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# Two threshold values of low pH block endocytosis at different stages

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**The influence of low extracellular pH on endocytosis was studied in baby hamster kidney cells. When the extracellular medium was adjusted to pH 5.7, the intracellular pH decreased within 2 min to pH 6.2 and the endocytosis of horseradish peroxidase (HRP) in the fluid phase dropped to an undetectable level. With an external pH of 6.3, the internal pH dropped to pH 6.8 and HRP was internalized at a normal rate for 5 min but accumulation during longer incubation times did not occur. Morphologically, HRP was visualized in the lumen of a sub-population of tubular and vesicular endosomes. These observations were confirmed by subcellular fractionation studies using free flow electrophoresis. Low extracellular pH also had an effect on the endocytosis of the membrane-spanning glycoprotein G of vesicular stomatitis virus which was implanted into the plasma membrane. The internalization of G-protein was quantitated by a surface fluoroimmunossay. The endocytosis of G-protein was not affected when the external pH was dropped to 6.3, but was reduced at an external pH of 5.7. The intracellular ATP was not depleted and the reduction of endocytosis was reversible upon return to physiological pH. Clathrin coated pits were detected by electron microscopy at the plasma membrane of the low-pH-treated cells. We conclude that different values of low external pH can be used to functionally dissect the endocytic pathway into at least two distinct subcompartments: (i) a small and saturable compartment which is accessible from the cell surface in <5 min and from which G-proteins and fluid phase are recycled to the cell surface; and (ii) a larger compartment which allows the net accumulation of solutes that have entered the cell for >5 min.**

*Key words:* coated pits/clathrin/fluid phase endocytosis/intracellular pH/VSV G-protein

## Introduction

Endocytosis is the process by which membrane proteins, lipids and solutes are internalized into cells. Receptors and their ligands collect into clathrin-coated pits which pinch off from the plasma membrane forming coated vesicles (Anderson and Kaplan, 1983; Steinmann *et al.*, 1983; Goldstein *et al.*, 1985; Wileman *et al.*, 1985). After the loss of their coat they fuse with tubular and vesicular endosomes (Geuze *et al.*, 1984; Marsh *et al.*, 1986). From this early endosome some receptors recycle back to the cell surface and others are routed to the lysosomes for degradation via later elements of the endocytic pathway. Little is known about the structural and functional distinction of the endosomal sub-compartments involved. One approach to investigating the endocytic pathway is to define experimental conditions that interfere with the rate of internalization, recycling and routing to degradation. The most commonly used perturbant is the reduction of temperature. Between 17 and 20°C endocytosis still proceeds at a slower rate but transfer of material to the lysosomes is blocked (Dunn *et al.*, 1980; Helenius *et al.*, 1983; Wolkoff *et al.*, 1984). This temperature arrest has been used to distinguish functionally the endosomal compartment from the lysosomes. An additional perturbant is the depletion of intracellular $K^+$ which prevents the internalization of low-density lipoprotein, epidermal growth factor and fluid phase markers into human fibroblasts, and inhibits the assembly of clathrin into newly formed coated pits (Larkin *et al.*, 1983, 1985, 1986). Low $K^+$ has also been reported to block the endocytosis of transferrin (Moya *et al.*, 1985), and human growth hormone (Ilondo *et al.*, 1986). The metabolic energy requirements for endocytosis have been tested. Low levels of cellular ATP are reported to have no effect on one cycle of asialoglycoprotein receptor internalization but to block subsequent rounds of recycling (Clarke and Weigel, 1985). Low ATP also reduced endocytosis of fluid phase markers (Sullivan *et al.*, 1987).

We investigated the effect of extracellular acidic pH on endocytosis. Endosomes are known to quickly acidify their lumen Tycko and Maxfield, 1982; Marsh *et al.*, 1983; Murphy *et al.*, 1984; Kielian *et al.*, 1986) which is topologically equivalent to the extracellular medium. The luminal pH decreases from pH 6.5 in early endosomes to pH 5.5 in later elements of the pathway, reaching a value of pH 5 or below in lysosomes (Kielian *et al.*, 1986; Roederer *et al.*, 1987). This acidification produced by a vacuolar ATPase (reviewed in Mellman *et al.*, 1986) represents an important property of the endosomal compartment responsible for the uncoupling of many receptor–ligand interactions and for the activation of hydrolases. In the present study we have investigated the effect of low extracellular pH on the endocytosis both of fluid phase and of a membrane glycoprotein, the G-protein of vesicular somatitis virus (VSV), which was implanted into the plasma membrane (Gruenberg and Howell 1986, 1987a). It was found that lowering the extracellular pH below 5.7, rapidly and reversibly blocks internalization in baby hamster kidney (BHK) cells. Intermediate values of low external pH around 6.3, allow endocytosis to proceed to an early endosomal sub-compartment from which recycling to the cell surface occurs, but do not allow transport of solutes towards a later compartment where accumulation occurs.

## Results

### *Low external pH inhibits fluid phase endocytosis*

To monitor the effect of low external pH on internalization, we first used horseradish peroxidase (HRP), a general marker of fluid phase endocytosis. Internalization of HRP was initiated by incubating the cells at 37°C either at physiological pH 7.4, or at the acidic pHs of 6.3 and 5.7 in the presence of 1 mg/ml HRP. The incubation was stopped at the desired time by returning the cells to ice-temperature. At pH 7.4, the kinetics of HRP uptake was typically non-linear (Figure 1A) because part of the endosomal content continuously recycles back to the extracellular

medium (Besterman *et al.*, 1981; Adam *et al.*, 1982; Swanson *et al.*, 1985). At pH 5.7, HRP uptake was dramatically reduced. Several buffer systems (20 mM succinate, 20 mM MES or 20 mM pyrophosphate) were used to achieve a pH of 5.7 and all produced the same extreme reduction of fluid phase uptake. At pH 6.3 the uptake is biphasic; endocytosis of HRP occurs at an approximately normal rate for the first 5 min but then levels off (middle curve of Figure 1A). The same biphasic kinetics were observed when cells were equilibrated at pH 6.3 without HRP and then allowed to endocytose HRP (Figure 1B).

The two pH thresholds in HRP endocytosis were better visualized by comparing the initial rate of uptake with the rate of net accumulation at different extracellular pHs (Figure 2). The initial rate (ng HRP/mg protein/min) was calculated from a linear regression of the HRP uptake data obtained at the time-points of 0.5 and 5 min (Figure 2, full squares). A sharp threshold of the initial rate of HRP uptake is observed at pH 5.7. At the intermediate external pH of 6.3–6.5, the initial rate of fluid phase endocytosis is normal but the rate of accumulation of HRP as measured after 10 min of continuous presentation is significantly lower than the normal (Figure 2, open squares). The second pH threshold related to the net accumulation of HRP is shifted by 0.6 pH units above the first one. Above an external pH of 7.0 the kinetics of HRP uptake are identical to the controls. Cell viability was not affected by 30 min of treatment at pH 5.7, the same number of cells were present 24 h after returning to pH 7.4, in comparison to untreated cells.

In order to test the reversibility of the inhibition of endocytosis on a shorter time scale, cells were treated for 15 min at pH 5.7 and then placed in the presence of HRP for 15 min at pH 7.4. During this reversion step, the presence of bicarbonate and of a 5% $CO_2$ atmosphere were required to restore endocytosis. The use of bicarbonate to regulate intracellular pH after an acid load has been described (L'Allemain *et al.*, 1985). Under these conditions the amount of HRP uptake during the first 15 min was 51 ± 4 ng/mg protein in the reversed cells compared with 64 ± 5 ng/mg protein in the untreated cells.

At an external pH of 6.3, the HRP uptake was normal for the first 5 min but subsequent accumulation did not occur. It was of interest to distinguish the compartments that contained the HRP at the early and later time points by electron microscopy (Figure 3) and to compare the general condition of low-pH-treated cells with control cells. A small number of HRP-positive tubular and vesicular elements are seen after 5 min of incubation both at pH 6.3 and at normal pH (Figure 3A and C). After 30 min of incubation, the labelling pattern of HRP-positive elements remained the same in the cells treated at pH 6.3 whereas there was a significant increase in HRP-positive elements at pH 7.4. The large vesicular and multivesicular structures, which remain unlabelled at pH 6.3, become labelled at physiological pH (Figure 3B and D).

In contrast to the results obtained with the $K^+$ depletion experiments (Larkin *et al.*, 1983, 1986), coated pits and coated vesicles are easily visualized at the plasma membrane of the low-pH-treated cells (Figure 4). A coated vesicle labelled with HRP after 5 min of incubation at pH 5.7 is shown in Figure 4D, providing evidence that a first round of internalization has occurred.

### *Fractionation of the endosomal compartments by free-flow electrophoresis*

Both the biochemical and morphological evidence show that the uptake of fluid phase becomes saturable after the first 5 min at an external pH of 6.3. The distinction between this early endosomal sub-compartment and a later endosomal sub-compartment or the lysosomes was analysed using subcellular fractionation by free-flow electrophoresis (Marsh *et al.*, 1987). After 5 min of internalization at pH 7.4, the HRP-positive peak corresponding to early endosomes is in fraction 46 (Figure 5B). Then by 10 min at pH 7.4, the HRP-positive peak shifts to fraction 40 (Figure 5C). These later endosomes are not resolved from the lysosomes since they comigrate with the lysosomal-enzyme-positive fractions. By 30 min at pH 7.4, the HRP-positive peak remains in fraction 40 (Figure 5D) representing later endosomes and lysosomes. In the pH 6.3 cell, the HRP-positive peak exactly parallels the peak of early endosomes in fraction 46.

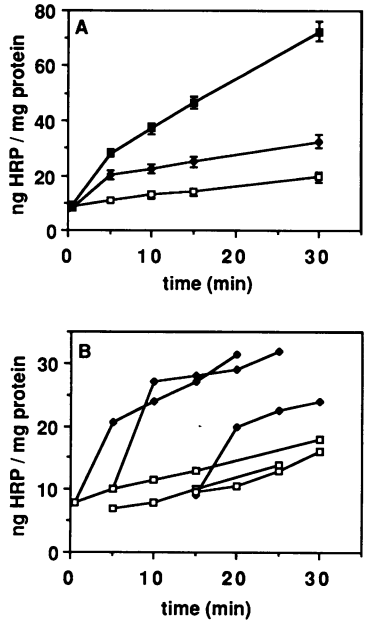


**Fig. 1.** Kinetics of fluid phase endocytosis. (**A**) The cells grown in plastic Petri dishes were incubated at 37°C in MEMb media containing HRP 1 mg/ml buffered to different pHs. At each time point the cells were cooled to 0°C washed extensively and then extracted in detergent. The cell-associated HRP enzymatic activity was assayed and expressed in ng enzyme/mg cell protein: pH 7.4 (full squares); pH 6.3 (full diamonds) or pH 5.7 (open squares). The first time point is a 30-s incubation. The error bar represents duplicate experiments. (**B**) The cells were first incubated for 0, 5 or 15 min (indicated on the right of the graph) at pH 6.3 in the absence of HRP and then in the presence of HRP up to 30 min. The equilibration of the cells at pH 6.3 did not alter significantly the kinetics of the HRP uptake.

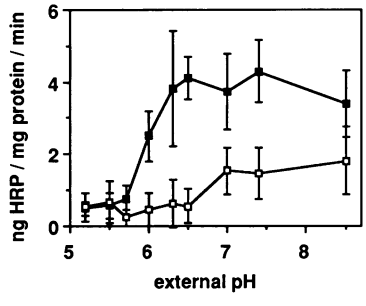


**Fig. 2.** Rate of fluid phase endocytosis as function of extracellular pH. At a given external pH, following the same conditions as in Figure 1, the amount of HRP uptake was determined for 0.5-, 5-, 10- or 15-min incubations at 37°C, in quadruplicate. The initial rate of HRP uptake was determined by linear regression of the 0.5- and 5-min data (full squares); the rate of net accumulation of HRP was determined by linear regression of the 10- and 15-min data (open squares). The error bar represents the 95% confidence interval on these linear rates determined with a Student's *t*-test.

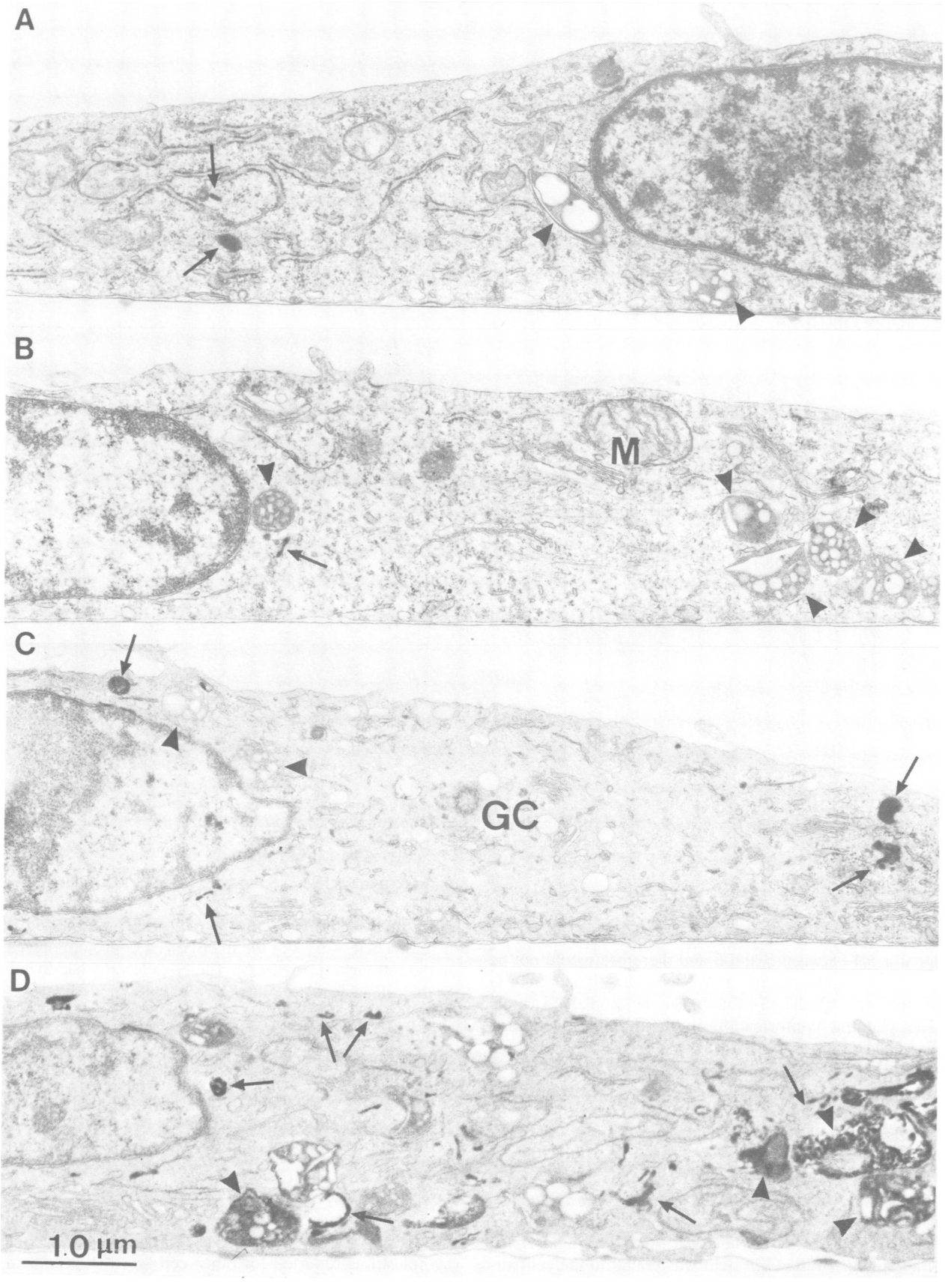


**Fig. 3.** Electron micrographs of BHK cell after internalization of HRP. The cells grown on a plastic support were incubated at 37°C for 5 (**A, C**) and 30 (**B, D**) min respectively in MEMb medium containing 5 mg HRP/ml and buffered at pH 6.3 (**A, B**) and 7.4 (**C, D**) respectively. The cells were then washed, fixed and processed for electron microscopy. Small tubular and vesicular elements of the endosomal compartment (arrow) are labelled at 5 min in both low-pH (**A**) and control cells (**C**). At 30 min, large multivesicular endosomes (arrow head) are heavily labelled in control cell (**D**), but remain unlabelled at low extracellular pH (**B**).

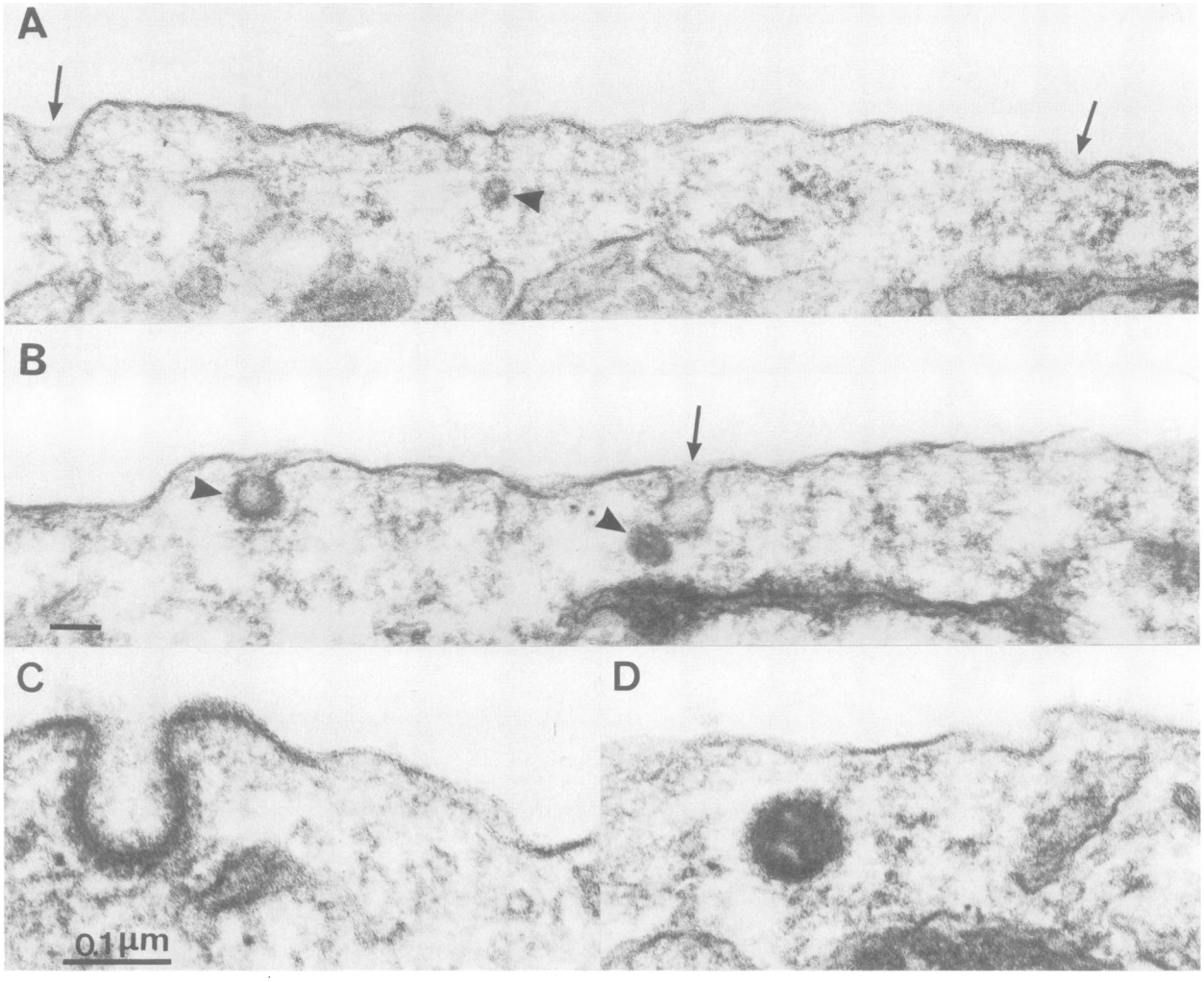


**Fig. 4.** Electron micrographs of BHK cell surface treated with low pH. High magnification micrographs of the surface of cells incubated as in Figure 3: 5 min pH 6.3 (**A**); 30 min pH 6.3 (**B**); 5 min at pH 5.7 (**C, D**). Coated pits at the cell surface (arrow) and coated vesicles in the peripheral cytoplasm (arrow head) are present in low-pH-treated cells. After 5 min of incubation at pH 5.7, a coated vesicle labelled with HRP is present close to the cell surface (**D**).

*Kinetics of G-protein endocytosis*

It was important to investigate the fate of a plasma membrane protein in the same conditions. Most ligands dissociate from their receptors at a pH between 5 and 6 and therefore would not be useful for following the receptors. We used the glycoprotein G of VSV because: (i) the G-protein can be implanted in its membrane-spanning conformation in the plasma membrane (Gruenberg and Howell, 1986, 1987b); and (ii) the implanted G-protein is restricted to the plasma membrane at 4°C and is internalized as a synchronous wave when the cells are warmed to 37°C (Gruenberg and Howell, 1986, 1987a).

Internalization of the G-protein was carried out for 0, 5, 15 and 30 min at 37°C at the different pH conditions and stopped by returning the cells to ice-temperature. The amount of G-protein present on the cell surface after the different times of internalization was quantitated with a new fluoroimmunoassay (Gruenberg and Howell, 1987b). A monoclonal antibody against an exoplasmic epitope of the G-protein was bound to the cell surface and detected with a Europium (Eu)-labelled antibody against the Fc domain of mouse IgG. The delayed fluorescence of this lanthanide was counted by time-resolved fluorometry. This labelling technique provides an extremely low background and a dynamic range wider than any other labelling technique (Soini and Hemmilä, 1979; Soini and Kojola, 1983; Hemmilä *et al.*, 1984).

At pH 7.4 the G-protein was quickly internalized; 70% in 5 min. Then ~30% of the internalized G-protein recycled back to the cell surface (Figure 6). The G-molecules that do not recycle to the cell surface are routed to the lysosomes via later elements of the pathway and are degraded (Gruenberg and Howell, 1987a,b). At an external pH of 6.3, the same kinetics of G-protein internalization and recycling were observed (Figure 6). However, at pH 5.7, G-protein internalization was reduced within the first 5 min to 15% of the total G-protein present and then internalization ceased (Figure 6). As shown below, it is likely that the intracellular pH does not drop quickly enough to prevent G-protein endocytosis.

*Cytoplasmic pH and intracellular ATP*

Decreasing the extracellular pH generated a pH gradient across the plasma membrane. This causes a gradual acidification of the cytoplasm as reported for other cell types (L'Allemain *et al.*, 1984). Since endocytosis was blocked within the first few minutes after the low pH treatment, it was essential to determine whether cytoplasmic acidification paralleled the arrest of endocytosis. The cytoplasmic pH was estimated by the partitioning of trace amounts of $^{14}$C-labelled benzoic acid across the plasma membrane

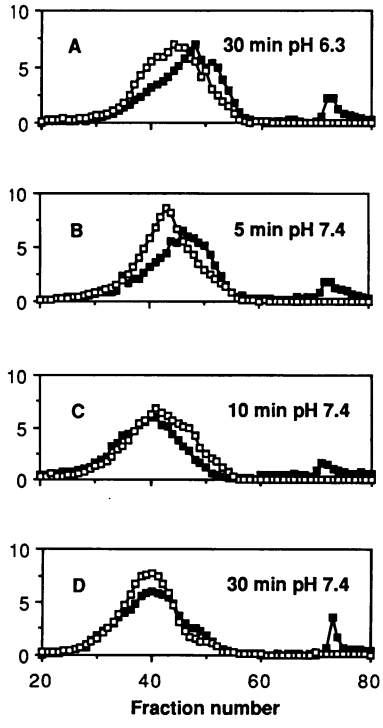


**Fig. 5.** Fractionation of the endosomal compartment by free-flow electrophoresis. The cells grown in plastic Petri dishes were incubated at 37°C for various time intervals in MEMb media containing 2 mg/ml HRP buffered to different pHs (indicated on the upper right of the graphs). The cells were cooled to 0°C, washed extensively,scraped, homogenized, a post-nuclear supernatant was prepared and trypsinized under mild conditions (0.05% mg TPCK inactivated trypsin/mg of protein). The post-nuclear supernatant was loaded in the free-flow electrophoresis chamber at the position of fraction 75. Enzymatic activities were assayed in all fractions and expressed as a percentage of the total: HRP, the fluid phase marker (full squares); GlcNac, a lysosomal marker (open squares). The protein distribution not presented on these graphs was identical in each experiment and gave a broad peak around fraction 50. Most of the HRP activity was latent within the lumen of vesicles and was deflected towards the anode, left. A small fraction of non-latent HRP remains at the site of injection.

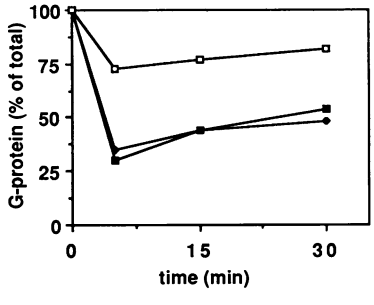


**Fig. 6.** Internalization of implanted G-protein. Following implantation the G-protein is restricted to the plasma membrane at ice-temperature. Cells with implanted G were transferred to MEMb at pH 7.4 (full squares), 6.3 (full diamonds) and 5.7 (empty squares), incubated at 37°C for the indicated times and then returned to ice-temperature. The amount of G-protein present on the cell surface was quantitated using a time-resolved fluoroimmunoassay with a monoclonal antibody against the G-protein exoplasmic domain followed by Eu-labelled antibody against the Fc domain of mouse IgG.

(L'Allemain *et al.*, 1984). The intracellular pH dropped within 2 min after decreasing the extracellular pH to 5.7 or to 6.3 (Figure 7A) and then reached a stable value ~0.5 pH units more alkaline than the extracellular pH. This corresponded to a 3-fold proton concentration gradient across the plasma membrane. This shows

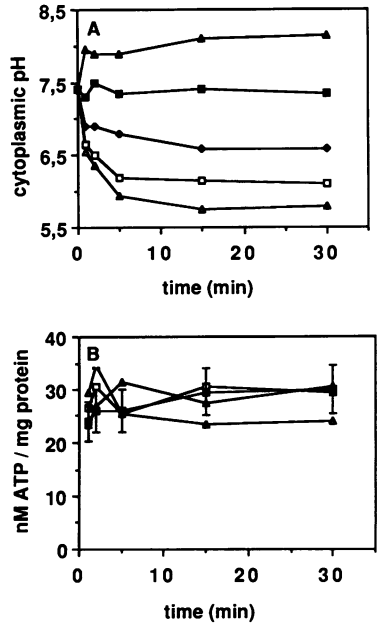


**Fig. 7.** Intracellular pH and intracellular ATP levels. (**A**) The intracellular pH was calculated from the intracellular accummulation of [$^{14}$C]benzoic acid. The cells grown on plastic Petri dishes at 37°C were incubated in the presence of MEMb buffered at the various pHs and supplemented with 0.5 $\mu$Ci/ml [$^{14}$C]benzoic acid and washed. Each measurement is in duplicate: $pH_{ext}$ 8.5 (full triangles); $pH_{ext}$ 7.4 (full squares); $pH_{ext}$ 6.3 (full diamonds); $pH_{ext}$ 5.7 (empty squares); $pH_{ext}$ 5.5 (empty triangles). (**B**) The intracellular ATP was measured in cells incubated at 37°C for the indicated times in MEMb buffered at the various pHs, using a luciferin–luciferase bioluminescence assay. The results are expressed as nmol ATP/mg of cell protein and each experiment was performed in quadruplicate. The error bar represents the mean standard deviation: $pH_{ext}$ 8.5 (full triangles); $pH_{ext}$ 7.4 (full squares); $pH_{ext}$ 6.3 (full diamonds); $pH_{ext}$ 5.7 (empty squares); $pH_{ext}$ 5.5 (empty triangles).

that the cells are able to actively regulate their intracellular pH when exposed to an extracellular low pH. Similar observations have been made for other cell types (L'Allemain *et al.*, 1984).

Reduction of intracellular ATP levels have been shown to inhibit endocytosis (Clarke and Weigel, 1985); therefore we have tested the effect of low extracellular pH on the cellular energy production. The intracellular levels of ATP were determined using the luciferin–luciferase bioluminescence assay (Figure 7B). At low pH, cellular ATP levels remained unchanged. This rules out the possibility that the decreased internalization seen at low pH was due to a reduction in intracellular ATP.

### *Intracellular distribution of clathrin*

Clathrin mediates the formation of coated pits and coated vesicles responsible for the internalization of receptor–ligand complexes (reviewed in Goldstein *et al.*, 1979, 1985) and fluid phase (Marsh and Helenius, 1980). The intracellular distribution of clathrin was visualized by immunofluorescence microscopy using a monoclonal antibody against clathrin heavy chain (Blank and Brodsky, 1986; Brodsky, 1987) and a rhodamine-labelled second antibody (Figure 8). When the cells were at pH 5.7 for 5 min (Figure 8C), the overall staining pattern was similar but slightly more punctate than in the control (Figure 8A). By 15 min at pH 5.7 (Figure 8D), larger clusters of clathrin had formed within the cytoplasm. In contrast, at pH 6.3 (Figure 8B), the distribution of clathrin remains similar to the control. It is difficult to determine what changes in clathrin distribution occurred at the plasma membrane because by immunofluorescence the staining pattern in the Golgi region dominates in the cell. Electron

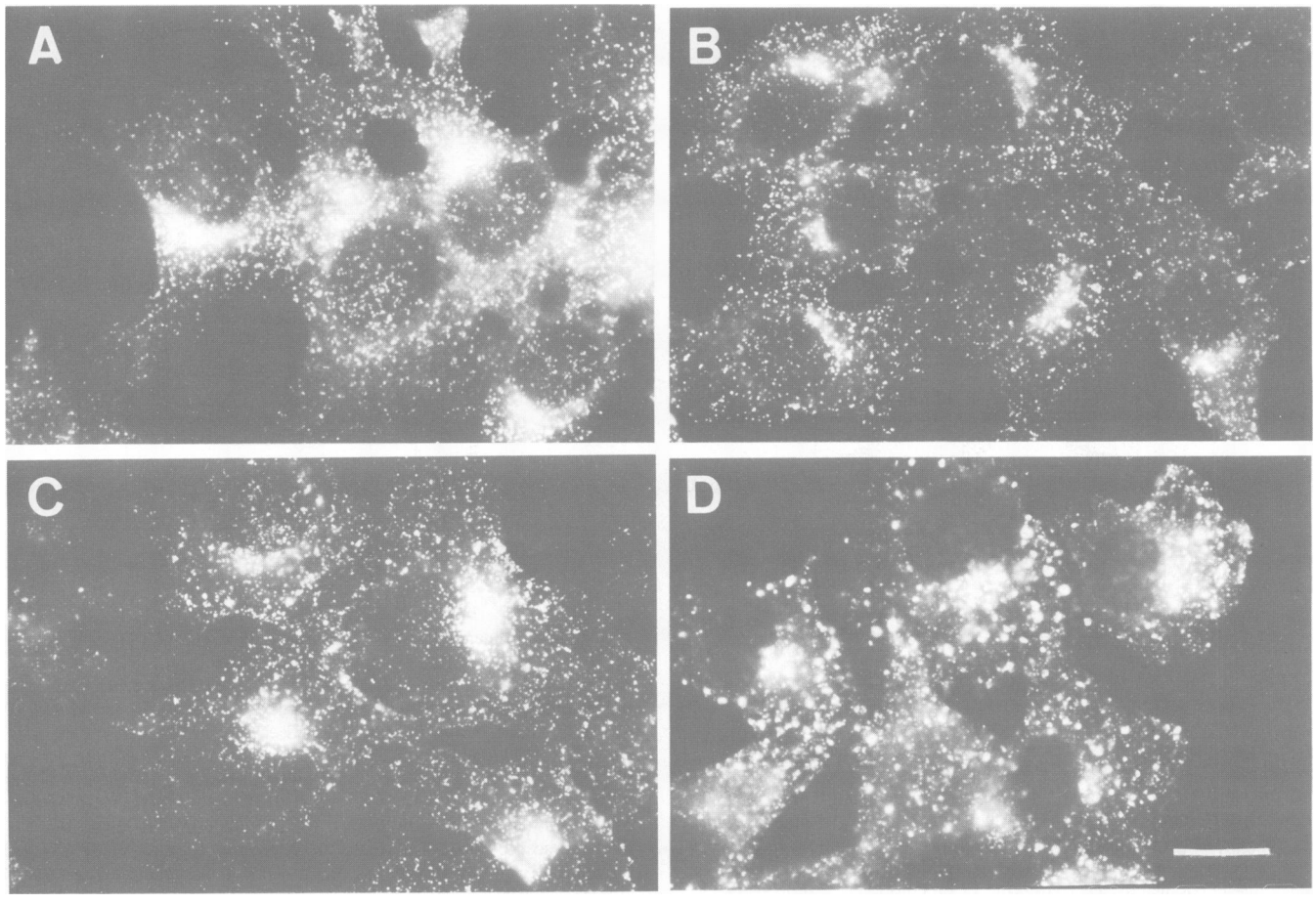


**Fig. 8.** Immunofluorescence staining of clathrin. BHK cells grown on glass coverslips were incubated at 37°C: pH 7.4 for 15 min (**A**), pH 6.3 for 15 min (**B**), pH 5.7 for 5 min (**C**), and pH 5.7 for 15 min (**D**). The coverslips were then processed for immunofluorescence using a mouse monoclonal anticlathrin heavy chain X22 antibody (Blank and Brodsky, 1986) and detected with an anti-mouse IgG rhodamine-labelled antibody. The bar equals 20 μm.

micrographs (Figure 4) confirmed that coated pits and coated vesicles were still present in low pH-treated cells.

## Discussion

The rapid acidification of endocytic vesicles (reviewed in Mellman *et al.*, 1986) plays an important role in the endocytic pathway. Specifically, the low pH causes dissociation of receptors from their ligands (Goldstein *et al.*, 1985). In a few well-established cases alkalinization of the luminal pH of the endosomes blocks the movement of molecules in transit (Basu *et al.*, 1981; Tietze *et al.*, 1982; Harford *et al.*, 1983). The importance of a pH gradient across membranes has prompted us to investigate whether the rate of endocytosis could be susceptible to a pH-dependent regulation in the acidic range. We have addressed this question by monitoring the effect of acidic extracellular pH on both the endocytosis of a solute in the fluid phase and the internalization of a membrane-spanning protein. Fluid phase endocytosis was studied using HRP, which has been widely used for biochemical and morphological analysis (reviewed in Steinman *et al.*, 1983). The G-protein of VSV was implanted into the plasma membrane and used as a reporter molecule for the endocytosis of membrane proteins. The pathway and the kinetics of G-protein endocytosis have been described previously in BHK cells (Gruenberg and Howell, 1987a).

Endocytosis of HRP and G-protein ceases at an extracellular pH below 5.7. The threshold value of the pH is very sharp since both HRP and G-protein are internalized at an external pH of 6.3. These values are in the range estimated for the lumen of the first compartment of the endocytic pathway, common to all internalized protein and receptors (Tycko and Maxfield, 1982; Tycko *et al.*, 1983; Murphy *et al.*, 1984). Receptors and ligands are normally exposed to a pH between 5 and 6 without denaturating these proteins. Indeed, in these experiments endocytosis resumes when the cells are returned to pH 7.4. This shows that irreversible alterations of cellular functions did not occur when the external pH is dropped to 5.7. Furthermore, the intracellular level of ATP is not depleted as a result of the low extracellular pH.

The distribution of clathrin was characterized using immunofluorescence microscopy and appeared as fine dots dispersed in the cytoplasm and larger clathrin-reactive elements concentrated in the Golgi region. The distribution of clathrin remained the same at pH 6.3 and after 5 min at pH 5.7, but after 15 min at pH 5.7, there was some redistribution of clathrin into larger clusters as seen in the Golgi region and through the cytoplasm. The significance of this alteration is not clear. At the ultrastructural level, however, coated pits were still detected at the surface of the low pH-treated cells. Therefore, the low pH treatment does not trigger the disappearance of coated pits and coated vesicles in the same fashion as $K^+$ depletion does (Larkin *et al.*, 1983; Moya *et al.*, 1985). Instead, lowering the cytoplasmic pH to 6.2 may somehow stabilize the existing coated pits and coated vesicles as has been shown for clathrin polymerization *in vitro* (Unanue *et al.*, 1980).

At the physiological external pH of 7.4, a wave of G-protein endocytosis occurred with a maximum of 70% internalization after 5 min. When the cells are exposed to the acidic pH of 5.7, 15% of the total surface G-protein was still internalized within the first 5 min and then stopped. At pH 5.7, the uptake of HRP detected either morphologically or biochemically was just significantly above background. Since it requires ~2 min for the intracellular pH to stabilize at 6.2, this delay probably accounts for the observed internalization of 15% of the G-protein. This supports the conclusion that the acidification of the cytoplasm is required to stop endocytosis. Converted into intracellular pH units, the initial rate of endocytosis would be arrested below 6.2 and remain normal above 6.8.

A large number of investigators have provided evidence for more than one sub-population of endosomes, generally corresponding to an early and a later stage of endocytosis before reaching the lysosomes. These have been characterized morphologically (for reviews see Helenius *et al.*, 1983; Hopkins, 1986), kinetically (for a review see Steinman *et al.*, 1983; also Clarke and Weigel, 1985; Swanson *et al.*, 1985; Murphy, 1985), by fractionation techniques (Berg *et al.*, 1985; Baenziger and Fiete, 1986; Mueller and Hubbard, 1986; Branch *et al.*, 1987; Courtoy *et al.*, 1987; Sullivan *et al.*, 1987) and by cell-free analysis of fusion competence (Gruenberg and Howell, 1987a).

We have observed that at an extracellular pH of 6.3, G-protein internalization occurs with the same kinetics as at pH 7.4. Under these conditions HRP is internalized with a normal initial rate but accumulation reaches a plateau after 5 min. Intracellular pH is then equal to 6.8. Morphologically, this early endosomal sub-compartment is primarily composed of tubular and vesicular elements. Subsequent internalization has be compensated for by recycling of the endosomal content in order to reach saturation in 5 min. This agrees well with the $t_{1/2} = 5$ min reported for the recycling of fluid phase markers (Besterman *et al.*, 1981; Adam *et al.*, 1982) and with the finding that recycling of the G-protein to the plasma membrane occurs from an early endosomal sub-compartment (Gruenberg and Howell, 1987a). At a similar extracellular pH, 6.5, the asialoglycoprotein could be normally internalized but failed to be degraded in rat hepatocytes (Samuelson *et al.*, 1986). We have found here that an intracellular pH close to physiological (>7.0) is needed to allow net accumulation of HRP into larger vesicular or multivesicular elements of the endocytic pathway. The fractionation of the endosomal compartment by free-flow electrophoresis confirmed that endocytosis is arrested at an early stage when the external pH is adjusted to 6.3. The sub-compartment loaded with HRP for 30 min at pH 6.3 comigrated with the sub-compartment loaded for up to 5 min at pH 7.4. These were clearly resolved from the late endosomal elements accessible after >10 min at pH 7.4 and the lysosome peak. The microtubles which play an important role in the transport of endocytic vesicles to be fused with lysosomes (Matteoni and Kreis, 1987) were not depolymerized in the acid-treated cells characterized by immunofluorescence microscopy (data not shown).

In summary our observations suggest that acidification of the cell cytoplasm experimentally produced by lowering the extracellular pH, is able to block endocytosis in two distinct steps: (i) the internalization of solutes and membrane proteins; and (ii) the delivery of endocytic markers from an early endosomal sub-compartment to later elements of the pathway. The transport between these two endosomal sub-compartments is more sensitive to acidic pH than the initial step of internalization to the early endosome. Reduction of cytoplasmic pH to 6.8 provides a means to block endocytosis at an early stage prior to the accumulation of solutes and it will be used to characterize the functional properties of this sub-population of early endosomes from which recycling to the cell surface occurs.

## Materials and methods

*Cell culture and media*

BHK-21 cells were grown in monolayers, cultured in plastic Petri dishes with Glasgow minimum essential medium (GMEM) supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine and 10 mM Hepes pH 7.4. Experiments were always carried out when cells had just reached confluency 40 h after passage.

The extracellular pH was varied with bicarbonate-free MEM supplemented with 0.2% bovine serum albumin (BSA, Sigma, St Louis, MO) and 0.1% glucose (MEMb) and buffered at 37°C as follows: (i) 20 mM succinate, 20 mM Mes, or 20 mM pyrophosphate at pHs from 5.2 to 5.7; (ii) 10 mM succinate and 10 mM bis Tris at pHs from 5.7 to 6.3; (iii) 10 mM Pipes, 10 mM Mops, and 10 mM $NaH_2PO_4$ at pHs from 6.5 to 7.0; (iv) 10 mM Tes, 10 mM Mops, 15 mM Hepes, and 2 mM $NaH_2PO_4$ at pH 7.4; (v) 15 mM Epps and 15 mM tricine at pH 8.5. All buffers were obtained from Sigma. Osmolarity of the media varied from 280 to 300 milliosmole and was routinely checked with a Roebling osmometer.

*Assay of HRP fluid phase endocytosis*

The cells grown on 5-cm-diameter plastic Petri dishes ($4 \times 10^6$ cells) were quickly rinsed in MEMb pH 7.4 and then incubated at 37°C in 3 ml of MEMb at the various pHs in the presence of 1 mg/ml HRP (Sigma) for various times. The cells were immediately cooled on ice, washed 5 × 5 min with 10 ml of ice-cold phosphate-buffered saline solution containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS+) supplemented with 0.2% BSA and washed five times with PBS+. After the washings, the cells were extracted at 0°C for 10 min with 500 μl of lysing buffer A, 10 mM Tris pH 7.4 plus 0.05% Triton X100, scraped, homogenized by 10 repetitive pipettings through 200-μl Eppendorf tips and aliquoted. HRP enzymatic activity was assayed at 460 nm using 0.342 mM O-dianisidine and 0.003% $H_2O_2$ in 0.5 M phosphate buffer pH 5.0 containing 0.3% Triton X100. The limit of detection was 2 ng of HRP internalized per mg of cellular protein. Protein was determined using a Biorad assay (Bradford, 1976).

*Electron microscopy and immunofluorescence*

The cells grown on plastic Petri dishes were incubated in MEMb pH 5.7, or 6.3 with 5 mg/ml HRP or in GMEM culture medium supplemented with 5 mg/ml HRP for defined times. The cells were then washed three times with ice-cold G MEM, twice with ice-cold PBS+, fixed *in situ* with 2% glutaraldehyde in 0.1 M cacodylate at a pH corresponding to the experiment, dehydrated, embedded in Epon and processed for electron microscopy. HRP was visualized with the reaction product of diaminobenzidine (Graham and Karnovsky, 1966).

For immunofluorescence detection of clathrin the cells were grown on 20 × 20 glass coverslips and incubated in MEMb at pH 7.4, 6.3 or 5.7 for 5, 15 and 30 min. The coverslips were then cooled on an ice plate, rinsed twice with cold PBS+ and fixed for 20 min at room temperature with 3% paraformaldehyde in PBS+. The cells were then reacted with the mouse monoclonal anti-clathrin heavy chain antibody X22 (kindly provided by F.Brodsky, University of California, San Fransisco), followed by an anti-mouse IgG rhodamine-labelled antibody (kindly provided by T.Kreis, EMBL).

*Fractionation of the endosomal compartment by free-flow electrophoresis*

The cells grown on plastic Petri dishes ($4 \times 10^8$ cells) were incubated at 37°C in MEMb at the various pHs in the presence of 2 mg/ml HRP and washed as indicated above for the quantitation of HRP uptake. The cells were scraped, resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM triethanolamine buffer pH 7.4 (TEA buffer) and washed twice by resuspension and centrifugation at 400 g for 5 min. The final pellet was resuspended with 5 vol TEA buffer, left 10 min on ice and homogenized 10 times through a 20-gauge syringe needle. The homogenate was pelleted at 2000 *g* for 10 min and the post-nuclear supernatant (20 mg protein/ml) was treated with TPCK inactivated trypsin (Worthington) at a concentration of 10 μg/ml for 10 min at 37°C. Then soybean trypsin inhibitor (Worthington) was added at a concentration of 20 μg/ml. The post-nuclear supernatant was pelleted again at 2000 *g* for 10 min and applied to the free-flow electrophoresis chamber above fraction 75 (Elphor Vap 11, Bender and Hobein, Munich). The separation was performed at 130 V/cm (180 mA) with a laminar flow rate of 4 ml/fraction/h. The injection of 4 ml post-nuclear supernatant was performed at 3 ml/h and 100 fractions of ~5 ml were collected. HRP enzyme activity and protein were assayed. β-*N*-acetylglucosaminidase (GlcNac), a lysosomal marker, was assayed according to Beaufay *et al.* (1974).

*Internalization of G-protein implanted in the plasma membrane*

Implantation of the membrane-spanning G-protein of VSV in the plasma mem-

brame and its subsequent internalization was performed as described previously (Gruenberg and Howell, 1986, 1987a). Briefly, $1.5 \times 10^7$ cells grown in a 75-$cm^2$ dish were first treated for 30 min at 4°C with 2.5 ml MEM pH 7.4 without BSA, but containing 0.25 mg wheat germ agglutinin which allows virus binding independent of potential receptor sites. The cells were then washed in PBS+ and 40 μg of VSV in 2.5 ml MEM pH 7.4 without BSA was allowed to bind for 1 h at 4°C. Fusion of the viral envelope with the plasma membrane was carried out after washing in PBS without divalent cations (PBS−) by incubating the cells for 30 s at 37°C in 15 ml MEM pH 4.9. The cells were quickly returned to 4°C and washed 2× in PBS−. The unfused virions were removed by treating the cells for 30 min at 4°C in MEMb containing 20 mM GlcNac. Internalization of the implanted G-protein molecules was carried out at various pHs, for 5, 15 and 30 min as described above.

After internalization the monolayer was returned to 4°C and washed with PBS+. Quantitation of the amount of G-protein at the cell surface was performed with a fluoroimmunoassay that uses time-resolved fluorescence of Eu as described (Gruenberg and Howell, 1987b). The monolayer was reacted with a monoclonal antibody against G-protein exoplasmic domain (17-2-21-4; K.Simons, EMBL) diluted to 0.5 μg/ml in MEMb for 30 min at 4°C. The cells were then washed $3 \times 5$ min with PBS+ containing 0.5% BSA and treated for 1 h at 4°C with 2.5 ml PBS− containing 0.2 μg affinity-purified sheep anti-mouse Fc antibody labelled with Eu (I.Hemmilä, Wallac Oy, Turku, Finland). The washing sequence was repeated and the monolayer was reacted with 0.5 ml Wallac enhancement solution (Wallac Oy) to free the bound Eu. Quantitation of the amount of Eu was performed by measuring the delayed fluorescence of 0.2-ml aliquots of the enhancement solution in a Wallac/LKB time-resolved fluorometer.

*Intracellular pH*

The intracellular pH was measured by using trace amounts of [$^{14}$C]benzoic acid labelled in the benzene ring, a weak acid employed for this purpose (L'Allemain *et al.*, 1984). The protonated form of this weak acid equilibrates between the extracellular medium and the cytoplasm in < 15 s at 37°C but the unprotonated form of the acid is impermeant and its accumulation will depend on the pH of the cytoplasm. Since the pK of benzoic acid is much lower than the actual intracellular pH, the acid is mainly present in the cytoplasm in its dissociated form. The intracellular pH can be calculated from the following equation.

$$\text{pH internal} = \text{pH external} + \log \frac{([^{14}\text{C}]\text{benzoic acid})_{\text{internal}}}{([^{14}\text{C}]\text{benzoic acid})_{\text{external}}}$$

The cells grown on 3-cm dishes were incubated at 37°C with MEMb buffered at the various pHs in the presence of 0.5 μCi/ml of [$^{14}$C]benzoic acid (Amersham) (50 mCi/mM). After an incubation time varying from 1 to 30 min, the dishes were quickly transferred to an ice plate and immediately washed four times with 2 ml of 140 mM NaCl buffered in the same way as for the incubation at 37°C. The cells were then extracted, scraped in 0.5 ml of lysing buffer A and homogenized by 10 repetitive pipettings. The amount of [$^{14}$C]benzoic acid taken up by the cells ($> 2 \times 10^3$ c.p.m.) was normalized to cell protein. Since the protonated form of benzoic acid can leak slowly at 0°C, the four washings and the last aspiration before the extraction, were performed in 10 s. In preliminary experiments, we have tested the rate of passive leak of [$^{14}$C]benzoic acid during these four washings for each condition from pH 5.5 to 8.5. The data were plotted on a semi-logarithmic plot; the efflux of [$^{14}$C]benzoic acid has a half-time of 27 s at 0°C which is independent of the external pH. The normalized counts were multiplied by a factor of 1.29, to account for the passive efflux of [$^{14}$C]benzoic acid during the washes.

In order to estimate the intracellular volume to cell protein ratio, the cytoplasmic pH was assumed to be 7.4 after 30 min of equilibration at 37°C in MEMb pH 7.4. The condition of an equal internal and external concentration of unprotonated benzoic acid gives an estimation of the intracellular volume accessible to benzoic acid as 7 μl per mg of cellular protein, consistent with the value found by other groups (L'Allemain *et al.*, 1984).

*Intracellular ATP levels*

The intracellular ATP levels were determined on cell monolayers using the luciferin−luciferase assay (Boehringer, Mannheim) as described by Larkin *et al.* (1985). After incubation in MEMb at the various pHs for 1−30 minutes, the cells were quickly transferred to ice-temperature. The medium was aspirated and replaced by 1 ml of 7% ice-cold trichloroacetic acid for 10 min at 0°C to precipitate cellular protein and to release the ATP. It was then neutralized by adding 0.8 ml of 1 M Tris base, collected and diluted 1:2, 1:5, 1:10 and 1:20 in buffer B consisting of 20 mM MgCl, 0.36 mM DTT, 0.3 mM AMP, 40 mM Hepes pH 7.75 and 4 mM EDTA. A standard solution of ATP was diluted in buffer B to a final concentration of 1 to 100 mg/ml. All the ATP-containing solutions were kept on ice. For the bioluminescence assay, scintillation vials were filled with 9.5 ml of buffer B plus 500 μl of reconstituted luciferin−luciferase solution yielding a final concentration of 80 μg/l of luciferase and 0.7 mM of D-luciferin. The enzyme solutions were allowed to equilibrate at room temperature before the addition of 100 μl of the ATP-containing samples. To quantitate bioluminescence, a Bekmann scintillation counter was used in the single photon counting mode out of coincidence. In buffer B, containing AMP, there was no decay of the counts during 30 min. The assay of ATP was linear from $2 \times 10^{-8}$ to $5 \times 10^{-11}$ M.

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## Note added in proof

van Deurs *et al.* (1987, *J. Cell Biol.*, **105**, 679–690) have shown in a recent report that cytosolic acidification inhibits endocytosis from coated pits as monitored by the uptake of transferrin and of epidermal growth factor. These findings are in good agreement with our observations. However the authors reported no effect of cytoplasmic acidification on the endocytosis of lucifer yellow in the fluid phase. This may reflect differences in cell type or in the experimental protocol used for acidification. We have further characterized the effect of low cytoplasmic pH on endocytosis using cell mutants lacking the $Na^+/H^+$ antiporter (L'Allemain *et al.*, 1984). Consistent with the present results, the fluid phase endocytosis of HRP and of lucifer yellow as well as the receptor mediated endocytosis of transferrin, are inhibited to a similar extent (5-fold) by lowering internal pH below pH 6.8. Endocytosis of the 3 markers resumes at its normal rate when the cytoplasmic pH is returned to a physiological value.