Calcium uptake via endocytosis with rapid release from acidifying endosomes

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A number of specific cellular Ca^{2+} uptake pathways have been described in many different cell types [1–3]. The possibility that substantial quantities of Ca^{2+} could be imported via endocytosis has essentially been ignored, although it has been recognized that endosomes can store Ca^{2+} [4,5]. Exocrine cells can release significant amounts of Ca^{2+} via exocytosis [6], so we have investigated the fate of Ca^{2+} taken up via endocytosis into endosomes. Ca^{2+}-sensitive and H^+-sensitive fluorescent probes were placed in the extracellular solution and subsequently taken up into fibroblasts by endocytosis. Confocal microscopy was used to assess the distribution of fluorescence intensity. Ca^{2+} taken up by endocytosis was lost from the endosomes within a few minutes, over the same period as endosomal acidification took place. The acidification was inhibited by reducing the extracellular Ca^{2+} concentration, and Ca^{2+} loss from the endosomes was blocked by bafilomycin (100 nM), a specific inhibitor of the vacuolar proton ATPase. Quantitative evaluation indicated that endocytosis causes substantial import of Ca^{2+} because of rapid loss from early endosomes.

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Results and discussion

Time courses of endosomal acidification and Ca^{2+} loss

Figure 1a and 1b shows fluorescence and transmitted light images, respectively, of a 3T3 Swiss fibroblast that has been incubated with the pH-sensitive probe fluorescein isothiocyanate (FITC)–dextran for 10 minutes at 37°C. Highly fluorescent endocytic vesicles can be seen in the peripheral part of the cell close to the plasma membrane, but no fluorescence or endosomes were observed in the nuclear area.

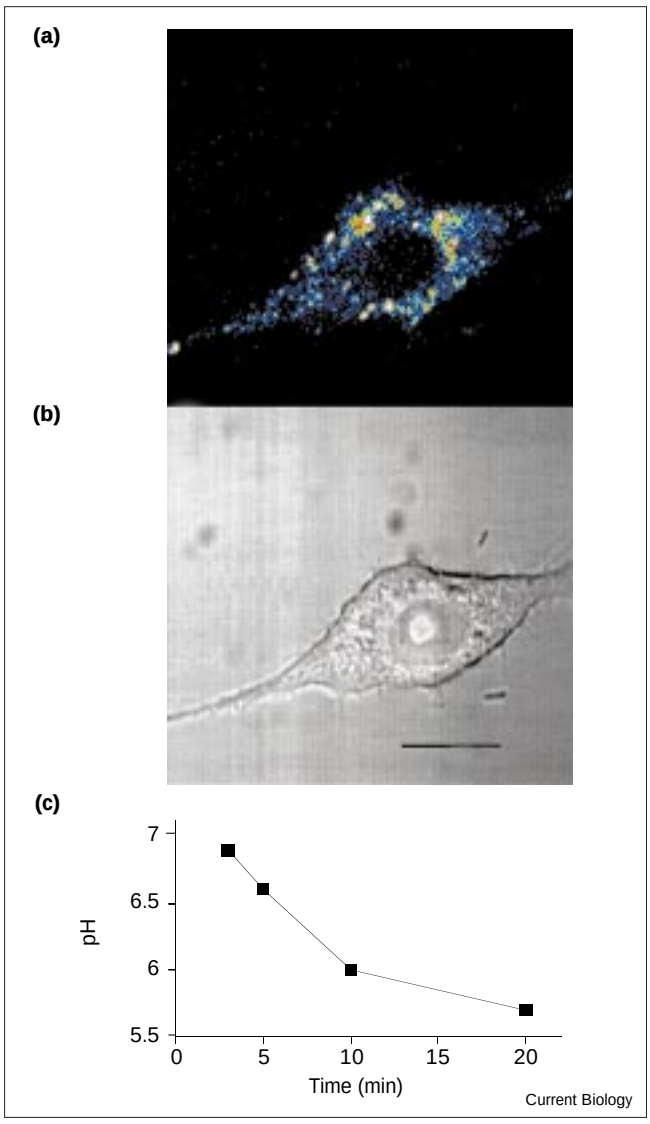
FITC–dextran has a pKa of about 6.4 with maximal sensitivity in the pH range 6.0–7.2 (Molecular Probes). The pH calibrations with the protonophore nigericin showed that after incubation of cells with FITC–dextran for 3 minutes the pH in the endosomes had decreased from the value of 7.4 in the external solution to 6.9 ± 0.2 (n = 40). After incubation for 5 minutes, the pH was 6.6 ± 0.2 (n = 10), after 10 minutes 6.0 ± 0.2 (n = 12) and after 20 minutes it had decreased to 5.7 ± 0.2 (n = 11; Figure 1c). These results are in agreement with previous data showing progressive acidification of endosomes [7].

In another series of experiments, the cells were incubated in a solution containing the Ca^{2+}-sensitive dye Oregon Green 488 BAPTA-5N. This dye is pH-insensitive at pH levels higher than 6.0 (Molecular Probes) and we found that its brightness decreased by about 10% when the pH changed from 6.0 to 5.0. The fluorescence image shown in Figure 2a was obtained after a 10 minute incubation of a fibroblast with Oregon Green 488 BAPTA-5N. Figure 2b shows a transmitted light image of the same cell. As in Figure 1, there is a peripheral distribution of round-shaped structures with a size less than 1 μm or clusters of such structures. No fluorescence or endosomal structures were seen in the nuclear region.

Oregon Green 488 BAPTA-5N has a dissociation constant (K_d) for Ca^{2+} of 20 μM (Molecular Probes). In order to assess the free Ca^{2+} concentration in the endosomes we calibrated by addition of the Ca^{2+} ionophore ionomycin in the presence of nigericin, first in a buffer with 2 mM $CaCl_2$ and then in a buffer with 2 mM of the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA). This was done after different periods of fluorescent dye incubation to assess the time course of changes in the endosomal Ca^{2+} concentration. The result is shown in Figure 2c. After incubating the cells for 3 minutes in a solution containing 2 mM $CaCl_2$, the free Ca^{2+} concentration in the endosomes was 28.6 ± 2 μM (n = 38). After 5 minutes of incubation the Ca^{2+} concentration was 8.5 ± 1.8 μM (n = 28), after 10 minutes it had decreased to 3.9 ± 1.2 μM (n = 27) and after 20 minutes was 3.0 ± 0.8 μM (n = 24).

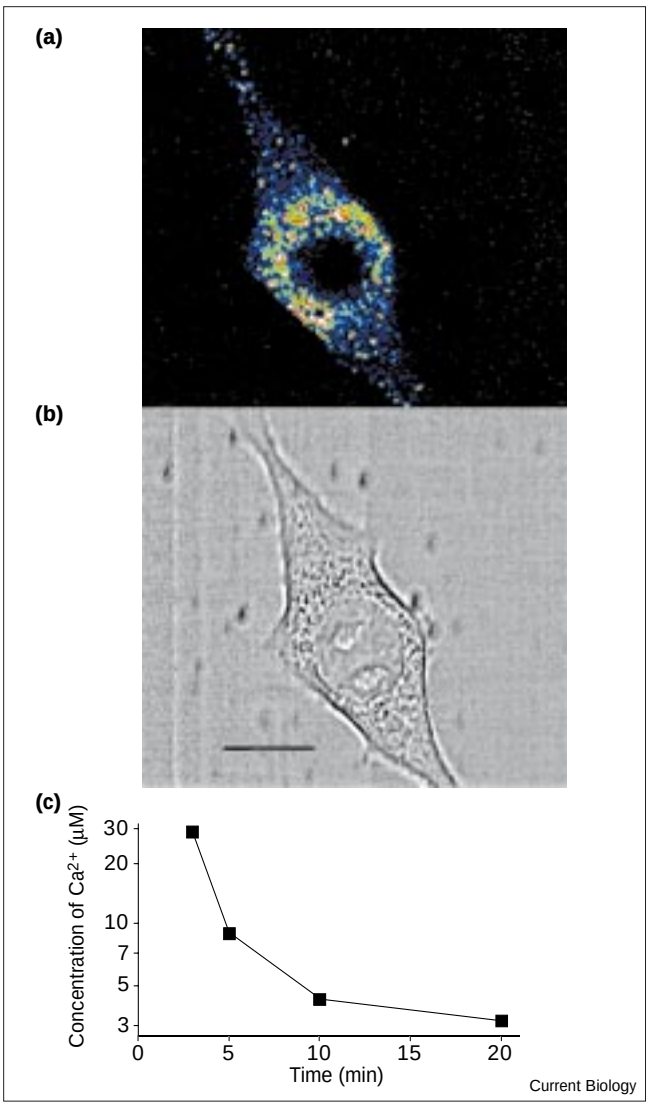
The results shown in Figure 2 indicate that most of the Ca^{2+} taken up by endocytosis had already been lost within 5 minutes. To check this important point, we also measured the endosomal Ca^{2+} concentration using the ratio of the fluorescence intensities of rhodamine B and Oregon Green 488 BAPTA-5N. We calibrated with Ca^{2+} and EGTA solutions in the presence of ionomycin and nigericin. The calculated free Ca^{2+} concentration in the

Figure 1



Distribution of the pH-sensitive probe FITC–dextran and pH measurement inside endosomes. (a) The localisation of FITC–dextran in the peripheral cytoplasm of a Swiss 3T3 fibroblast. The confocal image was taken following incubation for 10 min at 37°C. (b) Transmitted light picture of the same cell. Bar represents 10 μm. (c) The dependence of endosomal pH on the time of incubation with FITC–dextran.

Figure 2



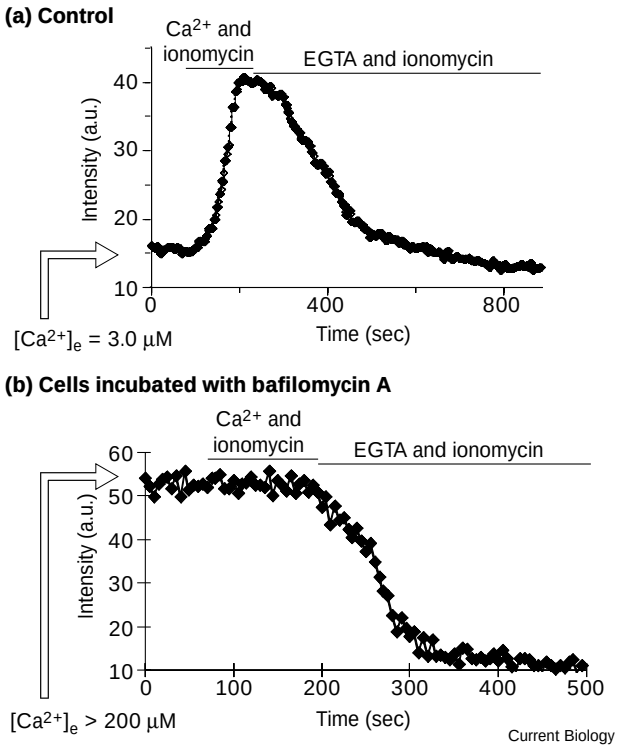
Ca^{2+} concentration in endosomes measured by the calcium-sensitive fluorescent dye Oregon Green 488 BAPTA-5N and its distribution. (a) Confocal image of the fluorescence of Oregon Green 488 BAPTA-5N in a fibroblast after incubation for 10 min at 37°C. (b) Transmitted light picture of the same cell. Bar represents 10 μm. (c) The dependence of the free Ca^{2+} concentration in the endosomes on the incubation time with Oregon Green 488 BAPTA-5N.

endosomes after a 20 minute incubation was 2.9 ± 1.2 μM (n = 5), which is in the same range as that measured simply using Oregon Green 488 BAPTA-5N fluorescence. These data show directly that Ca^{2+} taken up via endocytosis into endosomes is rapidly lost from these structures.

The relationship between endosomal acidification and Ca^{2+} loss

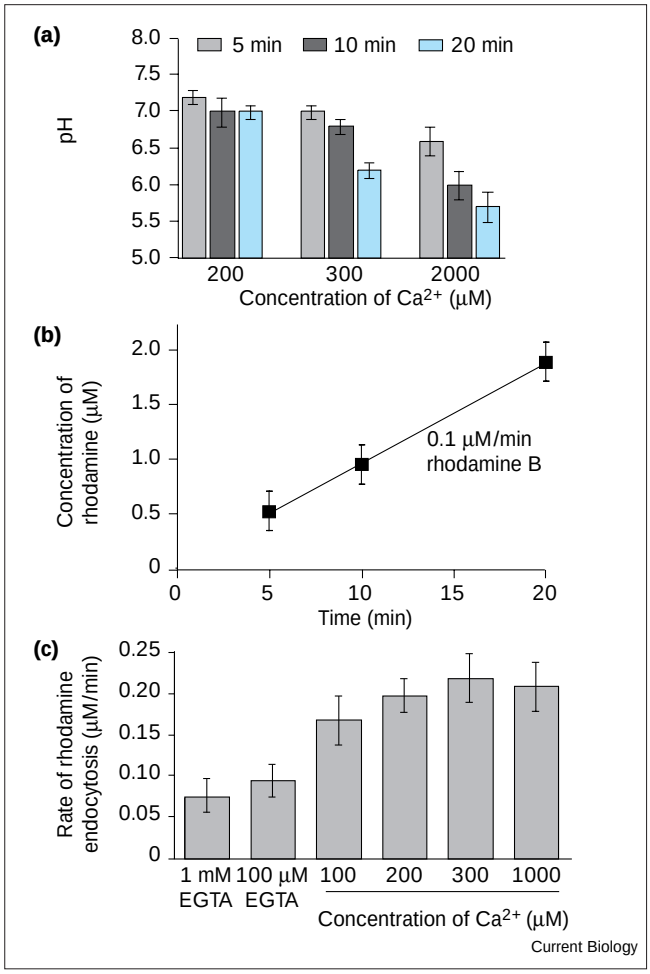
Bafilomycin, a specific inhibitor of the vacuolar-type proton ATPase [8,9], blocks endosomal acidification [8]. Bafilomycin has its maximal inhibitory effect on vacuolar ATPases at 100 nM. Even at a concentration of 1 μM it has no effect on other ATPases [8]. In control experiments, we confirmed that 100 nM bafilomycin abolished endosomal acidification (pH 7.4 ± 0.1, n = 8, after 20 minutes). We compared the endosomal Ca^{2+} concentration in cells incubated with 100 nM bafilomycin with those in control cells. Figure 3 shows the result of the calibration protocol in control (Figure 3a) and bafilomycin-incubated (Figure 3b) cells. In the control situation, the introduction of a high Ca^{2+} concentration in the presence

Figure 3



Calibration of the Ca^{2+} concentration in endosomes from Swiss 3T3 fibroblasts incubated with or without bafilomycin. Calibration of Oregon Green 488 BAPTA-5N fluorescence in the endosomes (shown in arbitrary units; a.u.) was performed using application of 20 μM ionomycin and 5 μM nigericin in a buffer with 2 mM $CaCl_2$ and then in a buffer with 2 mM EGTA. The incubation time with Oregon Green 488 BAPTA-5N at 37°C was 20 min. (a) Control. (b) Calibration after pre-treatment for 20 min with bafilomycin A (100 nM). The endosomal Ca^{2+} concentration ($[Ca^{2+}]_e$) at the start of the time course is shown.

Figure 4



(a) The effect of changing the external Ca^{2+} concentration on the endosomal pH in Swiss 3T3 fibroblasts. (b) The rate of endocytosis of rhodamine B in Swiss 3T3 fibroblasts. (c) Dependence of the rate of endocytosis of rhodamine B isothiocyanate dextran on the external Ca^{2+} concentration. Cells were incubated with rhodamine at 37°C.

of ionomycin and nigericin evoked a marked increase in the fluorescence intensity of the endocytosed Oregon Green 488 BAPTA-5N which could then be markedly reduced by the calcium chelator EGTA (Figure 3a). In contrast, Ca^{2+} and ionomycin (and nigericin) failed to evoke any increase in the fluorescence from the endocytosed marker in the cells that had been incubated with bafilomycin (Figure 3b), demonstrating that the indicator was already saturated with Ca^{2+}. Subsequent introduction of EGTA reduced the fluorescence (Figure 3b). The free Ca^{2+} concentration in the endosomes in the bafilomycin-treated fibroblasts was therefore high (> 200 μM; $n = 40$). These experiments indicate that blockage of the proton ATPase prevents loss of Ca^{2+} from endosomes.

We looked at the ability of endosomes to become acidic under different degrees of Ca^{2+} loading in a series of experiments in which cells were incubated in solutions with Ca^{2+} concentrations of 200 μM, 300 μM or 2 mM. As seen in Figure 4a, when the external Ca^{2+} concentration was 2 mM, the degree of endosomal acidification at 5, 10 and 20 minutes was similar to the control results shown in Figure 1. When the external Ca^{2+} concentration was reduced to 300 μM, the degree of acidification was considerably inhibited, and at an external Ca^{2+} concentration of 200 μM, there was virtually no acidification. These results indicate that acidification can occur only when there is an initially high Ca^{2+} concentration in the endosomes. One possibility is that H^+ uptake via the bafilomycin-sensitive H^+ pump is balanced by Ca^{2+} exit via endosomal Ca^{2+} channels. Another or additional possibility is that the Ca^{2+} inside endosomes is needed to keep other channels (K^+ and Cl^-, for example) open so that charge compensation can occur via K^+ and/or Cl^- transport. Several types of

Ca^{2+}-dependent K^+, Cl^- and non-selective cation channels have been characterized in cell membranes [10].

The dependence of the rate of endocytosis on the external Ca^{2+} concentration

We assessed the rate of endocytosis by measuring the uptake of rhodamine B into the fibroblasts (Figure 4b). The amount of endocytosed rhodamine increased linearly with time. The rate of uptake was about 0.1 μM per minute (at an external rhodamine concentration of 100 μM). As it is possible that substances of different sizes are taken up at different rates [11], we compared the uptake rates of rhodamine B with those of the larger, dextran-tagged rhodamine B isothiocyanate. The results were virtually identical. The data from Figure 4b indicate that, at an external calcium concentration of 2 mM, the calcium uptake might be sufficient to increase the total cellular calcium concentration by about 2 μM per minute. The endocytic pathway therefore allows substantial cellular Ca^{2+} uptake.

Further experiments of this type were carried out at different external Ca^{2+} concentrations (Figure 4c). The rate of endocytosis was not very sensitive to reductions of the external Ca^{2+} level. Thus, a reduction of the external Ca^{2+} concentration from 1 mM to 100 μM resulted in only a marginal attenuation of the endocytosis rate. When the external Ca^{2+} concentration was dramatically reduced by adding the Ca^{2+} chelator EGTA to a nominally Ca^{2+}-free solution, however, then the rate of endocytosis fell sharply (Figure 4c). This may relate to the previous finding that retrieval of granule membrane inserted into the plasma membrane during exocytosis is blocked by removing extracellular Ca^{2+} [12].

Cells can take up Ca^{2+} from their environment via a number of different types of more-or-less selective Ca^{2+} channels in the plasma membrane [1–3] and can extrude Ca^{2+} via plasma membrane Ca^{2+} ATPases [13]. We have recently shown that substantial amounts of Ca^{2+} can also be exported via exocytosis in exocrine cells [6]. Our new data, obtained in fibroblasts, demonstrate the presence of a novel Ca^{2+} uptake pathway via endocytosis and rapid release of endosomal Ca^{2+}. This release of Ca^{2+} is linked to endosomal acidification and the acidification is dependent on the initial Ca^{2+} concentration in the endosomes.

Materials and methods

Cell culture and endocytic labelling

Swiss 3T3 Albino fibroblasts (European Collection) were plated on 22 mm round glass coverslips in DMEM as previously described [14] and used 2–4 days after plating. For fluorescent labelling of the endocytic vesicles the cells were incubated in a medium containing 100 μM of the fluorescent dye FITC–dextran 9.3 kDa, rhodamine B, rhodamine B isothiocyanate–dextran 9.3 kDa (Sigma) or Oregon Green 488 BAPTA-5N (Molecular Probes), in a CO_2 incubator at 37°C. After incubation the coverslips were transferred to a 200 μl perfusion chamber where coverslips were placed in contact with the objective. The cells were superfused continuously with a physiological buffer (140 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 50 mM Hepes, 10 mM glucose, pH 7.4) at 37°C or at room temperature. Measurements of the pH in endosomes were done by a two-point calibration procedure where the cells were exposed to a buffer containing high K^+ (100 mM Na^+ replaced by K^+) and nigericin (5 μM) at pH 7.3 and 5.3 [15]. The calcium concentrations were calculated using calibration with ionomycin (20 μM) in the presence of nigericin (5 μM) with 2 mM $CaCl_2$ and then 2 mM EGTA in a physiological buffer.

Confocal microscopy measurements

The experiments were performed using a laser scanning confocal microscope (Noran Instruments, USA) with × 60 oil objective (NA 1.4, Nikon). The excitation wavelength was 488 nm and the emission wavelengths were 530 nm for Oregon Green 488 BAPTA-5N and FITC–dextran and 600 nm for rhodamine B and rhodamine B isothiocyanate–dextran. The slit was 100 μm and we used slow mode (sample time 3200 nsec) and integration 8 (0.5 frames per sec) in our experiments. The images were processed using TwoD analysis software (Noran Instruments). The Ca^{2+} concentrations were calculated assuming a K_d of 20 μM for Oregon Green 488 BAPTA-5N (Molecular Probes).

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