The use of permeabilized cells to study the ion requirements of receptor–ligand dissociation in endosomes

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The mannose receptor mediates the transport of high-mannose glycoproteins from the cell surface to lysosomes in macrophages. The binding of ligand to the receptor is dependent on both pH and Ca^{2+} . Upon internalization, ligands enter an acidic pre-lysosomal compartment where receptor–ligand dissociation takes place. Acidification is driven by an endosomal proton pump and anion transport is coupled to this acidification step. A permeabilized-cell assay has been designed to characterize the ionic requirements for receptor–ligand dissociation in endosomes. The plasma membrane of macrophages has been permeabilized selectively with digitonin without affecting endosomal membranes. Receptor–ligand dissociation in permeabilized cells required ATP and was blocked by proton ionophores. Di-isothiocyanostilbene-disulphonic acid and *N*-ethylmaleimide also blocked dissociation, but mitochondrial ATPase inhibitors and vanadate were ineffective. To explore the nature of the anion requirement for acidification, the ability of different anions to compensate for Cl^- was tested. For the halide series, Br^- was as equally effective as Cl^- in supporting receptor–ligand dissociation, but I^- was inhibitory. Citrate and gluconate were only partially effective, while SO_4^{2-} , NO_3^- and PO_4^{2-} blocked dissociation. Addition of Ca^{2+} to permeabilized-cell preparations impaired ATP-dependent dissociation without affecting endosome acidification. These results suggest that the endosomal membrane has a Ca^{2+} conductance that would permit the rapid efflux of Ca^{2+} from endosomes during acidification, and this would appear to be a necessary step for efficient sorting of Ca^{2+} -dependent receptors from their ligands.

INTRODUCTION

A growing list of protein ligands are known to enter cells by receptor-mediated endocytosis [1,2]. Receptors bind their respective ligands at the cell surface and, after clustering in coated pits, are internalized via coated vesicles. Receptor–ligand complexes are subsequently transferred to endosomes, a morphologically diverse vesicular structure which is thought to play a major role in sorting and targeting of internalized cellular proteins. Many ligands are, at this stage, targeted on to lysosomes to be degraded. The observation that many receptors return rapidly to the cell surface while their ligands are directed to secondary lysosomes has suggested that receptor–ligand dissociation and sorting take place soon after internalization [3].

For many receptor–ligand interactions, high-affinity binding requires a neutral pH and the presence of divalent cations. Lowering of pH or removal of divalent cations results in rapid receptor–ligand dissociation. Endosomes can actively lower their luminal pH by means of electrogenic ATP-dependent proton pumps [4,5]. This process has been shown to accelerate intravesicular receptor–ligand dissociation allowing the sorting of receptors and ligands [3,6]. Apart from proton transport, it has not been determined whether or how other ion fluxes across these membranes play a role in the dissociation step, although recent evidence suggests that internalized sodium pumps and Cl^- fluxes may play a role in modulating pH gradients [6,7]. Anions appear to

facilitate ATP-dependent acidification of endosomes by moving into the vesicle as a charge-compensating molecule.

Macrophages express a cell surface pinocytosis receptor that binds and internalizes mannose glycoproteins [8]. The binding of mannose-terminating glycoproteins to the macrophage mannose receptor requires Ca^{2+} and a neutral pH [9]. Mannose-receptor–ligand complexes are internalized to an endosomal compartment where sorting of receptor and ligand takes place. Receptors are recycled back to the plasma membrane for additional cycles of ligand internalization [10]. The ligand is delivered to lysosomes for storage or degradation.

In this study digitonin-permeabilized cells have been used to follow ATP-driven receptor–ligand dissociation within endosomes. Digitonin forms pore-like complexes when it binds to membrane cholesterol [11]. The plasma membrane contains a relatively high concentration of cholesterol. Cholesterol has also been shown to be present in the endosomal membrane of several cell lines [12–14]. In rabbit alveolar macrophages, however, the plasma membrane can be selectively permeabilized by low concentrations of digitonin without affecting the integrity of intracellular membranes. This allows the cytosolic content of these cells to be manipulated during experiments but leaves the endosomal membrane functionally intact.

With this permeabilized-cell assay the effect of anionic substitution on proton pump-dependent receptor–ligand dissociation in endosomes has been further characterized.

Abbreviations used: DIDS, di-isothiocyanostilbenedisulphonic acid; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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In addition, the effect of different Ca^{2+} concentrations on the pH dependence of receptor–ligand dissociation in endosomes has been investigated. Results from these studies define the anion requirement for acidification and suggest that Ca^{2+} efflux from the endosome may also occur during acidification. Ca^{2+} efflux would appear to greatly accelerate receptor–ligand dissociation.

EXPERIMENTAL

Materials and cells

Digitonin, Na_2ATP , ADP, AMP, Nigericin, di-isothiocyanostilbenedisulphonic acid (DIDS), *N*-ethylmaleimide (NEM), oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), sodium azide, Percoll, and the potassium salts of the anions tested were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). K_2SO_4 was obtained from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A. Sodium vanadate, EDTA disodium salt and CaCl_2 were from Fisher Scientific, Pittsburgh, PA, U.S.A. Chloramine-T was obtained from Eastman Kodak (Rochester, NY, U.S.A.).

Mannosylated bovine serum albumin (mannose–BSA) was prepared by the method of Lee *et al.* [15]. β -Glucuronidase was isolated from rat preputial glands using the method of Keller & Touster [16]. Ligands were radioiodinated as described previously using chloramine-T [17]. Binding and uptake of these ligands were performed using rabbit alveolar macrophages suspended in Hanks' balanced salt solution buffered with 10 mM-Hepes and 10 mM-Tes and supplemented with 1% BSA. Fluorescein dextran was a generous gift from Dr. Paul Schlesinger (Washington University, St. Louis, MO, U.S.A.).

Fluorescent measurements were made on a Perkin–Elmer spectrofluorimeter MPF43 equipped with a temperature-regulated cell holder.

Rabbit alveolar macrophages were induced and harvested by pulmonary lavage as previously described [18].

Permeabilization of cells

Cells ($10^7/\text{ml}$) were suspended in dissociation buffer (20 mM-Hepes / 5 mM- NaCl / 150 mM- KCl , pH 7.4) to which different concentrations of digitonin were added. Incubations were carried out for at least 30 min at 4 °C. Cells could be stored for several hours on ice before use.

ATP-dependent dissociation of receptor–ligand complexes

Rabbit alveolar macrophages ($10^7/\text{ml}$) were allowed to bind ^{125}I -mannose–BSA (5 $\mu\text{g}/\text{ml}$; 5×10^6 c.p.m./ μg) at 4 °C for 90 min. After washing, the cells were warmed to 37 °C for 5 min and then immediately cooled by dilution in ice-cold uptake buffer. After several washes, cells were treated with digitonin as described above. The assay for receptor–ligand dissociation and precipitation was carried out as described by Wileman *et al.* [6]. Briefly, a standard assay was carried out as follows: 50 μl of cells were added to 150 μl of dissociation buffer with ATP; addition of ATP was supplemented with equimolar amounts of MgCl_2 when dissociation buffer was used or magnesium acetate when another anion substituted for Cl^- . Incubations were performed at 37 °C for 5 min. After 5 min, 200 μl of 0.8% Triton X-100 in assay buffer (25 mM-Hepes, pH 7.4 / 1 M- NaCl / 0.6%

BSA / 50 mM- CaCl_2 / 100 μg of mannose–BSA / ml) were added, and the samples were kept on ice for 10 min. Samples were ammonium sulphate-precipitated by adding 200 μl of 0.5% γ -globulin in assay buffer followed by 400 μl of saturated ammonium sulphate. Precipitated radioactivity was collected on GF/C glass fibre filters, and the filters were washed twice with 40% ammonium sulphate before they were counted in a γ -counter. Two other conditions were always run concomitantly with each assay. Incubation of cells at 37 °C in the absence of ATP provided the total value for receptor–ligand complexes before dissociation. Incubation of cells at 37 °C with dissociation buffer (25 mM-Mes, pH 5.5 / 1.0 M- NaCl / 0.6% BSA / 15 mM-EDTA / 0.4% Triton X-100) provided the 100% dissociation value.

Buffers used for anionic substitution experiments were the following: KCl (155 mM), KBr (155 mM), KI (155 mM), potassium gluconate (155 mM), potassium citrate (78 mM), KNO_3 (175 mM), KH_2PO_4 (162 mM), and K_2SO_4 (125 mM); all buffered with 20 mM-Hepes to pH 7.4. When receptor–ligand dissociation assays were performed in one of the above buffers, macrophages were treated with digitonin in the same buffer. Buffers did not affect digitonin treatments.

Formaldehyde fixation of macrophages

Cells were treated as described [10]. Cells ($10^7/\text{ml}$) were incubated in 1% formaldehyde in 150 mM- NaCl / 30 mM- NaPO_4 (pH 7.0) for 30 min at 4 °C. They were then washed three times with buffered saline by centrifugation for 5 min at 900 *g* and resuspended in fresh medium.

Fluorescein dextran uptake and subcellular fractionation of macrophages

Rabbit alveolar macrophages ($2 \times 10^7/\text{ml}$) were incubated with fluorescein dextran (20 mg/ml) for 20 min at 37 °C in buffered Hanks' balanced salt solution with 1% BSA. Cells were then washed extensively and resuspended ($10^7/\text{ml}$) in homogenization media (250 mM-sucrose / 3 mM-imidazole, pH 7.4). Cell disruption was carried out using nitrogen cavitation (1725 kPa, 15 min). A post-nuclear supernatant was obtained which was subjected to centrifugation (40000 *g*, 15 min, Beckman JA-17 rotor) to obtain a particulate fraction. Vesicles from 10^8 cells were resuspended in 1 ml of homogenization buffer and layered over 35 ml of 1.07 g/ml self-forming Percoll gradients. This density separates secondary lysosomes from other membranes for rabbit alveolar macrophages [6]. Gradients were generated by centrifugation at 40000 *g* for 15 min at 4 °C (Beckman JA-17 rotor). Light fractions were pooled, diluted in dissociation buffer and kept at 4 °C before spectrofluorimetric measurements.

RESULTS

Permeabilization of alveolar macrophages

Incubation of rabbit alveolar macrophages at 4 °C with low concentrations of digitonin resulted in selective permeabilization of the plasma membrane. Digitonin (> 0.008% w/v) allowed > 96% of the macrophages to take up Trypan Blue dye and caused the release of 75% of the cellular protein including 80% of the cytoplasmic marker lactate dehydrogenase (Fig. 1). In contrast, this concentration of detergent released only a small proportion of both ^{125}I - β -glucuronidase that was previously

Fig. 1. Permeabilization of rabbit alveolar macrophages by digitonin

After a 5 min uptake of ¹²⁵I-β-glucuronidase followed by a 5 min chase to label endosomes, rabbit alveolar macrophages (10⁷ cells/ml) were incubated with different concentrations of digitonin in dissociation buffer at 4 °C for 30 min. For each concentration, total protein, ¹²⁵I-β-glucuronidase (¹²⁵I-β-Gluc) radioactivity, lactate dehydrogenase (LDH) and β-hexosaminidase (β-Hex) activity of the supernatant were measured after cells were pelleted by centrifugation (500 g, 5 min) and compared to total values for Triton-solubilized cells, taken as 100 %. Results are the means of at least two experiments.

internalized for 5 min to load the endosomal compartment and the endogenous lysosomal marker, β-hexosaminidase. Incubations with higher concentrations of digitonin caused the release of all the endosomal and lysosomal markers. Macrophages treated with digitonin remain visually intact even after overnight incubation at 4 °C. Homogenization and subsequent subcellular fractionation of freshly permeabilized cells on Percoll gradients showed that the buoyant densities of endosomes and lysosomes, and the concentrations of endocytic markers or marker enzymes, were unaffected by digitonin (results not shown). These results indicate that 0.008 % digitonin permeabilizes the macrophage plasma membrane selectively and leaves the limiting membrane of endosomes and lysosomes intact.

ATP-dependent receptor–ligand dissociation

A previous report from this laboratory describes a solubilization precipitation assay that can distinguish between receptor-bound ligand (mannose–BSA) and free ligand [6]; this assay takes advantage of the fact that mannose–BSA–receptor complexes can be precipitated with much lower concentrations of ammonium sulphate than free mannose–BSA. We used this assay to follow receptor–ligand dissociation in semi-intact cells. To verify that ATP could enter the cytosol of permeabilized macrophages and show that the endosomal membrane remained functionally intact, the ATP dependence of receptor–ligand dissociation in endosomes was determined. Cells that contained ¹²⁵I-mannose–BSA in

Fig. 2. Nucleotide dependence of receptor–ligand dissociation

¹²⁵I-Mannose–BSA was bound to the surface of rabbit alveolar macrophages at 4 °C. The cells were then washed and warmed to 37 °C for 5 min. Uptake was stopped by cooling the cells to 4 °C. Cells were washed again and incubated with 0.008 % (w/v) digitonin as described in the Experimental section. Digitonin-treated cells were incubated for 5 min at 37 °C with increasing concentrations of nucleotide and an equimolar concentration of MgCl₂, and subjected to the solubilization and precipitation assay. Values are reported as percentages and are the means of at least three experiments.

endosomes were permeabilized at 4 °C in 0.008 % digitonin in dissociation buffer. Fig. 2 shows that incubation of these cells with increasing concentrations of ATP for 5 min at 37 °C results in a concomitant increase in dissociated ligand. Receptor–ligand dissociation was dependent on the concentration of ATP present and was maximal at 5 mM. Receptor–ligand dissociation could not be driven by ADP or AMP. The incubation was restricted to 5 min to prevent any substantial degradation of mannose–BSA by endosomal proteases. Mannose–BSA is a good ligand for these proteases in alveolar macrophages [19]. Fig. 3 shows the effects of increasing concentrations of nigericin and CCCP on dissociation induced by 5 mM-ATP. Both compounds are proton ionophores that dissipate hydrogen-ion gradients across membranes. Previous work has shown that acidification of endosomes is inhibited by these compounds [6,20]. Receptor–ligand dissociation in permeabilized cells was also inhibited by these drugs. Inhibition was dose-dependent and maximal at 10 μM for both nigericin and CCCP.

Table 1 shows the effects of several ATPase inhibitors. A mitochondrial F₁, F₀-ATPase inhibitor, oligomycin, was ineffective at preventing receptor–ligand dissociation, as were sodium vanadate and sodium azide. Fig. 3 shows the dose-response to *N*-ethylmaleimide, an inhibitor of vacuolar proton pump ATPase [21], and DIDS, reported to inhibit both the proton pump and Cl[−] transport [22]. These compounds also inhibited endosomal acidification. The maximal response to these agents (10 mM for NEM, 1 mM for DIDS) required much higher concentrations than reported earlier [5,6]. The protein content of our preparations is high when compared with that of the subcellular fractions used for those studies. This probably explains the discrepancy. Both drugs irreversibly modify functional groups on proteins and excess concentrations of each may be necessary to

Fig. 3. Inhibition of ATP-dependent receptor-ligand dissociation

Cells containing receptor-ligand complexes were prepared as described in the legend to Fig. 1 and warmed to 37 °C for 5 min in the presence of 5 mM-ATP and increasing concentrations of the indicated inhibitor. Cells were then subjected to the solubilization and precipitation assay to determine the extent of ligand dissociation.

Table 1. Inhibition of ATP-dependent receptor-ligand dissociation

¹²⁵I-Mannose-BSA (5 μg/ml) was incubated with rabbit alveolar macrophages at 4 °C for 90 min. The cells were washed twice in ice-cold Hanks' balanced salt solution containing 1 % BSA (HBSA) and then warmed to 37 °C for 5 min in the same buffer. Uptake was stopped by rapidly cooling the cells to 4 °C. After washing the cells twice with HBSA, they were incubated with 0.008 % (w/v) digitonin in dissociation buffer as described in the Experimental section. Permeabilized cells were incubated with 5 mM-ATP in dissociation buffer at 37 °C for 5 min in the presence or absence of inhibitor. The cells were then assayed for receptor-ligand complexes by solubilization and precipitation. The numbers of experiments are reported in parentheses. Values are the means of these experiments.

Inhibitor	Receptor-ligand dissociation (%)	Inhibition of dissociation (%)
None	66.0 (8)	—
Oligomycin (10 μg/ml)	64.3 (3)	2.6
Azide (0.3 mM)	64.7 (2)	2.0
Vanadate (0.3 mM)	65.4 (3)	< 1.0

overcome the presence of these reactive groups on the additional cellular proteins present in permeabilized cells.

Effect of anionic substitution on receptor-ligand dissociation

To study the specificity of the anion requirement on ATP-dependent receptor-ligand dissociation, the ability of various anions to compensate for Cl⁻ was studied. Care was taken to ensure that all buffers had the same osmolarity (300 ± 10 mosmol/kg). The results are presented

Table 2. Anionic requirement of ATP-dependent receptor-ligand dissociation

Cells were allowed to internalize ¹²⁵I-mannose-BSA as described in Table 1. They were then treated with 0.008 % (w/v) digitonin in each of the listed buffers and incubated with 5 mM-ATP at 37 °C for 5 min. Cells were then assayed for intact receptor-ligand complexes. Values are reported as percentage inhibition of the dissociation obtained with KCl buffer and are the means of at least three experiments. The S.D. is reported for the percentage dissociation obtained in each condition.

Ionic composition	Receptor-ligand dissociation (%)	Inhibition of dissociation (%)
KCl	70.5 ± 2.8	0
KBr	72.3 ± 3.0	0
KI	1.8 ± 0.9	97.4
K gluconate	31.0 ± 5.0	56.0
K ₃ citrate	25.9 ± 2.9	63.3
KNO ₃	8.7 ± 2.7	87.7
KH ₂ PO ₄	18.1 ± 4.2	74.3
K ₂ SO ₄	0.0 ± 2.7	100.0

in Table 2. For the halide series, Cl⁻, Br⁻, and I⁻ were studied. F⁻ was not compatible with the buffers used for the assay. Both Br⁻ and Cl⁻ supported receptor-ligand dissociation equally well; in contrast, I⁻ almost completely inhibited dissociation. NO₃⁻, PO₄²⁻ and SO₄²⁻ failed to substitute for Cl⁻ and inhibited receptor-ligand dissociation, with SO₄²⁻ showing the strongest inhibition. Substitution with citrate and gluconate resulted in 63 % and 56 % reductions in receptor-ligand dissociation respectively. None of the anions tested affected the binding affinity of the receptor.

Effect of Ca²⁺ on receptor-ligand dissociation

Binding of ligand to the mannose receptor requires Ca²⁺. To characterize the effect of Ca²⁺ on pH-dependent dissociation of ligand from the mannose receptor, the dissociation of mannose-BSA from the surface of macrophages at 37 °C was first studied. Macrophages were fixed with formaldehyde to prevent endocytosis. Fixed cells were then allowed to bind mannose-BSA at 4 °C (fixation had no effect on receptor affinity as determined by Scatchard plots). The cells were first washed and then warmed by resuspension in buffers of decreasing pH at 37 °C. Fig. 4 depicts the relative degree of dissociation observed for each concentration of Ca²⁺ after a 5 min incubation, the standard time of incubation for the permeabilized-cell assay. When ligand dissociation is carried out at neutral pH, the degree of dissociation is relatively unaffected by Ca²⁺. Ca²⁺ has a pronounced effect on receptor-ligand dissociation at pH 6.0 and pH 6.5, the calculated pH of early endosomes. The inhibitory effect of Ca²⁺ on dissociation was less prominent when incubations were carried out at pH 5.5. These results indicate that at an endosomal pH of approx. 6.0, receptor-ligand dissociation is markedly slowed by the presence of Ca²⁺. Incubation in the presence of EDTA is used as a control for absence of Ca²⁺; however, complete dissociation of ligand from receptor is not observed at most pHs tested. Longer times of

Fig. 4. Effect of Ca^{2+} on pH-dependent receptor–ligand dissociation

Cells were fixed in 1% formaldehyde for 30 min at 4 °C. They were then washed three times with $10\times$ their incubation volume. The cells were allowed to bind ^{125}I -mannose–BSA at 4 °C, washed, and warmed to 37 °C in dissociation buffer adjusted to the indicated pH (pH 5.5–6.5, Mes buffer; pH 7.0, Hepes buffer) for 5 min in the presence of different concentrations of CaCl_2 ; 0.3 mM (□), 1.0 mM (▲) and 3.0 mM (●). EDTA (5 mM) was added to control cells (■). Cells were then subjected to the solubilization and precipitation assay to determine the extent of ligand dissociation.

incubation (60 min) are required to see full dissociation in the presence of EDTA.

The potential importance of the effect of Ca^{2+} on dissociation becomes apparent when one considers that the endocytosis of ligand by macrophages *in vivo* normally takes place in extracellular fluid containing 3 mM- Ca^{2+} , and these ions are presumably present within the lumen of primary endocytic vesicles. Since Ca^{2+} inhibits receptor–ligand dissociation, endocytosis may become more efficient or perhaps regulated if the endosome were able to allow Ca^{2+} to leave the endosome selectively during acidification. There is a high concentration gradient between the endosomal lumen ($[\text{Ca}^{2+}] = 3 \text{ mM}$) and the cytoplasm ($[\text{Ca}^{2+}] = 1\text{--}10 \mu\text{M}$); in addition, there should be a positive luminal membrane potential generated by the pumping of hydrogen ions into the endosome. Thus, the electrochemical gradient would favour passive efflux of Ca^{2+} from the endosome. Experimentally, this efflux might be inhibited by increasing the Ca^{2+} concentration in the cytosol in order to reduce the net Ca^{2+} efflux from the endosome. To test this possibility, two experiments were performed using permeabilized cells. In the first experiment, the effect of increasing cytoplasmic Ca^{2+} concentrations on pH-dependent receptor–ligand dissociation in endosomes was studied. Macrophages containing ^{125}I -mannose–BSA in endosomes were permeabilized with digitonin and

transferred to dissociation buffers but without added ATP. The buffers were adjusted to different pH values ranging from pH 5.5 to 7.0 in the absence or presence of Ca^{2+} . Dissociation was allowed to proceed at 37 °C for 5 min. Fig. 5 shows that receptor–ligand dissociation is accelerated by lowering the pH and that this dissociation, as seen at the cell surface, can be markedly inhibited by the addition of Ca^{2+} to the cytosol. The inhibitory effect of Ca^{2+} was markedly dose-dependent between pH 6.0 and 7.0, and Ca^{2+} concentrations as low as 0.3 mM impaired receptor–ligand dissociation. In a second experiment, the effect of a Ca^{2+} gradient across the endosomal membrane on ATP-driven dissociation was tested. Permeabilized cells containing ^{125}I -mannose–BSA in endosomes were incubated with increasing concentrations of Ca^{2+} and exposed to 10 mM-ATP at pH 7.0 to acidify the endosomal lumen via the ATP-dependent proton pump. Receptor–ligand complexes remaining after 5 min incubation at 37 °C were determined. Fig. 6 shows that the presence of Ca^{2+} outside the endosome markedly inhibits ATP-dependent receptor–ligand dissociation. Inhibition was maximal at 3 mM- Ca^{2+} in the cytosol.

To show that the inhibitory effect of Ca^{2+} on receptor–ligand dissociation was not due to a decrease in endosome acidification, acidification of subcellular fractions containing macrophage endosomes was measured directly. ATP-dependent acidification of these vesicles was followed by measuring the pH-dependent

Fig. 5. Effect of Ca^{2+} on pH-dependent receptor–ligand dissociation in endosomes

Cells containing receptor–ligand complexes prepared as described in the legend to Fig. 1 were incubated in dissociation buffer adjusted to the indicated pH (pH 5.5–6.5, Mes buffer; pH 7.0, Hepes buffer) for 30 min at 4 °C. Before the incubation, CaCl_2 was added at the indicated concentrations: 0.3 mM (□), 1.0 mM (▲) and 3.0 mM (●). EDTA (5 mM) was added to control cells (■). Cells were then assayed for the extent of receptor–ligand dissociation. Ligand leakage was minimal in all instances.

Fig. 6. Effect of Ca^{2+} on ATP-dependent receptor-ligand dissociation in endosomes

Cells containing receptor-ligand complexes prepared as described in the legend to Fig. 1 were incubated in dissociation buffer with 10 mM-ATP at 37 °C for 5 min in the presence of an increasing concentration of Ca^{2+} . Cells were then subjected to the solubilization and precipitation assay to determine the extent of receptor-ligand dissociation.

fluorescence quenching of fluorescein dextran [23]. This ligand is internalized by fluid phase endocytosis, and its uptake is kinetically less efficient because fluid phase ligands preferentially use noncoated pits of the plasma membrane for internalization [24]. The intracellular pathways followed by both receptor-mediated and fluid phase ligands have been shown to share the same intracellular compartments, since ligands that use coated pits as means of internalization co-localize in endosomes with ligands that use noncoated pits [25]. To allow endocytic vesicles, including those containing receptor-ligand complexes, to be loaded with fluorescein dextran, alveolar macrophages were incubated with this ligand for 20 min at 37 °C. The cells were then washed and homogenized. A crude particulate fraction was obtained and fractionated on a 1.07 g/ml Percoll gradient to remove secondary lysosomes. The light fraction from this gradient containing endosomal vesicles was collected and analysed in a spectrofluorimeter for its ability to acidify in response to ATP. Fig. 7 shows the effect of ATP and Ca^{2+} on fluorescein dextran fluorescence. Addition of ATP at 37 °C results in endosome acidification which is detected by the quenching of fluorescein emission (Fig. 7a). When ATP is added in the presence of 3 mM- Ca^{2+} , the same degree of fluorescence quenching is observed (Fig. 7b). Although the presence of Percoll in these measurements made the calibration of pH rather

Fig. 7. Effect of Ca^{2+} and ATP on endosome acidification

A particulate fraction prepared from cells which were allowed to internalize fluorescein dextran (20 mg/ml) for 20 min were fractionated on a 1.07 g/ml Percoll gradient. The light membrane fraction was pooled and diluted in dissociation buffer. Aliquots (2 ml) were taken for fluorescence measurements. The tracings show the uncorrected excitation spectra at the peak of fluorescein dextran emission fluorescence (520 nm) after 10 min incubation at 37 °C with or without 3 mM-ATP in the absence (a) or the presence (b) of 3 mM- Ca^{2+} in a typical experiment.

difficult, the drop of pH is estimated to be between 0.4 and 0.8 units. Addition of Ca^{2+} ions, therefore, had no direct effects on endosome acidification.

DISCUSSION

The mannose receptor has been well characterized for its role in receptor-mediated endocytosis of high-mannose glycoproteins. This macrophage-specific receptor has been recently purified and reported to be a 175 kDa membrane glycoprotein [9]. Ligand binding to the mannose receptor is both Ca^{2+} - and pH-dependent; Ca^{2+} and neutral pH are required for binding while low pH and EGTA favour receptor-ligand dissociation. Receptor-ligand complexes traverse a pre-lysosomal endocytic compartment during the internalization cycle. As a consequence, ligand is transported to secondary lysosomes while the receptor recycles back to the cell surface. Sorting of ligand from the mannose receptor and other recycling receptors is thought to occur in endosomes; however, the steps that lead to compartmental sorting of receptor and ligand are poorly understood. Morphological evidence suggests that the sorting of the asialoglycoprotein receptor from its ligand takes place in CURLs (compartments for the uncoupling of receptor and ligand) [26]. These are specialized endosomes with arm-like extensions from which receptors are thought to pinch off in small vesicles while ligands remain in the lumen of the organelle. For receptor-ligand sorting to take place by this mechanism, dissociation of ligand from the receptor has to occur. Dissociation is known to occur in an acidic environment and endosomes maintain an acidic lumen by means of a proton pump ATPase [5]. Amines and proton ionophores dissipate pH gradients across endosomal membranes and block the transfer of

ligand from endosomes to lysosomes [17,27]. In previous studies, endosomal fractions containing receptor–ligand complexes have been used to show that receptor–ligand dissociation required ATP as an energy source [3,6].

In this study, a permeabilized-cell assay has been designed to analyse receptor–ligand dissociation within endosomes. In particular, the role of anion and Ca^{2+} transport during receptor–ligand dissociation has been explored. For this purpose, we have used digitonin to selectively permeabilize the plasma membrane so as to gain access to the cytoplasmic side of endosomes. Digitonin permeabilization appears to be innocuous to a variety of cellular processes that have been studied, e.g. exocytosis of chromaffin granules [11,28] and vesicle motility [29]. Weigel & Oka have used digitonin for the quantification of ligand binding to internal pools of asialoglycoprotein receptors in isolated rat hepatocytes [30]. In this study, high concentrations of digitonin (0.055%) permeabilize both plasma membrane and endosomal membranes. Although endosomal membranes have been shown to contain cholesterol when analysed in whole cells [12] or in isolation [13,14], cells exposed to low levels of digitonin exhibit a selective permeabilization of the plasma membrane. Yashamiro *et al.* used low concentrations of digitonin to permeabilize the plasma membrane of mouse fibroblasts without affecting the integrity of endosomes in their studies of endosome acidification using fluorescent probes [20]. In the present study, the concentration of digitonin required for plasma membrane permeabilization was very sensitive to the concentration of cells (result not shown). An increase in cell concentration required a parallel increase in digitonin concentration. This probably indicates that the surface of membrane exposed to digitonin determines the extent of permeabilization obtained. Cholesterol molecules on the plasma membrane of whole cells may bind sufficient digitonin to cause a drop in the concentration of free digitonin molecules in solution prior to their access to the endosomal membranes. This would lead to a differential permeabilization of these two types of membranes even though both may contain comparable levels of cholesterol.

The permeabilized-cell assay confirmed that receptor–ligand dissociation required ATP and was inhibited by proton ionophores (i.e. nigericin and CCCP). ATP-dependent dissociation was not affected by oligomycin or sodium vanadate (inhibitors of mitochondrial F_1F_0 -ATPase and plasma membrane Na^+K^+ ATPase respectively) but was inhibited by NEM. NEM has been shown by others to be a selective inhibitor of vacuolar proton pump ATPases [5,20,31]. We conclude that this permeabilized-cell assay makes possible the study of the effects of cytosolic content of the function of endosomes.

Endosome acidification requires the presence of Cl^- [5,6,31]. It is thought that Cl^- transport into the endosome is coupled to endosome acidification. Acidification did not require an anion conductance when valinomycin was present in the media (results not shown). Presumably this K^+ ionophore allows the K^+ efflux to compensate for Cl^- influx suggesting that the endosomal proton pump, as well as those characterized in coated vesicles [22], multivesicular bodies [31] and lysosomes [32], is electrogenic. Recent studies using proton pumps reconstituted within liposomes have shown that the membrane potential generated by proton transport

inhibits the ATPase, thereby inhibiting endosome acidification [33–35]. Anion transport would collapse such a membrane potential and increase the efficiency of acidification. A channel may fulfil this anion conductance. DIDS was tested for its ability to specifically inhibit this putative anion channel. Although we have shown that DIDS inhibited receptor–ligand dissociation, its effect could not be reversed with valinomycin, suggesting that DIDS inhibited the proton pump directly (results not shown).

In this study we analyse the ability of a series of anions to substitute for Cl^- during endosomal acidification. For the halide series, both Br^- and Cl^- were equally effective in the dissociation assay. I^- , on the other hand, did not substitute for Cl^- . The presence of I^- and Cl^- together did not affect dissociation (results not shown). These results suggest that halide ion size is likely to provide the selectivity of anion conductance. Other anions showed a varying ability to compensate for Cl^- . For example, replacing Cl^- with gluconate or citrate only slowed receptor–ligand dissociation. These anions are presumably able to pass to some degree into the endosome, indicative, perhaps, of their partial membrane permeability across endosomal membranes. In crude homogenates containing endosomes that were never exposed to detergent the same partial inhibition of receptor–ligand dissociation has been observed in the presence of gluconate ions [6]. PO_4^{2-} , SO_4^{2-} and NO_3^- prevented dissociation. These anions are either unable to enter the anion channel or, alternatively, may affect the proton pump ATPase directly. NO_3^- is thought to be membrane permeable [31]; nevertheless, it still inhibited ATP-dependent receptor–ligand dissociation. NO_3^- may either (1) increase the permeability of endosomal membrane to protons which in turn would decrease acidification, or (2) inhibit proton pump activity directly. Van Dyke [36] has shown that NO_3^- inhibits acidification of hepatocyte multivesicular bodies by a direct inhibition of the proton pump. Moriyama & Nelson [37] have shown that SO_4^{2-} and NO_3^- inhibited, and Cl^- stimulated, both ATPase activity and proton pumping in purified chromaffin granule proton pump complex. Recently, Van Dyke [38] observed that Cl^- affected both the rate of acidification and the membrane potential of rat liver multivesicular bodies but could not conclude that Cl^- affected proton pump turnover. Unfortunately, the presence of both proton pump and anion channels in our vesicles and the present inability to specifically inhibit the anion conductance made the study of anion effects on the proton pump as assessed by the receptor–ligand dissociation assay difficult. Thus, anions may play a dual role in the regulation of endosome acidification by modulating pumping activity and serving as charge-compensating ions during the acidification step.

Several years ago, Steinman and co-workers calculated that macrophages internalize a volume of fluid by pinocytosis equivalent to a third of their total volume every hour [39]. Subsequently, Besterman *et al.* [40] showed that as much as 50% of pinocytosed fluid internalized during a 5 min incubation was quickly released to the media by an exocytic process. Nevertheless, a considerable amount of fluid is internalized constitutively by endocytosis. Thus, the ionic composition of the early endocytic compartment may resemble that of the extracellular compartment if it is not regulated by the cell. The present studies have shown

that the pH-dependent dissociation of ligand from receptor is inhibited by Ca^{2+} . Receptor–ligand dissociation at pH 6.0 was quite substantial when ligand was bound either to receptors in the plasma membrane (~80%) or to receptors in endosomes (~65%), whereas dissociation in the presence of 3 mM- Ca^{2+} was inhibited to less than 10% in both instances. Endosomal concentrations of Ca^{2+} may be as high as those present in the extracellular medium (3 mM). Presence of Ca^{2+} in the endosome may, therefore, retard the dissociation step. A calcium channel or transporter in endosomes could facilitate the extrusion of Ca^{2+} ions into the cytosol down their electrochemical gradient. Experimentally, one might reason that if Ca^{2+} transport were mediated by a channel or a protein carrier, an increase in Ca^{2+} concentration in the cytosol would inhibit Ca^{2+} transport out of the endosome and, consequently, would lead to inhibition of receptor–ligand dissociation. When receptor–ligand dissociation was evaluated in the presence of increasing concentrations of Ca^{2+} in the cytosol, a concomitant decrease in dissociation was observed. Direct measurements of endosomal pH showed that the decrease in ATP-dependent receptor–ligand dissociation by extravesicular Ca^{2+} was not due to inhibition of endosome acidification. These results, when taken together, suggest that the endosomal membrane has a Ca^{2+} conductance.

The endosomal membrane appears to contain different proteins that function together to regulate the ionic composition of the endosome. A nucleotide-dependent proton pump in conjunction with an anion channel acidify the endosome which leads to the dissociation of ligands from a variety of receptors that tranverse this compartment (e.g. mannose, mannose 6-phosphate, and asialoglycoprotein receptors). A calcium channel may allow Ca^{2+} to leave the endosome, providing a means of reducing its concentration in endocytic compartments. There may be, in addition, other proteins with unknown regulatory functions. Both proton pumps and channels may be either permanent residents of endosomes or, like endocytic receptors, present only transiently *en route* to other compartments.

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