co-internalized (BR + BTf). Lower but significant ATP-dependent fusion was observed when PNS fractions were prepared from Vero cells incubated with either B-ricin or B-transferrin. The fusion activity of the PNS fraction from CHO cells incubated with B-ricin was intermediate between that of Vero and K562 cells. K562 cells were used to prepare B-ricin-loaded vesicles for further studies to permit direct comparisons of the fusion properties of vesicles containing B-ricin with those of vesicles loaded in parallel or simultaneously with B-transferrin.

### Fusion Activity of Both B-Ricin and B-Transferrin PNS Fractions Requires Cytosol and is Inhibited by GTP-γ-S and NEM

To obtain fusion-competent vesicles depleted of nucleotides and cytoplasmic factors, PNS fractions loaded with B-ricin were subjected to gel filtration on small columns of Sepharose 6B. Material excluded by the gel was turbid and had

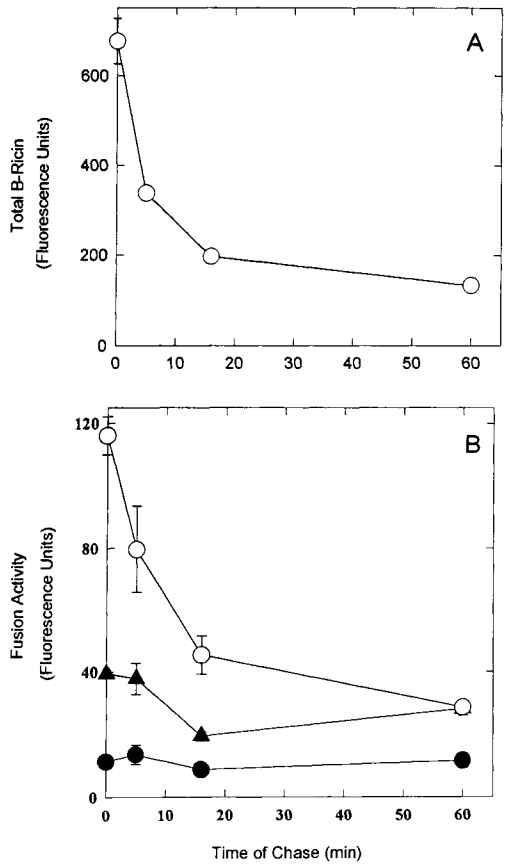


**Fig. 2.** Dependence of fusion activity and B-ricin content of PNS fractions on time of chase in B-ricin free medium. The chase was carried out with CHO cells that had internalized B-ricin for 5 min as in Figure 1. The cells were washed three times each with 3 ml of cold PBS containing 0.1 M galactose and once with PBS at 37°C. Prewarmed uptake medium without B-ricin was added, and the cells were incubated for various times at 37°C. The chase was stopped by washing twice with cold PBS and once with cold homogenization buffer before collecting the cells by scraping. The resulting B-ricin PNS fractions (79 μg protein) were assayed (as described in Fig. 1) (**A**) for total B-ricin and (**B**) for fusion activity with $A_{enz}$ PNS (65 μg protein) after 40 min incubation in the presence of ATP at 37°C (○) or at 4°C (●), or in the absence of ATP at 37°C (▲).

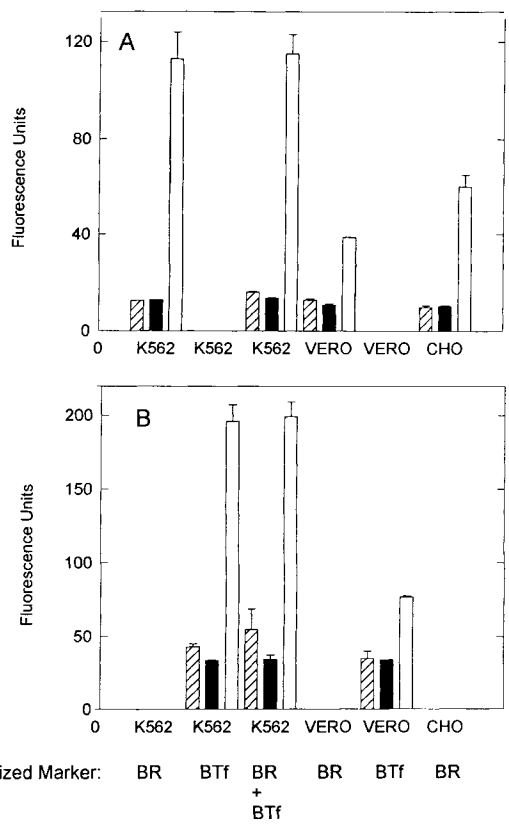


**Fig. 3.** Fusion activity of vesicles from CHO, K-562, and VERO cells loaded with B-ricin, B-transferrin, or with both ligands. PNS fractions were prepared from cells that had internalized either B ricin (BR) or BTf or both ligands (BR + BTf). Fusion assays were carried out in parallel under standard conditions. Incubations were for 20 min at 37°C in the presence (□) or absence (■) of ATP or in the presence of ATP on ice (▨). Aliquots were analyzed on microtiter wells coated with antibody specific for (**A**) ricin or (**B**) transferrin.

fusion activity dependent on the addition of cytosol (Fig. 4A). Fusion activity was maximal in the presence of 2 mg/ml of cytosol. Preincubating cytosol with 40 μM GTP-γ-S reduced by 30% its ability to stimulate fusion of Sepharose 6B-excluded vesicles. The same experiment carried out in parallel with Sepharose 6B-excluded vesicles containing B-transferrin is shown in Figure 4B. Clearly, the dependence on cytosol and its sensitivity to GTP-γ-S were essentially identical for the vesicles containing the two biotinylated ligands.

Fusion activity was also sensitive to NEM, as shown for in vitro fusions involving other ligands. To determine whether the NEM-sensitive factor(s) was soluble or was associated with the vesicles, Sepharose 6B-excluded vesicles and cytosol were pretreated separately with NEM, then tested in the fusion assay (Fig. 5). NEM pretreatment of either cytosol or vesicles reduced fusion activity, but complete elimination of activity required NEM pretreatment of both fractions. Similar results were observed using Sepharose 6B-excluded vesicles containing B-transferrin (data not shown).

### Monensin Stimulates the Rate of Fusion

Monensin is an ionophore that increases ricin cytotoxicity [Sandvig and Olsnes, 1982; Ghosh and Wu, 1988; Wellner et al., 1994] and disrupts

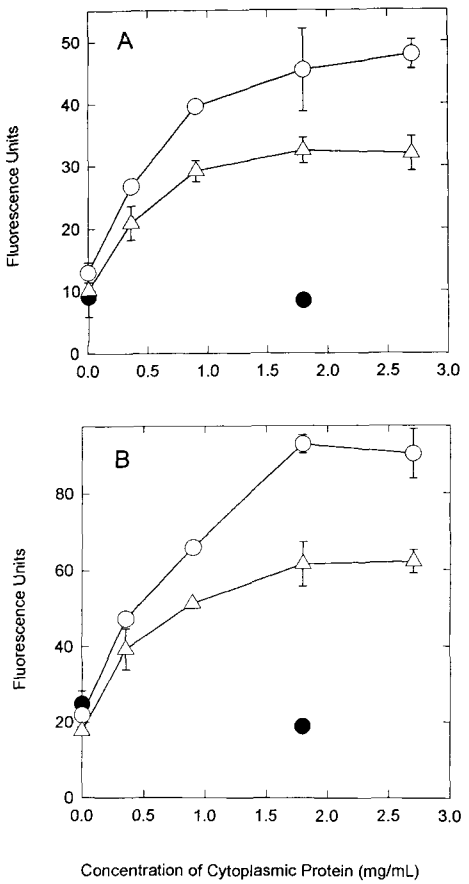


**Fig. 4.** Cytosol dependence of fusion of sepharose 6B excluded vesicles. Separate Sepharose 6B were used to prepare excluded fractionations from $A_{enz}$ PNS, B-ricin PNS, and B-transferrin PNS. Different amounts of cytosol were preincubated with homogenization buffer (○, ●) or with 40 μM GTP-γ-S (△) for 10 min at 37°C. After chilling, cofactors and two complementary Sepharose 6B-excluded vesicle preparations, $A_{enz}$ and either B-ricin (**A**) or B-transferrin (**B**), (54 μg total vesicle protein), were added reducing the concentration of GTP-γ-S to 20 μM in the final reaction volume of 50 μl. After incubation for 40 min at 37°C (○, △) or 4°C (●), clarified supernatants were analyzed for fusion complex by incubation with microtiter wells coated with antibody specific for (**A**) ricin or (**B**) transferrin.

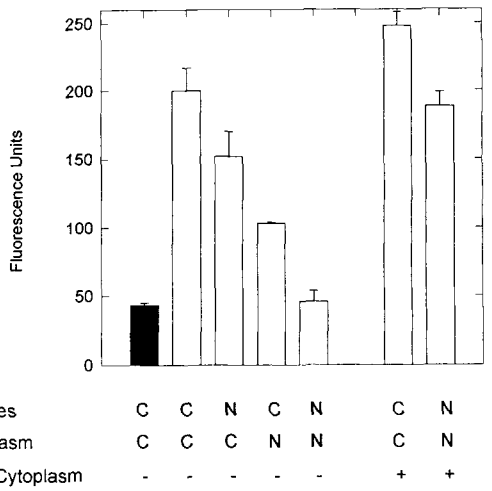


**Fig. 5.** NEM sensitivity of cytoplasm and sepharose 6B-excluded B-ricin vesicles. Sepharose 6B-excluded $A_{enz}$ vesicles were mixed with B-insulin then with Sepharose 6B-excluded B-ricin vesicles. NEM-treated vesicles (*N*) were prepared by adding NEM to a final concentration of 1.6 mM, incubating 15 min on ice, then adding dithiothreitol to 2.2 mM. For control vesicles (*C*), dithiothreitol and NEM were added in the opposite order. NEM-treated cytosol and control cytosol were prepared in the same way. Fusion activity was measured after a 25 min incubation at 37°C (□) or at 4°C (■). Each assay contained a total of 12.4 μg of Sepharose 6B-excluded protein and 45 μg cytosolic protein, or 90 μg where additional cytosolic protein was added.

Golgi stucture at low concentrations (10 to 100 nM) [Sandvig et al., 1986; Mollenhauer et al., 1990]. At higher concentrations, monensin increases the pH of acidic vesicles by exchanging external $Na^+$ or $K^+$ for internal $H^+$ [Mellman et al., 1986]. The time course of fusion in the presence and absence of 50 μM monensin is shown in Figure 6. In this experiment, Sepharose 6B-excluded vesicles were prepared from cells that had cointernalized both B-ricin and B-transferrin. For both ligands, monensin stimulated the rate of fusion (10 min incubation), but did not change the extent of fusion after a 50 min incubation. As shown in Figure 7, stimulation by monensin showed the same concentration dependence with either B-ricin or B-transferrin, in this case using PNS fractions. When the fusion incubations were supplemented with cytosol (1.8 mg/ml), 2 to 50 μM monensin increased fusion to 150% of the control. Without additional cytosol, 2 to 20 μM monensin stimulated somewhat less, and higher concentrations had no effect or were inhibitory. In other experiments, 0.5 μM monesin had no effect on the rate of fusion, and standard fusion assays containing 10 or 50 μM monensin gave background fluorescence when kept on ice or when ATP was absent at 37°C (data not shown).

### Chloroquine Alters Fusion Activity

Another reagent that increases endosomal pH is the weak base chloroquine. It has been reported that chloroquine, like other weak bases, increases the cytotoxicity of ricin [Thompson et al., 1995]. We confirmed this in Vero cells and found that the ricin concentration required for 50% inhibition of protein synthesis was reduced six-fold in the presence of 0.5 mM chloroquine, which alone caused a small (11%) decrease in the rate of protein synthesis. The effects on

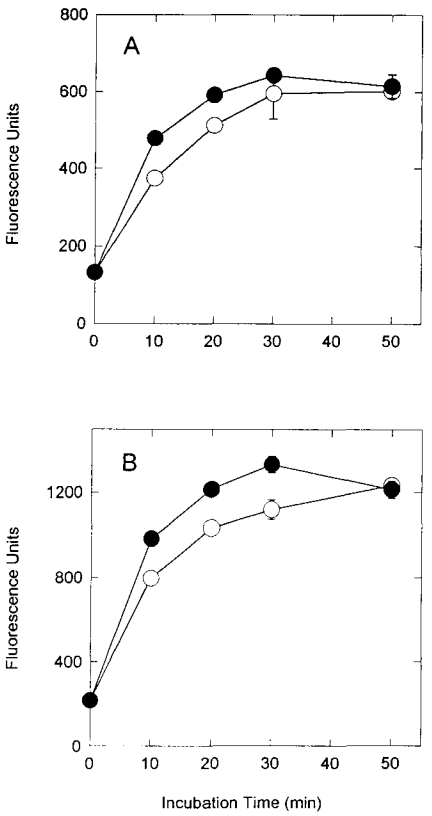


**Fig. 6.** Time course of fusion in the presence or absence of monensin. Sepharose 6B-excluded fractions were isolated from PNS containing $A_{enz}$ and from PNS containing cointernalized ligands (BR + BTf) then assayed for fusion activity in the presence (●) or absence (○) of 50 μM monensin. Duplicate aliquots from a 100 μl assay mixture were incubated with microtiterwells coated with antibody against (**A**) ricin or (**B**) transferrin.

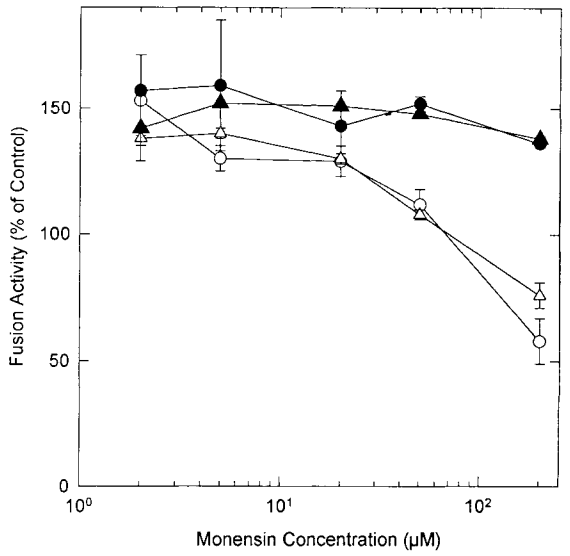


**Fig. 7.** Concentration dependence of monensin stimulation of the rate of fusion. PNS containing cointernalized ligands (BTf + BR) was assayed for fusion activity by incubating with $A_{enz}$ PNS under standard conditions for 10 min at 37°C with different monensin concentrations in the presence (●, ▲) or absence (○, △) of 1.8 mg/ml cytosol. Duplicate aliquots from a 100 μl incubation mixture were incubated with microtiter wells coated with antibody against ricin (○, ●) or transferrin (△, ▲). Activity is reported as a percentage of the corresponding control incubation with no monensin.

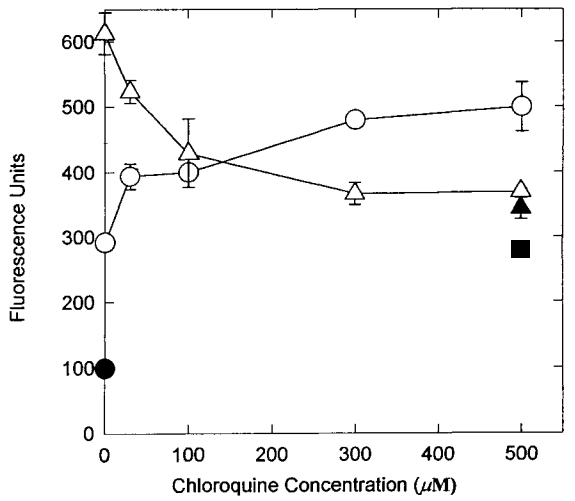


**Fig. 8.** Effect of chloroquine concentration on the rate and extent of fusion. PNS containing B-ricin was assayed for fusion after incubation with $A_{enz}$ PNS at 37°C for 10 min (○) or 60 min (△) or at 4° (●) in the presence of various concentrations of chloroquine. Incubations were also carried out for 60 min in the presence of 0.5 mM chloroquine and either 1 mM PMSF (▲) or a protease inhibitor mix (■).

apparent rate and extent of fusion were examined as a function of chloroquine concentration using incubations for 10 and 60 min, respectively (Fig. 8). Significant stimulation of the rate of fusion occurred with 30 μM chloroquine; stimulation was maximal in the presence of 300 μM chloroquine. In contrast, after a 60 min incubation, the amount of fusion complex declined with increasing concentrations of chloroquine. The chloroquine concentration dependence was similar for stimulation of the rate of fusion and for subsequent loss of fusion complex, suggesting that both effects may be consequences of the same initial action of chloroquine.

The time course of the fusion reaction in the presence of chloroquine is shown in Figure 9. After 10 min, fusion in the presence of chloroquine was stimulated relative to the buffer control. However, detectable fusion complex reached a maximum at 20 min and then appeared to fall by 50% or more. The surprising decline in fluorescence signal presumably indicates that the fusion complex, [β-gal-Av.B-ricin], was degraded or inactivated during the incubation due to the

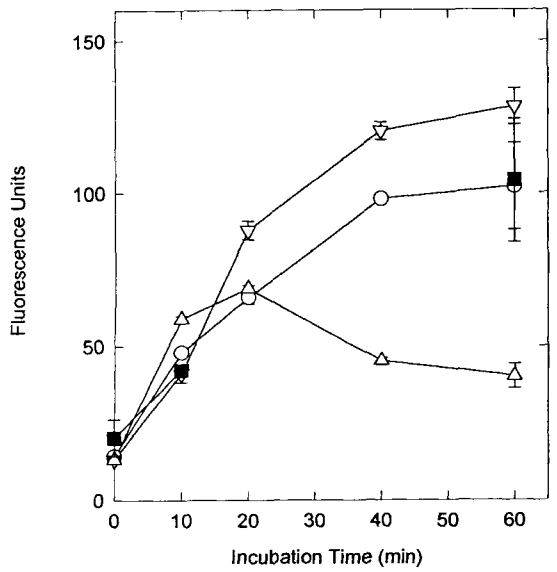


Fig. 9. Time course of fusion in the presence of bafilomycin $A_1$ and/or chloroquine. PNS containing B-ricin was assayed for fusion activity by incubating with $A_{enz}$ PNS at 37°C for the time indicated under standard conditions (○), or in the presence of 0.1 μM bafilomycin $A_1$ (▽), 0.5 mM chloroquine (△), or both bafilomycin $A_1$ and chloroquine (■). Similar results were observed in two additional, independent experiments.

addition of chloroquine. Control incubations of chloroquine with soluble avidin-β-galactosidase, or with the soluble, preformed complex, [β-gal-Av.B-ricin] were carried out in each case with or without added PNS. The results gave no evidence that incubation with chloroquine inhibited β-galactosidase activity or decreased the amount of [β-gal-Av.B-ricin] that was competent to bind microtiter wells coated with anti-ricin antibody. We also attempted to address the possibility that the fusion complex was degraded within sealed vesicles, but the results were not conclusive. Adding PMSF or a mixture of protease inhibitors (Fig. 8) or leupeptin (not shown) to the fusion assay alone or in the presence of 0.5 mM chloroquine had no significant effect on the amount of fusion complex detected after 10 or 60 min incubations.

### Effects of Bafilomycin $A_1$ on Fusion Activity

Bafilomycin $A_1$ is a specific inhibitor of the vacuolar $H^+$-ATPases [Bowman et al., 1988]. It dissipates the endosomal pH gradient due to leakage of protons across the membrane. The time course of fusion in the presence of 0.2 μM bafilomycin $A_1$ is also shown in Figure 9. Bafilomycin $A_1$ did not significantly change the rate of fusion, but it increased the extent of fusion to 130% ± 15% of the control (n = 17) with PNS fractions containing B-ricin. In contrast, the extent of fusion in the presence of bafilomycin $A_1$ was the same as the control when PNS containing cointernalized ligands (BR + BTf) was assayed after 60 min incubation for fusion complexes containing either B-ricin (93% ± 13%, n = 11) or B-transferrin (102% ± 8%, n = 5). Thus, the fusion of vesicles containing B-ricin was enhanced by bafilomycin $A_1$, but only when B-transferrin was absent and only after the reaction approached a plateau.

Also shown in Figure 9 are results of incubations containing both chloroquine and bafilomycin $A_1$. The stimulation of fusion at 10 min observed in the presence of chloroquine alone was not observed when both chloroquine and bafilomycin $A_1$ were present. Moreover, the reduction of fusion product caused by chloroquine after 60 min (to 30% ± 15% of the control, n = 9) was nearly eliminated when bafilomycin $A_1$ was also present (83% ± 21% of the control, n = 9). These results suggest that the vacuolar $H^+$-ATPase activity is required for both the early and late effects of chloroquine on fusion. Essentially identical results were observed using PNS fractions containing cointernalized (BR + BTf).

To clarify the effects of monensin, chloroquine, and bafilomycin on the rate of fusion, we examined several other reagents that increase endosomal pH. Nigericin is an ionophore similar to monensin. FCCP and valinomycin are electrogenic ionophores specific for protons and $K^+$, respectively. To confirm that these reagents did indeed cause pH increases in our vesicle preparations, PNS was prepared from cells that had internalized FITC-ricin for 5 min at 37°C [Galloway et al., 1988]. The PNS fraction was diluted in homogenization buffer to 0.2 mg/ml protein, and the fluorescence changes caused by ATP and the other reagents were examined at room temperature. The results (not shown) confirmed that adding ATP resulted in acidification of the vesicles containing FITC-ricin, and that the acidification was rapidly reversed by FCCP, $NH_4Cl$, monensin, nigericin, or bafilomycin $A_1$. Chloroquine quenched the fluorescence of fluorescein, but we did not observe a shift of the excitation peak and were therefore unable to correct for the quenching as reported by Ohkuma and Poole [1978].

**TABLE I. Effects on Fusion Activity of Agents That Increase Endosomal pH[a]**

| Addition | Fusion activity(% of Control) | |
|---|---|---|
| | B-Ricin | B-Transferrin |
| None | 100 ± 10 (15) | 100 ± 10 (6) |
| 0.2 μM Bafilomycin | 99 ± 16 (12) | 96 ± 25 (8) |
| 20 mM $NH_4Cl$ | 123 ± 20 (4) | 136 ± 8 (1) |
| 10 μM Monensin | *134 ± 15 (15) | *133 ± 16 (6) |
| 1 μM Nigericin | *129 ± 18 (10) | *141 ± 24 (6) |
| 500 μM Chloroquine | *169 ± 26 (14) | *173 ± 37 (4) |
| 10 μM Monensin + 0.2 μM Bafilomycin | 103 ± 2 (1) | 102 ± 1 (1) |
| 1 μM Nigericin + 0.2 μM Bafilomycin | 97 ± 21 (2) | 103 ± 10 (2) |
| 500 μM Chloroquine + 0.2 μM Bafilomycin | 98 ± 12 (4) | 124 ± 9 (4) |
| 1 μM Valinomycin | 97 ± 6 (3) | 106 ± 11 (2) |
| 10 μM FCCP | 84 ± 10 (3) | 82 ± 3 (2) |
| 1 μM Valinomycin + 10 μM FCCP | 88 ± 7 (2) | 115 ± 13 (2) |

[a]The fusion assay was carried out under standard conditions for 10 min at 37°C with PNS containing B-ricin or cointernalized (BTf + BR). Results are means ± SD. Numbers of experiments (each in duplicate) are given in parentheses.
*Indicates significant difference ($P < 0.05$) from no addition.

The results of a number of experiments are summarized in Table I. Nigericin, monensin, and chloroquine significantly increased the rate of fusion. Stimulation by $NH_4Cl$ was marginal and was not pursued. Control incubations in which an ATP-depleting system replaced the ATP-generating system were unchanged by the addition of chloroquine, $NH_4Cl$, monensin, or nigericin (data not shown). In contrast to the other reagents, bafilomycin $A_1$ by itself did not significantly change the rate of fusion. However, bafilomycin $A_1$ reduced or eliminated the stimulation by monensin, nigericin, and chloroquine. Neither 1 μM valinomycin nor 10 μM FCCP, individually or together, stimulated the rate of fusion. In all cases, assays for fusion complexes containing B-ricin or B-transferrin gave very similar results.

## DISCUSSION

During the last several years, ricin has been used by many investigators as a tool for studying receptor-mediated uptake and intracellular trafficking processes in eukaryotic cells [Chazaud et al., 1994; Hudson and Grillo, 1991; Magnusson et al., 1993; Melby et al., 1991; Oda and Wu, 1993; Sandvig and van Deurs, 1994; Wellner et al., 1994; Wellner et al., 1995; Yoshida et al., 1991]. Various agents are known to alter intracellular trafficking of ricin and transferrin and to increase the pH of acidic compartments including endosomes. In this paper, we characterized the effects of such compounds on the fusion of endosomes containing ricin and transferrin, and we propose a mechanism by which some of them stimulate endosome fusion.

Because in vitro fusion of ricin-containing early endosomes had not been described, we first characterized these fusions and compared them to previously reported fusions of endosomes containing transferrin [Wessling-Resnick and Braell, 1990a, 1990b]. The fusion activity of vesicles (PNS fractions) prepared from K562, CHO, or Vero cells incubated with ricin or transferrin for 5 min at 37°C was similar. This result was not necessarily expected as ricin internalization is reported to be relatively slow [van Deurs et al., 1990; Sandvig and van Deurs, 1994]. However, the number of binding sites for ricin (reported to range from 1 to 100 million per cell) [van Deurs et al., 1990] may greatly exceed the number of transferrin receptors ($1.6 \times 10^5$ per K-562 cell) [Klausner et al., 1983]. Vesicles from cells incubated with B-ricin on ice had no fusion activity, and a 5 min internalization at 37°C resulted in vesicles with maximal fusion activity as observed with other markers [Braell, 1987; Gruenberg and Howell, 1989]. When internalization was followed by a chase at 37°C, both the fusion activity and the biotinylated ricin content of the resulting PNS fractions rapidly declined. At the acidic pH of the endosome (pH 5.5 in K562 cells) [Sipe et al., 1991], ricin remains largely bound to galactose-terminated glycoproteins and glycolipids that recycle to the plasma membrane during the chase [van Deurs et al., 1988; Magnusson et al., 1993]. Previous reports have shown that fusion activity declines as markers are chased into more distal compartments of the endosomal pathway [Braell, 1987; Wessling-Resnick and Braell, 1990a; Gruenberg et al., 1989].

The fusion properties of vesicles containing B-ricin were identical to those of vesicles containing B-transferrin by several criteria. In both cases, fusion depended on time, temperature, and ATP, and vesicles excluded from Sepharose 6B required addition of cytosol for fusion activity and were similarly inhibited by NEM and

GTP-γ-S. Similar requirements for fusion of vesicles containing transferrin have been reported previously [Woodward and Warren, 1988; Wessling-Resnick and Braell, 1990b; Rodriguez et al., 1994]. The results are consistent with delivery of both ricin and transferrin to the same early endosomal compartment.

Bafilomycin $A_1$ caused a significant increase of the extent of fusion (plateau reached after 60 min incubation) but only in vesicles loaded with B-ricin; this increase was not observed if the vesicles were loaded with B-transferrin alone or with both B-ricin and B-transferrin. We note that at pH 5.5, transferrin releases ferric ions, which are then translocated from the endosome to the cytoplasm. The translocation of iron may involve the endosomal, vacuolar $H^+$-ATPase, possibly altering its proton transport activity [Li et al., 1994] and perhaps also its interaction with bafilomycin $A_1$.

The cytotoxicity of ricin is enhanced by bafilomycin $A_1$ [Yoshida et al., 1991], ammonium chloride [Sandvig and Olsnes, 1982; Esworthy and Neville, 1984; Ghosh and Wu, 1988; Chazaud et al., 1994], and chloroquine [Thompson et al., 1995] at concentrations similar to those used in this study. Cells are also sensitized to ricin by monensin and nigericin [Sandvig and Olsnes, 1982; Ghosh and Wu, 1988; Yoshida et al., 1991; Wellner et al., 1994] at concentrations ($<100$ nM) that alter the Golgi region without changing endosomal pH. Because of their ability to raise the pH of endosomes [Mellman et al., 1986], these reagents have been used to establish that a pH gradient across the endosomal membrane is not required for fusion of early endosomes since neither chloroquine, $NH_4Cl$ (alone or with methylamine), monensin, nigericin, nor bafilomycin $A_1$ inhibit fusion in vitro [Braell, 1987; Diaz et al., 1988; Beaumelle and Hopkins, 1989; Beaumelle et al., 1993; Wessling-Resnick and Braell, 1990a,b; Clague et al., 1994]. Several recent studies suggest that an acidic lumenal pH may be required for sorting of certain proteins from early endosomes to late endosomes, to the plasma membrane, or to the TGN [Clague et al., 1994; Stein and Sussman, 1986; Felder et al., 1990; Chapman and Munro, 1994]. Other reports differ or suggest more complex interpretations [Melby et al., 1991; van Weert et al., 1995].

We demonstrated that monensin, nigericin, and chloroquine significantly increased the rate of fusion. Stimulation occurred only during the early, linear portion of the fusion reaction, however, which may explain why it was not reported in previous studies. Moreover, stimulation by monensin, nigericin, or chloroquine occurred only when the vacuolar $H^+$-ATPase was active as stimulation was reduced or eliminated by bafilomycin $A_1$. Bafilomycin $A_1$ and FCCP failed to increase the rate of fusion. Bafilomycin $A_1$ and FCCP, as well as $NH_4Cl$, monensin, and nigericin all increased the pH of early endosomes containing FITC-ricin, as expected. Thus, stimulation of the fusion rate did not correlate with changes of endosomal pH. Rather, stimulation occurred with reagents that promoted accumulation of cations within the vesicles, presumably accompanied by permeable counterions, probably chloride [Lukacs et al., 1992].

Nigericin and monensin mediate an electroneutral, 1 for 1 exchange of intravesicular protons for $K^+$ in the medium. Nigericin is selective for $K^+$, but monensin will exchange either $Na^+$ or $K^+$ with a ten-fold preference for $Na^+$ [Mellman et al., 1986; Mollenhauer et al., 1990]. Chloroquine and $NH_4Cl$ increase the pH of acidic compartments by equilibration of the uncharged amine into the vesicle, where it is trapped by protonation [Mellman et al., 1986]. The extent to which base accumulates depends on many factors, including the pH of the vesicle, the pK of the base, and on the permeability of the protonated form of the base [Ohkuma and Poole, 1981]. Compared to the doubly charged chloroquine cation, the ammonium ion is likely to be much more permeable, limiting its accumulation [Maxfield and Yamashiro, 1991; Okuma and Poole, 1981]. This may account for the low stimulation observed with $NH_4Cl$. Thus, both the carboxylic ionophores and some weak bases can cause significant accumulation of cations dependent upon influx of counter ions and driven by the activity of the vacuolar $H^+$-ATPase.

By contrast, bafilomycin $A_1$ and FCCP increase the pH of acidic vesicles without accumulation of cations. Bafilomycin $A_1$ inhibits the vacuolar $H^+$-ATPase, preventing acidification [Bowman et al., 1988; Yoshimori et al., 1991]. The lumenal pH of already acidic vesicles increases when the proton pump is blocked by addition of bafilomycin $A_1$ due to a finite permeability of the vesicle membrane to protons [Mellman et al., 1986]. The protonophore FCCP increases the rate of proton equilibration across the vesicle membrane such that even a fully

active vacuolar $H^+$-ATPase cannot maintain a pH gradient. Thus, adding FCCP or bafilomycin $A_1$ is not expected to cause ions to accumulate within the vesicle. Furthermore, bafilomycin $A_1$ would block the ability of monensin, nigericin, and chloroquine to promote uptake of ions.

The effects of these reagents are summarized in the diagram shown in Figure 10. We propose that monensin, nigericin, and chloroquine stimulate fusion by causing an accumulation of ions within the vesicle that would drive an influx of water. The fluid uptake would result in vesicle swelling and distortion of the membrane, altering its fusion properties. This interpretation builds upon models proposed previously to explain the effects of monensin on chromaffin granules [Geisow and Burgoyne, 1982] and Golgi [Mollenhauer et al., 1990] and accumulation of weak bases in lysosomes [Ohkuma and Poole, 1981; Mellman et al., 1986].

Due to the short internalization time, B-ricin in our preparations should be restricted to the earliest endocytic compartments, coated and uncoated vesicles and early endosomes. One interpretation consistent with our results is that monensin, nigericin, and chloroquine stimulate homotypic fusion between early endosomes. Alternatively, as these reagents alter all acidic compartments, they may promote fusion of the early endosomes containing the complementary probes to a common, third compartment present in the heterogeneous PNS fraction used for this study. It will be interesting to examine the effects of these reagents using purified early endosomes.

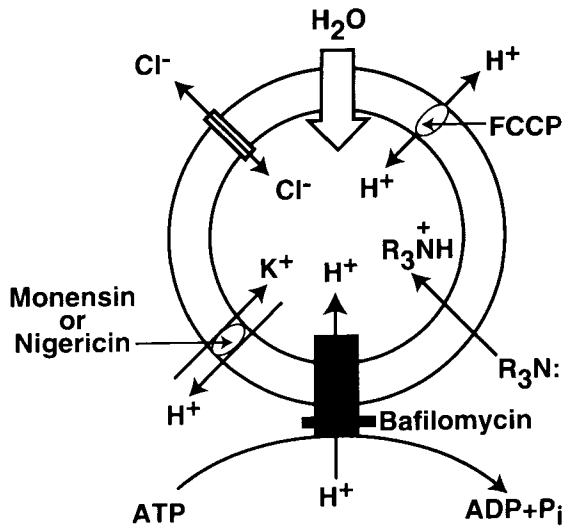


**Fig. 10.** Changes of vesicle ion content caused by ionophores, weak bases, and bafilomycin $A_1$. Vacuolar $H^+$-ATPase catalyzes accumulation of protons (normal conditions) or $K^+$ ion (in the presence of monensin or nigericin) or protonated amine (in the presence of weak base). Cation accumulation is accompanied by influx of anions (to maintain electroneutrality) and water, causing the vesicles to swell. Bafilomycin inhibits the vacuolar $H^+$-ATPase, and FCCP in effect reverses its activity by making the vesicle freely permeable to protons.

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