J. Membrane Biol. 134, 1–13 (1993)

The Journal of
Membrane
Biology

Triggered Exocytosis and Endocytosis Have Different Requirements for Calcium and Nucleotides in Permeabilized Bovine Chromaffin Cells

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Received: 16 October 1992

Abstract. The intracellular requirements for membrane recapture in permeabilized chromaffin cells were compared to the requirements for exocytosis from the same cells.

In permeabilized bovine chromaffin cells, calcium-driven exocytosis also triggers, with a short delay, uptake of extracellular horseradish peroxidase (HRP). This internalized HRP remains compartmentalized within the cell and migrates to a low density band on a Percoll gradient which is distinct from the heavier chromaffin granules.

The amount of horseradish peroxidase internalized is similar in intact and leaky cells and is approximately equivalent to the volumes secreted. Endocytosis in both preparations is blocked by botulinum toxin, operates in a collapsed membrane potential, and is inhibited by low temperature. In permeabilized cells, exocytosis and coupled endocytosis are activated by the same concentrations of Ca^{2+} and MgATP. Although secretion requires Ca^{2+} and MgATP, once exocytosis has occurred the subsequent endocytosis can proceed in the virtual absence of Ca^{2+} or MgATP, and is largely unaffected by a variety of nucleotide triphosphates (including nonhydrolyzable analogues), and cyclic nucleotides.

These data suggest that endocytosis can proceed, once exocytosis has been triggered, under conditions that are quite different from those necessary to support exocytosis, and that the specific requirements for Ca^{2+} and MgATP in secretion are for the exocytotic limb of the secretory cycle rather than for the associated endocytotic pathway.

Key words: Endocytosis—Exocytosis—Calcium—Adrenal medulla

Introduction

Exocytosis involves the incorporation of vesicular membrane into the plasma membrane, thus increasing the surface area of the cell, while endocytosis leads to a decrease in membrane area. The surface area of the cell is kept constant by one balancing out the other. Such cycles of exocytosis and endocytosis have been described in many cell types and can proceed either constitutively or in response to an external stimulus (*for review see* Tartakoff, 1987). It is not clear, however, how exocytosis is coupled to endocytosis of an equivalent area of membrane. In bovine adrenal medullary cells cholinergic agonists trigger, in a calcium-dependent way, the secretion of catecholamine. Cholinergic stimulation also triggers the uptake of extracellular fluid phase markers, e.g., horseradish peroxidase. Electron microscopy has shown that the marker is located in the cell within small intracellular vesicles (Baker & Knight, 1981; Knight & Baker, 1983). Endocytosis rapidly follows the release of catecholamine, and continues after secretion is stopped abruptly (von Grafenstein, Roberts & Baker, 1986; von Grafenstein & Knight, 1992). Although endocytosis, which follows exocytosis, proceeds in the absence of extracellular calcium, the permeability barrier of the plasma membrane prevents control of the intracellular milieu, and no conclusions can be reached about the calcium dependence of endocytosis, or for that matter, about any other intracellular factors controlling endocytosis and its coupling to exocytosis.

In isolated bovine adrenal medullary cells rendered permeable by either high voltage electric fields or by detergents, this barrier is bypassed and the chemical composition of the intracellular environment can be accurately defined. Using such leaky

preparations it has been shown that in the presence of MgATP, micromolar levels of Ca^{2+} trigger catecholamine secretion (Knight & Baker, 1982; Dunn & Holz, 1983). Associated with triggered secretion is the uptake of extracellular horseradish peroxidase (HRP) into small intracellular vesicles (Baker & Knight, 1981; Baker, Knight & Roberts, 1982; Knight & Baker, 1982). This suggests that secretion from permeabilized cells might involve the same cycle of exocytosis and endocytosis as occurs in intact cells. These experiments do not shed any light, however, as to where within the secretory exocytosis/endocytosis cycle Ca^{2+} and MgATP are needed, nor how tightly coupled these two events are.

In this paper we describe results of experiments which separate the two events and so allow a comparison to be made of the intracellular factors necessary for each event. Our conclusions are that Ca^{2+} and MgATP are required for exocytosis, and that coupled endocytosis proceeds independently of Ca^{2+} and a variety of nucleotides once exocytosis has been triggered.

Materials and Methods

Solutions and Chemicals

The following solutions were used: Physiological saline (mM): NaCl, 150; KCl, 5; glucose, 10; $CaCl_2$, 2.5; $MgCl_2$, 1.8; Bovine serum albumin (BSA), 0.1%, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10; pH 7.3. In solutions containing zero calcium, Ca was replaced by Mg and 2 mM Ethylene glycol-O, O'-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) was added.

High potassium solution: The NaCl in the physiological saline solution was replaced iso-osmotically by KCl. Intracellular solution (mM): Potassium glutamate, 150; 2-deoxyglucose, 1; KCN, 1; Mg acetate, 2; EGTA, 2; piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), 20; pH 6.6. Cell fractionation buffer (mM): Sucrose, 250; EGTA, 2; HEPES, 10; pH 7.0; 0°C. Washing solution for permeabilized cells (mM): KCl, 150; PIPES, 2; pH 6.6 EGTA, 1; digitonin, 0.01; 0°C. Washing solution for intact cells: Ca-free physiological saline; 0°C. All chemicals, except for botulinum toxin, were obtained from Sigma. Purified botulinum toxin type D was obtained from Wako, Japan.

Preparation of Cells

Cells were isolated and cultured in multiwell plates at a density of 5×10^5 cells/well (von Grafenstein & Knight, 1992). For experiments in which the enzyme dopamine β-hydroxylase was assayed, cells were cultured in 9 cm petri dishes (2×10^7 cells/dish). Cultures (3–5 day) were used for experiments. In some experiments cultured cells were incubated with botulinum toxin type D as previously described (Knight, 1986; von Grafenstein, Borges & Knight, 1992).

Catecholamine Secretion and Horseradish Peroxidase Uptake Intact Cells

Secretion of catecholamine and endocytosis of HRP were monitored as described previously (von Grafenstein & Knight, 1992).

Leaky Cells

Cultured cells were rendered leaky by incubating them for 10 min at room temperature in intracellular solution containing 2 mM MgATP together with 10 μM digitonin. This treatment produces large holes in the plasma membranes thus rendering the cells leaky to macromolecules (Dunn & Holz 1983; Grant, Aunis & Bader, 1987). Digitonin was then removed by a brief wash (1 min) with intracellular solution containing 0.1% BSA (not delipidated) and 2 mM MgATP.

In some experiments cells were rendered leaky by electropermeabilization (described below). Catecholamine release and HRP uptake were similar in electropermeabilized and digitonin-permeabilized cells. However, as the time needed to electropermeabilize a culture plate of cells was much longer than the permeabilization procedure with digitonin, the latter method was used routinely. The experiments described in this paper which investigate endocytosis of HRP into leaky chromaffin cells refer only to measurements of HRP uptake into cells permeabilized with digitonin.

Permeabilized cells, bathed in "intracellular solution" together with 2 mM MgATP, were stimulated with various levels of Ca^{2+} buffered with EGTA (Knight & Baker, 1982). In some experiments HRP (1 mg/ml) was added together with the CaEGTA Buffer. In other experiments the cells were challenged first with Ca^{2+} for 10 min, the supernatant removed for assay of catecholamine, and the leaky cells washed twice (within 3 min) in intracellular solution containing various levels of Ca^{2+} or various nucleotides. HRP was then added as a marker to follow endocytosis. In experiments in which the concentration of Ca^{2+} was changed, the calcium buffer concentration (CaEGTA) was increased from 2 to 10 mM. Among the nucleotides tested were adenosine 5'-[β,γ-imido]triphosphate (AMPPNP), adenosine 5'-[β,γ-methylene]triphosphate (AMPPCP), adenosine 5'-O-[3-thiotriphosphate] (ATPγS), guanosine 5'-[β,γ-imido]triphosphate (GMPPNP), guanosine 5'-O-[3-thio]triphosphate (GTPγS). When cyclic nucleotides were used, 5 μM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was included. Experiments were performed at room temperature.

HRP uptake into leaky or intact cells was terminated by removing the bathing medium containing HRP and washing the 24-well culture plates or dishes containing the cells three times at 0°C in washing solution. The total washing period at 0°C took 15 min. Digitonin-permeabilized cells were washed in a washing medium containing 10 μM digitonin to ensure the plasma membrane remained leaky and thus facilitate the removal of any freely diffusible HRP within the cytosol. The HRP left in the cells after such a washing procedure is therefore expected to be trapped within intracellular vesicles.

Introducing Horseradish Peroxidase Directly into the Cytosol

HRP was introduced directly into the cytosol of cells at 0°C by electropermeabilizing the cultured cells in the presence of the enzyme. Very little HRP could enter or leave the cells within a

few minutes after electropermeabilization (*see* Fig. 4*c*), consistent with the plasma membrane being rendered transiently leaky to such a protein (Weaver et al., 1988).

Monolayers of cultured cells were electropermeabilized at 0°C in intracellular solution using a modified version of the technique used to electropermeabilize cells in suspension (Knight & Baker, 1982). Briefly, this involved applying eight 2 kV discharges from a 2 μF capacitor across two stainless steel electrodes, immersed in intracellular solution positioned 10 mm apart and 0.5 mm above a cell monolayer. The electrodes were rotated after each discharge. The time constant of each pulse was approximately 200 μsec. The time to give eight discharges was 25 sec, and the time taken to electropermeabilize all 24 wells was 12 min. Cells were electropermeabilized in the presence of 1 mg/ml^{-1} of HRP. Fifteen minutes after the last well was electropermeabilized, the extracellular label was removed by three washes, at 0°C over a further 15 min in washing solution lacking digitonin. This solution did not make the plasma membrane leaky and thus the freely diffusible HRP was retained in the cytosol. This procedure seemed to effectively and uniformly permeabilize the cells as judged by the loss, within 45 sec, of over 90% of $^{86}Rb^{+}$ previously loaded into the cells (2 hr loading in culture medium), and the uniformity of trypan blue staining 5 min after electropermeabilization.

Cell Fractionation and Percoll Density Gradient Centrifugation

After incubation with 1 mg/ml HRP, electropermeabilized or digitonin-permeabilized cells were washed in washing solution. The digitonin-permeabilized cells were washed in washing solution containing 10 μM digitonin, whereas the electropermeabilized cells were washed in buffers lacking digitonin. In the case of the electropermeabilized cells, this ensured that the diffusible cytosolic HRP was not lost during the washing procedure. Cells were suspended and homogenized in cell fractionation buffer as described elsewhere (von Grafenstein & Knight, 1992). The supernatant of the homogenate, containing subcellular organelles and membrane fragments, was mixed with Percoll to give a final Percoll concentration of 50%, and the suspension centrifuged at 4°C for 30 min at 65,000 × *g* in a Sorvall ultracentrifuge (T865 fixed angle rotor). The distributions of catecholamine, HRP and DβH throughout gradients were determined as described before (von Grafenstein & Knight, 1992). The freely diffusible solutes would be expected to remain evenly distributed throughout the gradient, whereas solutes compartmentalized within cell organelles would be expected to segregate into bands according to the bouyant densities of the organelles (Winkler, 1977; von Grafenstein & Neumann, 1983). Thus endocytosed HRP would be expected to migrate at a lower density, and the catecholamine associated with the heavy chromaffin granules would be expected to migrate towards the bottom of the tube. In order to determine the DβH distribution throughout the gradient, more material and larger sample volumes of the gradients were used. Less EGTA was used in the solution making up the gradient because of its inhibitory nature on the DβH assay (Knight & Baker, 1982). The cells were homogenized in a fractionation buffer containing only 0.2 mM EGTA and the final concentration in the Percoll gradient was 0.07 mM. Typically, 14 petri dishes of cells were used to determine the DβH, HRP and catecholamine distribution throughout Percoll gradients, 7 for cells challenged with Ca^{2+} and 7 for cells maintained in low Ca^{2+}. To keep the background levels of catecholamine and soluble DβH levels low throughout the gradient, Percoll gradients used for DβH assays were generated in two steps. First, the homogenates of cell fractions were mixed with Percoll to give a final Percoll concentration of 25% and centrifuged as described above. This level of Percoll generated a density gradient which showed only an appreciable density gradient at the bottom of the tube. Consequently, the chromaffin granules migrated to the bottom of the gradient, whereas a very broad HRP peak, containing very little contaminating catecholamine, was seen over the top half of the gradient. To sharpen up the bands of light organelle markers, the top half of this gradient was removed, Percoll added to give a final concentration of 45% and another density gradient was generated by centrifuging as described before. The resultant density gradient profile showed a marked increase in the density at the top end of the tube and thus a clearer resolution of the lighter bands.

Assays

Catecholamine was assayed and the activities of HRP and DβH determined as described previously (von Grafenstein & Knight, 1992).

Results

The Time Course of HRP Uptake in Leaky Cells during Triggered Membrane Recapture

Cells that had been permeabilized with digitonin took up HRP when incubated with this fluid phase marker. This proceeded linearly with time. When challenged with calcium, secretion was triggered and was accompanied by an increase in HRP uptake. Figure 1 shows the time course and extent of this uptake compared to catecholamine secretion. The data show that for leaky cells, triggered HRP uptake has a slower rate of onset compared to that of triggered catecholamine secretion. Catecholamine release increases mainly during the first ten minutes of release, whereas HRP uptake increases mainly during the subsequent ten-minute period. These data suggest that in experiments designed to investigate the coupling of exocytosis and endocytosis, catecholamine secretion is best stimulated first for ten minutes and the HRP uptake monitored during a subsequent ten-minute period.

Comparison between the Properties of Uptake into Leaky Cells and those into Intact Cells

Figures 2 and 3 compare some properties of triggered HRP uptake into leaky cells with that into intact cells.

Figure 2*a*, *b*, *c* summarizes the uptake of HRP

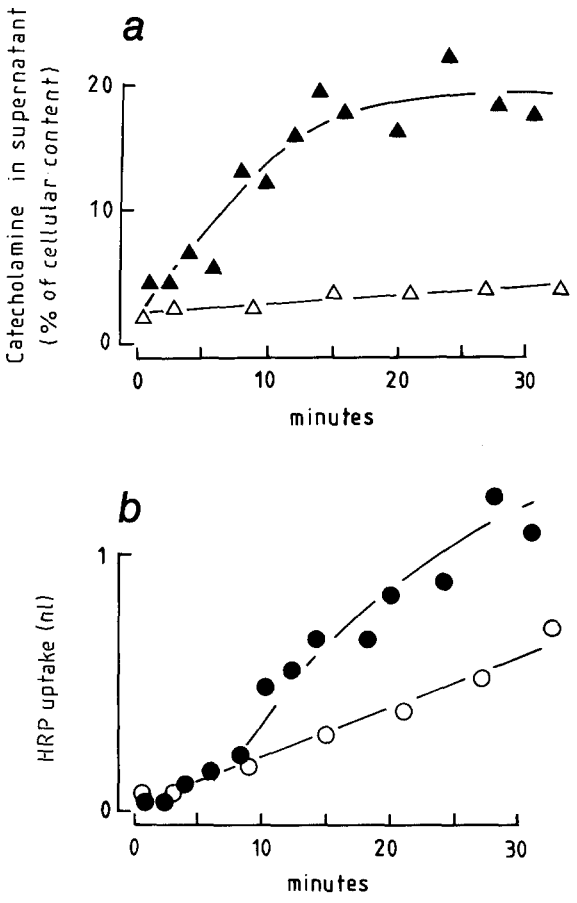

Fig. 1. Time course of catecholamine secretion and HRP uptake. Cultured chromaffin cells were permeabilized and treated with either 0.01 μM Ca^{2+} (open symbols) or 10 μM Ca^{2+} (filled symbols) in the presence of HRP for the various times shown. (*a*) Catecholamine secreted from cells expressed as a percentage of the cellular content. (*b*) The HRP content of the same cells described in *a* above, expressed as a volume of extracellular fluid taken up (1 mg of HRP corresponding to 1 ml).

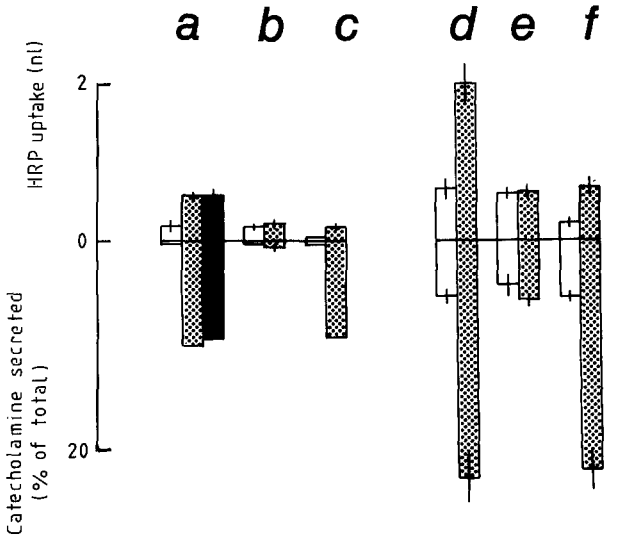

Fig. 2. Effect of depolarization, low temperature and botulinum toxin on triggered uptake of HRP into intact and permeabilized cells. (*a–c*) Intact cells: Cells were incubated in physiological saline solution for 3 min with 100 μM carbamylcholine (shaded bars), and the secretory response was measured. Cells not stimulated (open bars). Following this 3-min stimulation period, secretion was stopped, HRP was added to the extracellular fluid and the amount taken up into the cells over the next 10 min was measured. In some experiments 80 mM K^+ was included in exchange for an equimolar amount of Na^+, and Ca^{2+} was replaced by an equimolar amount of Mg^{2+} during this incubation period with HRP (filled bar). (*a*) Control cells. (*b*) Cells were pretreated with 50 μg/ml of botulinum toxin type D for three days before the experiment. (*c*) The temperature of the cells during the HRP uptake period was reduced to 0°C. In all other cases the temperature was 23°C. (*d–f*) Permeabilized cells. Cells were permeabilized in intracellular solution containing 2 mM MgATP and digitonin and stimulated with 10 μM Ca^{2+} for 10 min (shaded bar) or left unstimulated at 0.01 μM Ca^{2+} (open bar). The amount of catecholamine secreted over this 10-min period was measured. Following this, HRP was added to the extracellular fluid and the amount of HRP taken up into the cells was measured during a further 10 min. (*d*) Control cells. (*e*) Cells were pretreated with botulinum toxin (as in *b*). (*f*) The temperature of the cells during the HRP uptake period was reduced to 0°C. In all other cases it was 23°C. Catecholamine secreted from cells is expressed as a percentage of the cellular content, and the HRP content of the same cells is expressed as a volume of extracellular fluid taken up (1 mg of HRP corresponding to 1 ml). Data are means of four determinations. The error bars are the SEM.

into intact cells challenged with carbamylcholine. The amount of HRP taken up into the cells was independent of whether the cells were bathed in physiological saline or in a solution containing high concentrations of potassium, a condition that would depolarize the cell. Such data indicate that a maintained membrane potential is not essential for membrane recapture. Consistent with an earlier finding (von Grafenstein et al., 1992), carbamylcholine failed to trigger both catecholamine secretion and the associated HRP uptake in cells which had been pretreated with botulinum toxin (Fig. 2*b*). Fluid phase endocytosis following triggered exocytosis in intact chromaffin cells has previously been shown to be temperature sensitive (von Grafenstein et al., 1986) and Fig. 2*c* illustrates this by showing that although catecholamine was secreted in response to a carbamycholine challenge, the subsequent HRP uptake was inhibited by lowering the temperature.

Figure 2*d–f* summarizes the uptake of HRP, under similar conditions, into digitonin-treated cells which had been challenged with Ca^{2+}. Uptake occurred into these cells which, by virtue of having leaky plasma membranes, had collapsed membrane potentials. Pretreatment with botulinum toxin inhibited not only Ca^{2+}-driven catecholamine secretion but also HRP uptake. Lowering the temperature

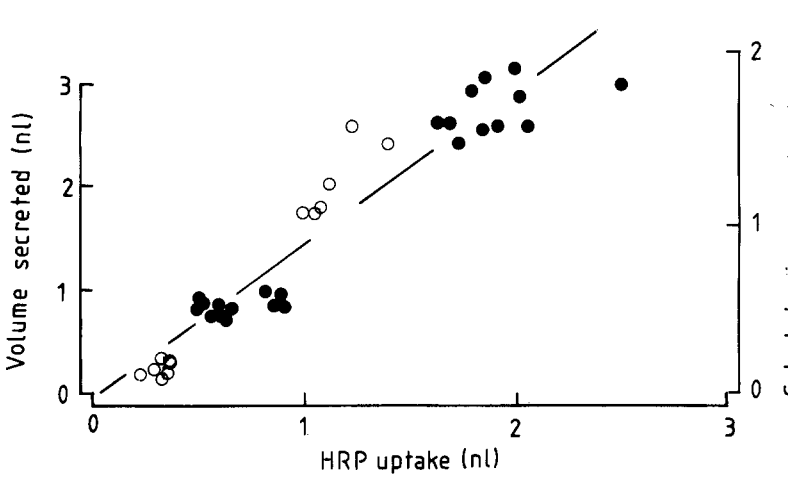

Fig. 3. The volume of triggered fluid phase uptake associated with secretion from intact and permeabilized chromaffin cells. The amount of HRP uptake into cell monolayers measured as a volume of extracellular fluid (1 ml corresponding to 1 mg of HRP) is plotted against the amount of catecholamine secreted from the monolayers. The amounts secreted are expressed as nmol of catecholamine, and also in terms of a volume (assuming the catecholamine is stored in the chromaffin granules at a concentration of 0.6 M). The graph shows data of the secretory response from, and associated uptake into, intact chromaffin cells challenged with various concentrations of carbamylcholine (○), and permeabilized cells challenged with various concentrations of Ca^{2+} (●).

after secretion had been triggered from leaky cells also inhibited the subsequent HRP uptake.

Figure 3 shows that the amounts of HRP taken up into stimulated leaky cells were very similar to the amounts taken up into stimulated intact cells. In both cases the uptake was proportional to the amount of catecholamine secreted. If the concentration of catecholamine stored in chromaffin granules is known then the amount of catecholamine secreted can be expressed in terms of a volume secreted (von Grafenstein et al., 1986). Assuming a catecholamine concentration in the granules of 0.6 M (Winkler & Westhead, 1980; Phillips, 1982) this calculation shows that the volumes of fluid phase taken up into both stimulated intact and stimulated leaky cells are close to the volumes secreted by these cells.

The Compartment Associated with Triggered HRP Uptake is Vesicular and Distinct from High Density Chromaffin Granules

The results of the subcellular fractionation studies on leaky chromaffin cells are shown in Fig. 4. Figure 4*a* shows that in a Ca^{2+}-free medium, the HRP equilibrated in the gradient at a low buoyant density (corresponding to 1.055 g/ml). This was distinct from the catecholamine peak (corresponding to 1.12 g/ml) associated with the heavier chromaffin granules. After secretion was triggered by exposure to a Ca^{2+} challenge, the increased HRP uptake into cells was seen as an increase in the magnitude of the low density peak (Fig. 4*b*). This narrow distribution of HRP on the Percoll gradient was in marked contrast to the distribution of HRP when the marker was directly introduced into, and trapped within, the cytosol. This was achieved by making use of the property that on electropermeabilization, the plasma membrane is transiently leaky to large molecules such as proteins. Figure 4*c* shows that electropermeabilization of cells at 0°C in the presence of HRP allowed some of the enzyme to enter the cells, which after several minutes could not be removed by repeated washing in a digitonin-free solution. Far less HRP was associated with cells when the marker was presented several minutes after electropermeabilization. Cell fractionation studies on cells which had HRP introduced directly into the cytosol by such a method showed the HRP to be equilibrated uniformly throughout the Percoll density gradient (Fig. 4*d*). This control experiment provides evidence that the single low density peak of HRP associated with triggered secretion from permeabilized cells is not an artifact of the fractionation process and the generation of Percoll gradients, but rather reflects a compartmentalization of the HRP within the cell.

The Secretory Granule Membrane Marker Dopamine β-Hydroxylase is Associated with HRP Following Triggered Endocytosis

Figure 5 shows the distribution of HRP, DβH, and catecholamine in Percoll gradients for leaky cells which have either been held at low Ca^{2+} or challenged to secrete catecholamine by 10 μM Ca^{2+}. Percoll (50%) often gave rise to a relatively high background catecholamine level throughout the gradient (*see* Fig. 4*a* and *b*) and was probably a consequence of lysis by the Percoll of some of the chromaffin granules isolated from digitonin-treated cells. The background was not as high when less Percoll was used. To reduce the background levels in the

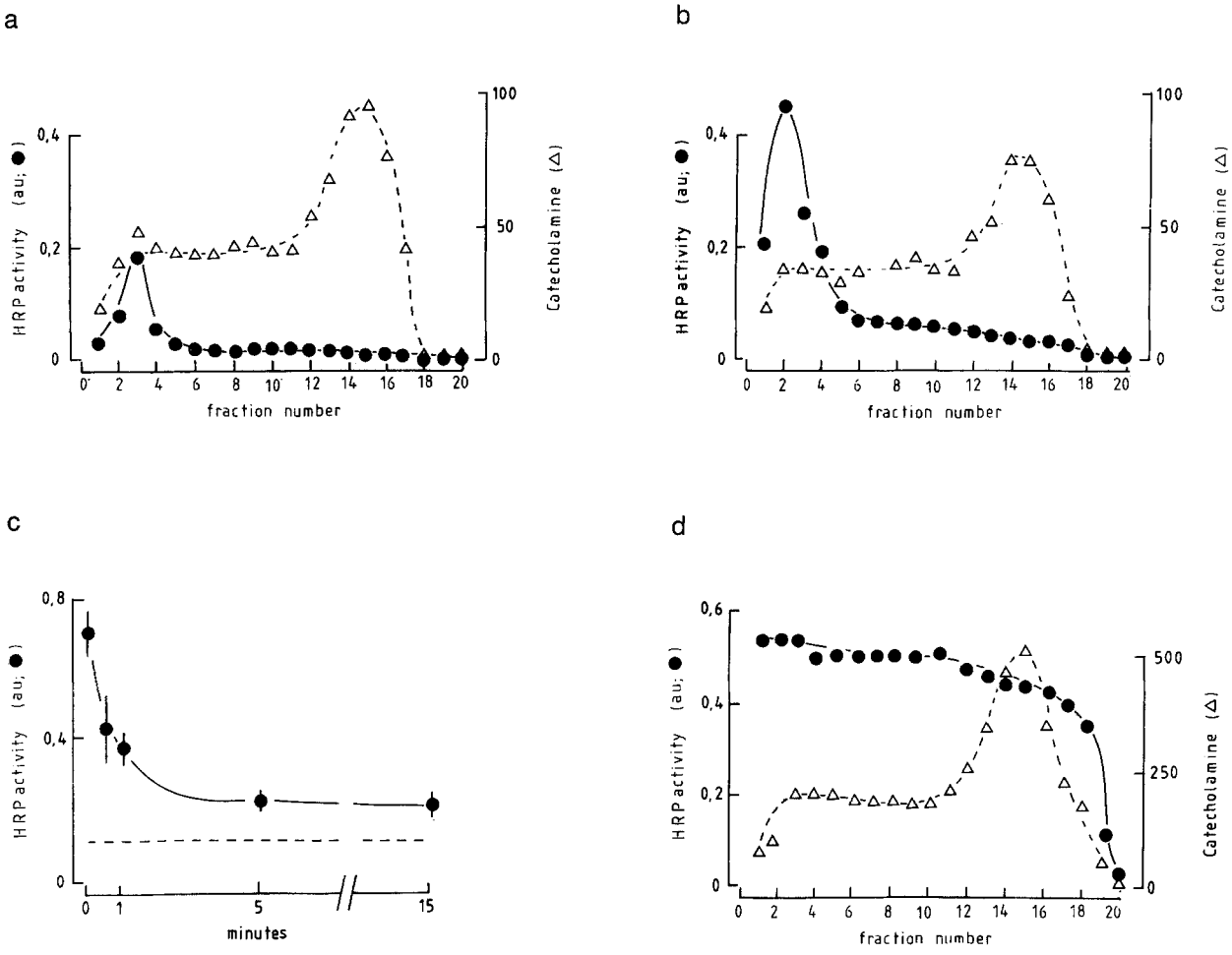

Fig. 4. Percoll density gradients showing the HRP taken up into stimulated leaky cells is in a compartment distinct from the catecholamine compartment, whereas HRP introduced directly into the cytosol is not compartmentalized. Permeabilized cells were challenged with 10 μM Ca^{2+} in the presence of 2 mM MgATP for 10 min and subsequently incubated at 23°C with 1 mg/ml HRP for a further 10 min. The same concentrations of Ca^{2+} and MgATP were maintained throughout. Permeabilized cells that were not challenged to secrete catecholamine were incubated at 23°C in a medium containing HRP and at a Ca^{2+} concentration close to 0.01 μM. The cells were then homogenized and centrifuged with Percoll to generate density gradients in which the subcellular organelles separated according to their buoyant densities. (*a*) Percoll density gradient distribution of HRP and catecholamine in permeabilized cells exposed to 0.01 μM Ca^{2+}. (*b*) Percoll density gradient distribution of HRP and catecholamine in permeabilized cells challenged to secrete by exposure to 10 μM Ca^{2+}. (*c*) Accessibility of HRP to the cytosol of electropermeabilized cells. Cultured cells were rendered leaky by electropermeabilization at 0°C and afterwards, at the various times shown, were exposed to 1 mg/ml HRP for 15 min before being washed free of the extracellular HRP in a digitonin-free washing solution. The ordinate shows that amount of HRP associated with the electropermeabilized cells, the error bars being the SEM of between six and three determinations. The dotted line shows the amount of HRP associated with intact cells treated in a similar fashion. (*d*) Percoll density gradient distribution of HRP and catecholamine in the cells which had been electropermeabilized at 0°C in the presence of HRP, 2 mM MgATP and 0.01 μM Ca^{2+}. The HRP (●) and catecholamine (Δ) content of the Percoll density fractions are shown in arbitrary units.

low density region of the gradient, the gradients were generated in two steps. First, the supernatant of the cell homogenate was mixed with 25% Percoll and a gradient generated. Figure 5*a* and *b* show the HRP, DβH and catecholamine distribution throughout two such gradients for leaky cells incubated in low Ca^{2+} (Fig. 5*a*) and for cells triggered to secrete with 10 μM Ca^{2+} (Fig. 5*b*). The gradients have a slowly changing low density profile, the density only rising from 1.025 to 1.06 g/ml over the first seven fractions. The fairly narrow HRP band seen in the 50% Percoll gradient, corresponding to a density of 1.055 g/ml, therefore appeared as a broad band over the top half of a 25% Percoll gradient. The catecholamine level over this gradient range was very low because the chromaffin granules migrated to a narrow band at the bottom

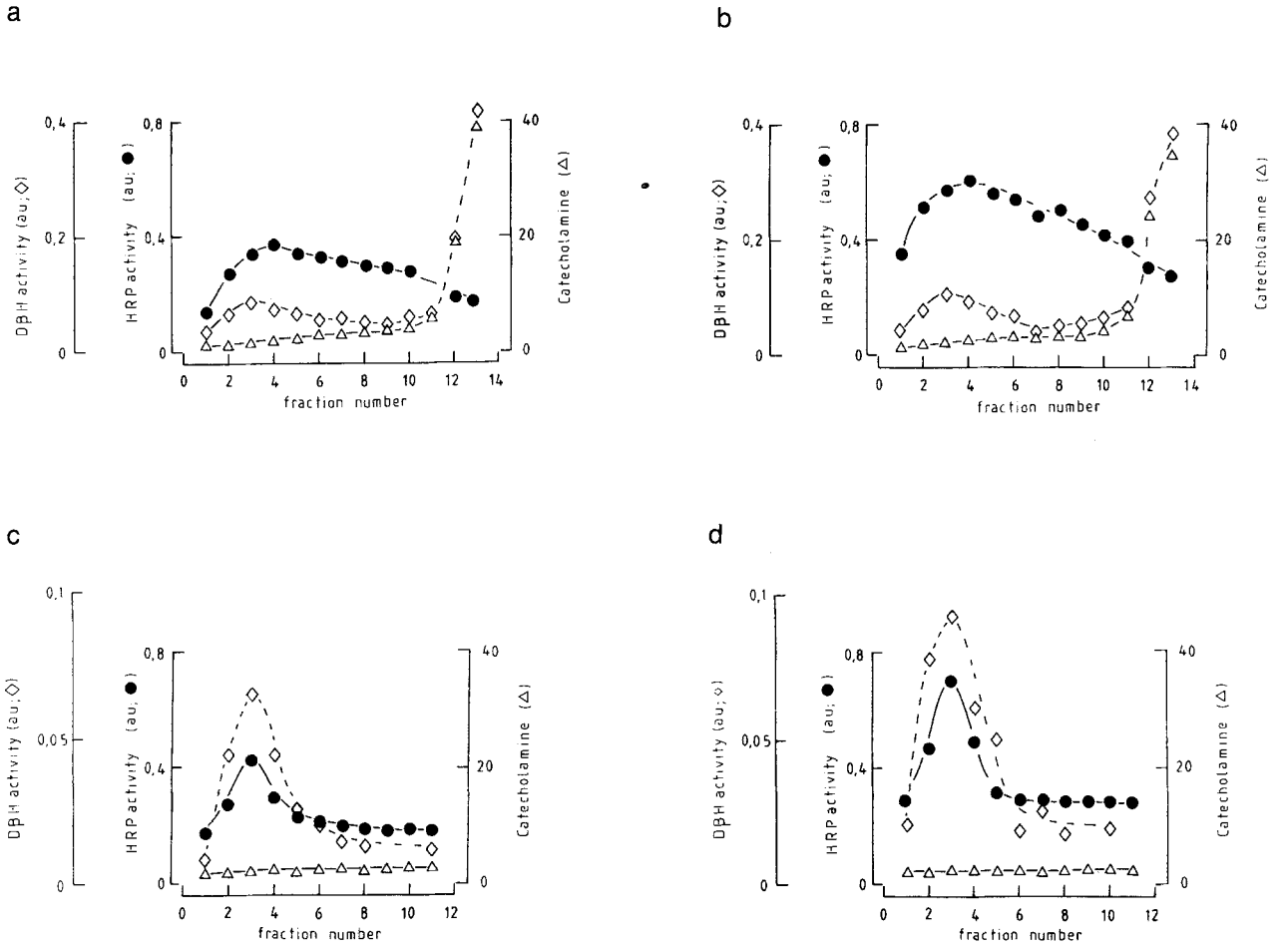

Fig. 5. Percoll density gradients showing that the cellular compartment of triggered endocytosis of HRP in leaky cells is associated with the secretory granule marker dopamine β-hydroxylase. Cells, cultured on 9 cm petri dishes were permeabilized with digitonin, and challenged in the presence of 2 mM MgATP and 1 mg/ml HRP with (*a, c*) 0.01 μM Ca^{2+} or with (*b, d*) 10 μM Ca^{2+} for 20 min before being washed three times, fractionated, mixed with 25% Percoll and the density gradient generated (*a, b*). The top half of each of these density gradients, i.e., the first seven fractions, were mixed with 45% Percoll and new density gradients generated (*c, d*). The data show the activities of HRP (●), DβH (◇) and catecholamine (Δ) throughout the gradients and are given in arbitrary units.

of the gradient where the density increased steeply from 1.07 at fraction 11 to about 1.16 at fraction 13. DβH was distributed throughout the gradient mainly as a dense narrow band associated with the chromaffin granules, and also as a low density broad peak. The relative distributions in the gradient of HRP, DβH and catecholamine remained unchanged for stimulated or unstimulated leaky cells, the only apparent difference being in the larger HRP and the low density DβH signals in stimulated cells. The increase in both low density DβH and HRP is more clearly seen in Fig. 5*c* and *d*. Here the material of the first seven fractions of the 25% Percoll gradient was re-equilibrated on 45% Percoll gradients. Both HRP and DβH migrated to narrow bands, the peaks corresponding to 1.055. The amounts of HRP and DβH associated with the peaks increased for the stimulated cells (Fig. 5*d*). Three other experiments yielded similar results.

Consistent with the findings of triggered endocytosis in intact chromaffin cells, these data provide evidence that following triggered exocytosis in leaky cells, the secretory vesicle membrane is recaptured trapping the low density extracellular fluid.

The Dependence of Endocytosis on Ca^{2+}-Activated, ATP-Dependent Exocytosis

The dependence of endocytosis on Ca^{2+}-activated exocytosis in leaky cells is shown in Fig. 6. Exocytosis and coupled endocytosis were both activated

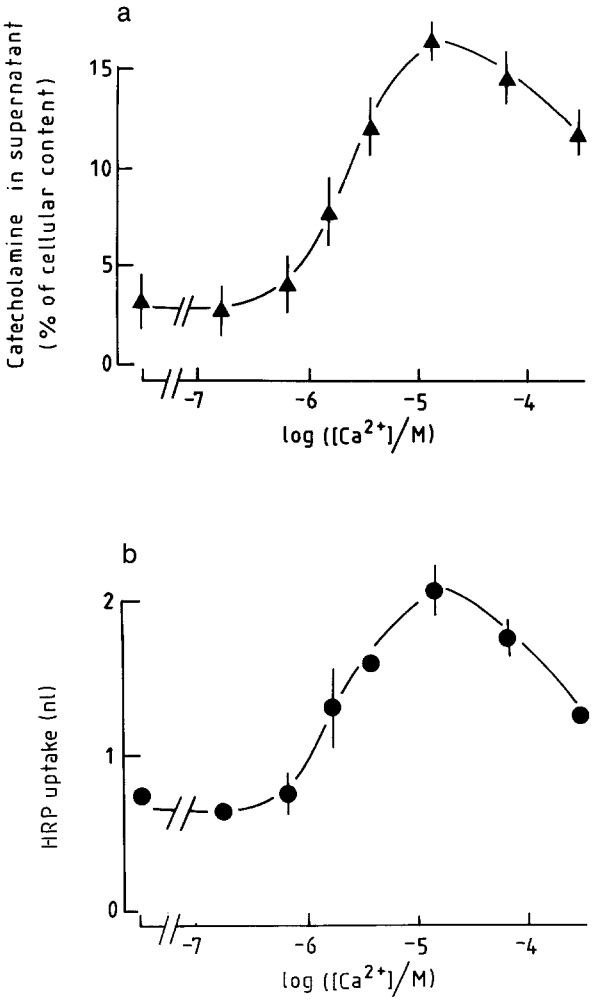

Fig. 6. Ca dependence of the exocytosis/endocytosis secretory cycle. Cells were permeabilized and challenged with various concentrations of Ca^{2+} in the presence of 2 mM MgATP. After 10 min, the medium was removed for assay of secreted catecholamine, and intracellular solution containing 1 mg/ml HRP was added to the cell monolayers. The cells were incubated for a further 10 min at 23°C. The same concentrations of Ca^{2+} and MgATP were maintained throughout. The cell monolayers were washed and assayed for catecholamine content and HRP activity. The data are means of three determinations (SEM) and are plotted against the concentrations of Ca^{2+} used. (*a*) Catecholamine secreted from cells expressed as a percentage of the cellular content. (*b*) The HRP content of the same cells expressed as a volume of extracellular fluid taken up into the cell monolayers, 1 mg of HRP corresponding to 1 ml.

by μM levels of Ca^{2+}, half-maximal secretion and the associated fluid phase uptake occurring close to 1–2 μM Ca^{2+}, and maximal secretion and uptake occurring at 10 μM Ca. When the Ca^{2+} concentration was exceeded 10 μM Ca^{2+}, the extents of both catecholamine secretion and fluid phase uptake decreased. It has been shown elsewhere that catecholamine secretion requires mM levels of MgATP, nonhydrolyzable analogues such as AMPPNP failing to replace MgATP. Figure 7 shows that coupled endocytosis occurred at the same levels of MgATP and, like secretion, was not triggered in the presence of AMPPNP.

The data presented so far suggest that fluid phase uptake following triggered exocytosis show common features in intact and permeabilized cells, and support the idea that exocytosis drives endocytosis. Although these data support a role for Ca^{2+} and MgATP in triggered exocytosis, they do not shed any light on possible involvement of these agents in endocytosis because the solutions have not been changed after triggering exocytosis and thus the apparent Ca^{2+} and ATP dependence of endocytosis may simply reflect its dependence on exocytosis.

The Ca^{2+} and Nucleotide Dependence of Endocytosis Following Triggered Exocytosis

To investigate the Ca^{2+} requirements of endocytosis following triggered exocytosis, leaky cells were first challenged to secrete catecholamine by exposure to 10 μM Ca^{2+} for 10 min, and the Ca^{2+} concentration then changed before monitoring subsequent endocytosis. Figure 8*a* shows the results of such an experiment. Here the initial calcium challenge elicited secretion of 19% of the cellular content of catecholamine. Although the Ca^{2+} level was subsequently reduced to levels that did not support secretion (Fig. 8*b*) HRP uptake proceeded over the following 10 min in an almost Ca^{2+}-independent manner. Cells that had not previously been challenged with calcium secreted about 6% of the cellular content of catecholamine in response to this second challenge and also took up a small amount of HRP during this time, consistent with the slow uptake shown in Fig. 1.

A similar protocol was used to investigate the nucleotide dependence of endocytosis following triggered exocytosis, and the results are shown in Fig. 9. After stimulating secretion for 10 min with 10 μM Ca^{2+} in the presence of 2 mM MgATP, which led to a 13% release of catecholamine, exocytosis was blocked by reducing Ca^{2+} to 0.01 μM. The solutions were changed by briefly washing the cells with intracellular solution containing various levels of MgATP or AMPPNP (the nonhydrolyzable ATP analogue) before HRP uptake was monitored during the following 10 min. Figure 9 shows that once exocytosis had been activated, endocytosis proceeded in the apparent absence of MgATP and even in the presence of the nonhydrolyzable ATP analogue. Figure 9*b*, which is effectively an in situ control, showed

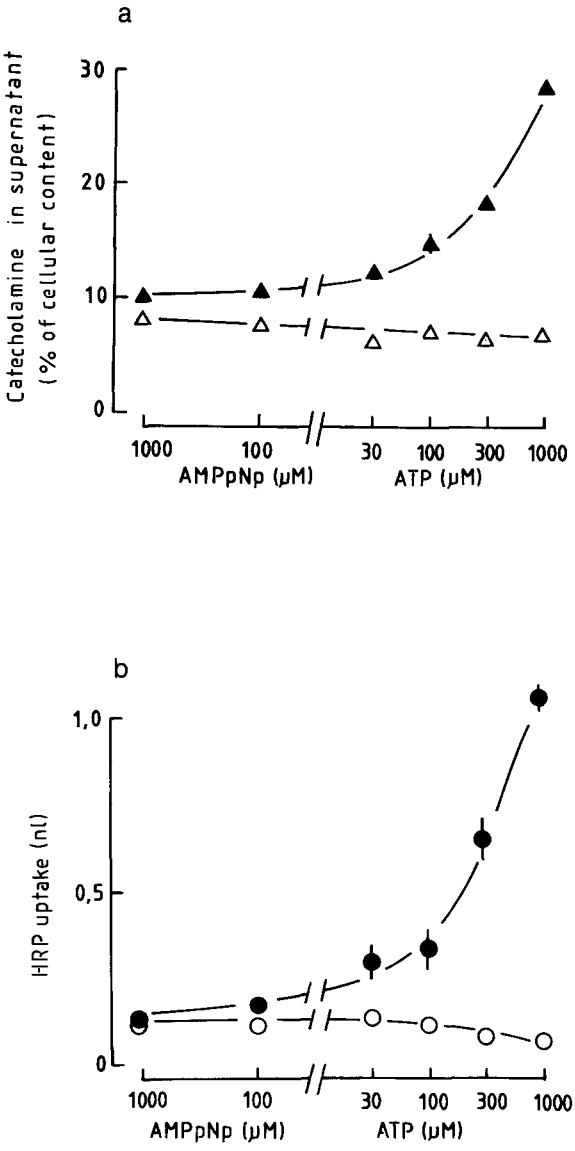

Fig. 7. MgATP-dependence of the exocytosis/endocytosis secretory cycle. Cells were permeabilized and equilibrated with the various concentrations of MgATP or MgAMPPNP shown for 5 min before being challenged with 10 μM Ca^{2+} (filled symbols) or kept close to 0.01 μM Ca^{2+} (open symbols) for 10 min. After this time, the supernatants were removed to measure the catecholamine secreted, and 1 mg/ml HRP added to the cell monolayers for a further 10 min. The same concentrations of Ca^{2+}, MgATP and AMPPNP were kept constant throughout. Cells were then washed and assayed for catecholamine content and HRP enzyme activity. Temperature throughout was 23°C. (*a*) Catecholamine secreted from cells expressed as a percentage of the cellular content. (*b*) The HRP content of the same cells expresed as a volume of extracellular fluid taken up into the cell monolayers, 1 mg of HRP corresponding to 1 ml. Data are means of three determinations (SEM) and are plotted against the concentrations of MgATP and MgAMPPNP used.

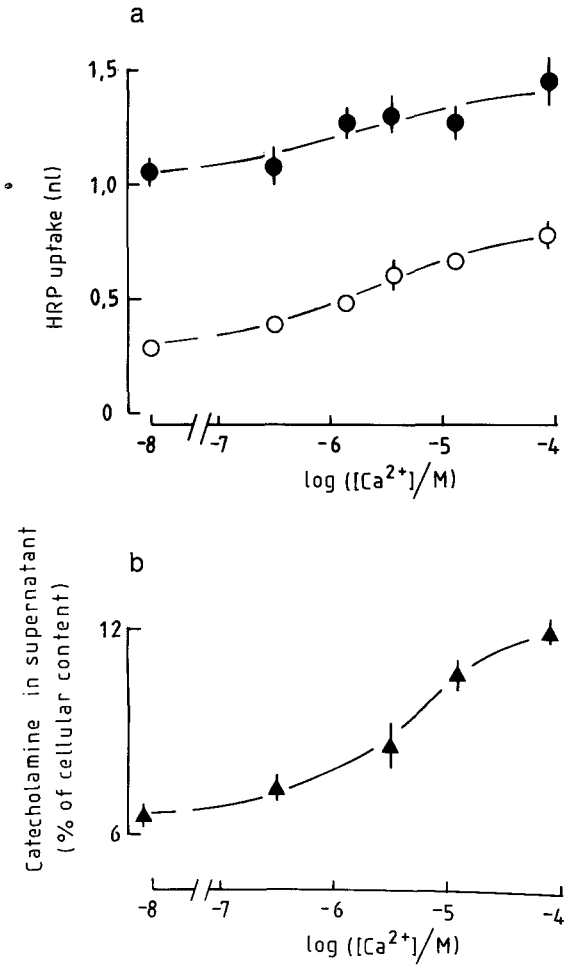

Fig. 8. The Ca^{2+}-dependence of endocytosis, following triggered exocytosis, is distinct from that of exocytosis. Cells were permeabilized and stimulated with 10 μM Ca^{2+} (buffered with 2 mM EGTA) for 10 min in the presence of 2 mM MgATP (prestimulated cells). Unstimulated cells were incubated in the presence of 0.01 μM Ca^{2+}. The supernatant was removed from the monolayers for assay of secreted catecholamine. The medium was replaced by intracellular solution containing the indicated concentrations of Ca^{2+} (buffered with 10 mM EGTA) and 2 mM MgATP. To ensure a complete solution change, the cells were washed twice, within 3 min, with the new solutions before HRP (1 mg/ml) was then added and the extent of HRP uptake measured after a further 10 min. Data are means of three determinations (SEM) and are plotted against the concentrations of Ca^{2+} used. Temperature 23°C. (*a*) HRP uptake into prestimulated cells (●); HRP uptake into cells that had not been prestimulated (○). The HRP content is expressed as a volume of extracellular fluid taken up into the cell monolayers, 1 mg of HRP corresponding to 1 ml. (*b*) Ca^{2+} dependence of catecholamine secretion: Cells, earlier held at 0.01 μM Ca^{2+} in the presence of 2 mM MgATP, were washed with solutions containing the various concentrations of Ca^{2+} shown, and the catecholamine secreted over 10 min was measured (▲). The catecholamine released over this period is shown, expressed as a percentage of the cellular content.

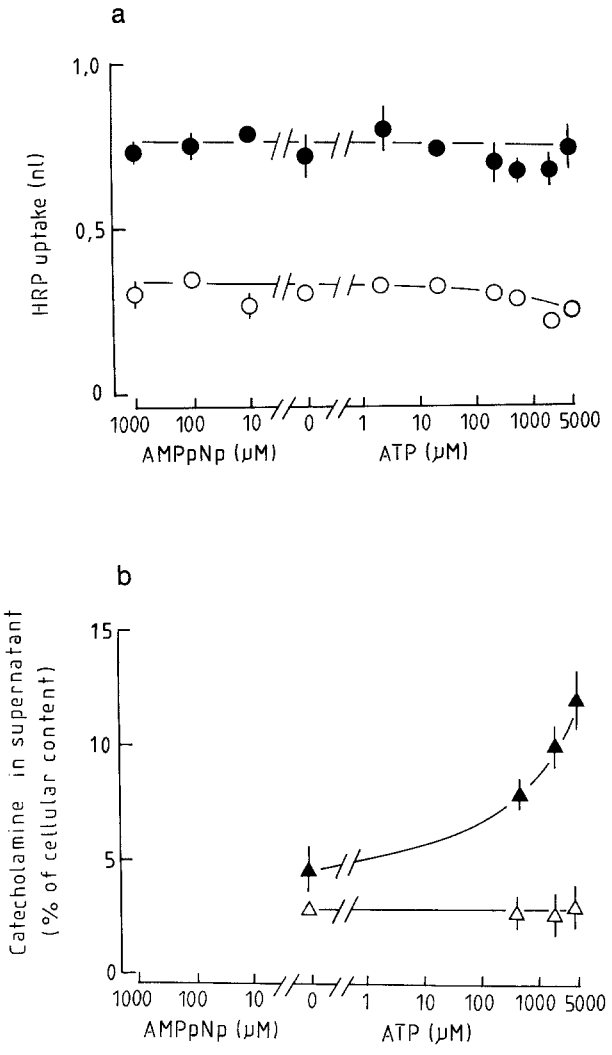

Fig. 9. The MgATP-dependence of endocytosis, following triggered exocytosis, is distinct from that of exocytosis. Cells were permeabilized and stimulated for 10 min with 10 μM Ca^{2+} in the presence of 2 mM MgATP (prestimulated cells). Unstimulated cells were incubated in the presence of 0.01 μM Ca^{2+} and 2 mM MgATP. The medium was then replaced by intracellular solution containing the indicated concentrations of nucleotide in the presence of 0.01 μM Ca^{2+}. To ensure a complete solution change, the cells were washed twice, within 3 min, with the new solutions. HRP (1 mg/ml) was then added and the extent of HRP uptake measured after a further 10 min. (*a*) MgATP dependence of endocytosis: The HRP content expressed as a volume of extracellular fluid taken up into the cell monolayers, 1 mg of HRP corresponding to 1 ml. HRP uptake into prestimulated cells (●); HRP uptake into cells that had not been prestimulated (○). (*b*) MgATP dependence of catecholamine secretion: Cells, earlier held at 0.01 μM Ca^{2+} in the presence of 2 mM MgATP, were washed with the various concentrations of the nucleotides shown (as described above) and treated for 10 min with either 0.01 μM Ca^{2+} (△) or 10 μM Ca^{2+} (▲). The catecholamine released over this period is shown, expressed as a percentage of the cellular content. This secretory response shows that the procedure of washing the cells reduces the MgATP concentration to a level unable to support exocytosis. Temperature 23°C. Data are means of three determinations (SEM).

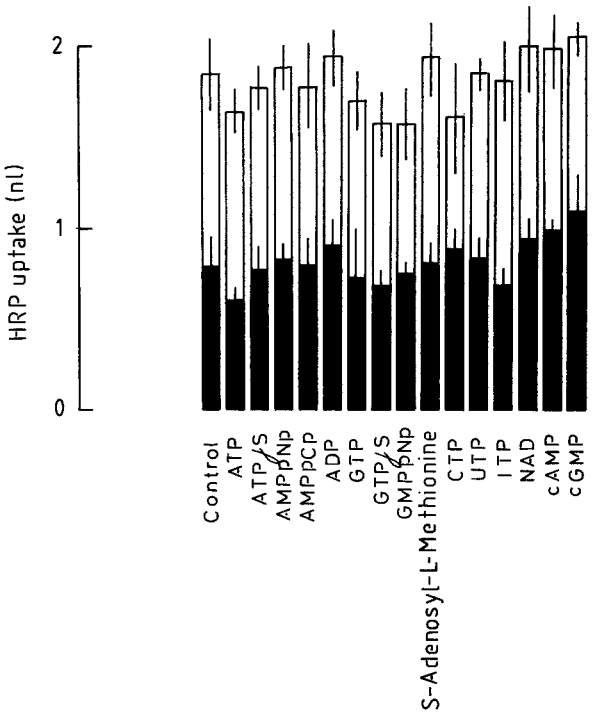

Fig. 10. The effect of nucleotides on endocytosis following triggered exocytosis. Cells were permeabilized and incubated, in the presence of 2 mM MgATP, with either 0.01 μM Ca^{2+} or 10 μM Ca^{2+}. After 10 min the extracellular fluid was replaced by intracellular solution containing the 100 μM of the various nucleotides shown and at a Ca^{2+} level of 0.01 μM. To ensure a complete solution change, the cells were washed twice with the new solutions before HRP was added to the extracellular medium and the extent of fluid phase endocytosis monitored after a further 10 min. The HRP content is expressed as a volume of extracellular fluid taken up into the cell monolayers, 1 mg of HRP corresponding to 1 ml. The filled bars show the uptake, measured in the presence of the various nucleotides, into cell monolayers that had only been exposed to 0.01 μM Ca^{2+}. The uptake of extracellular HRP into cells challenged to secrete catecholamine by 10 μM Ca^{2+} was larger than this background level for all nucleotides used. This increase in the uptake is shown by the open bars. No nucleotides were added to controls. Temperature throughout was 23°C. Data are means of six determinations, the error bars are the SEM.

that this brief washing and nucleotide replacement procedure led to levels of nucleotide which did not support calcium-dependent secretion.

The significance of these results, therefore, is that endocytosis can proceed, once exocytosis has been triggered, at Ca^{2+} and MgATP levels that will not support exocytosis. Figure 10 summarizes the apparent lack of effect of a variety of nucleotides on triggered uptake and includes the nucleotide triphosphates UTP, GTP, ATP, CTP and ITP, the non-hydrolyzable nucleotides AMPPNP, AMPPCP, ATPγS, GMPPNP and GTPγS, the nucleotide di-

phosphate ADP, cyclic nucleotide monophosphates 3′,5′cAMP and 3′,5′cGMP, S adenysyl L methionene and NAD. Reducing the temperature to 4°C was the only maneuver that clearly inhibited endocytosis following triggered exocytosis (Fig. 2).

The nature of this Ca^{2+}- and nucleotide-"independent" uptake of HRP seems to be by endocytosis as the trapped HRP is again found associated with vesicles of low buoyant density on a Percoll gradient (*data not shown*).

Discussion

Endocytosis in intact bovine chromaffin cells follows exocytosis evoked by carbamylcholine, even in the presence of depolarizing levels of extracellular potassium. A normal membrane potential therefore seems not to be essential for endocytosis following triggered exocytosis.

In permeabilized cells, where the membrane potential is collapsed, HRP uptake follows Ca^{2+}-driven catecholamine secretion. Several findings suggest that this uptake is due to endocytosis. First, the uptake of HRP into leaky cells cannot be washed out in solutions containing digitonin, a condition that generates sufficiently large holes in the plasma membrane of chromaffin cells to allow free passage of diffusible cytosolic proteins at least as large as HRP (Grant et al., 1987). Second, HRP taken up into cells is recoverable on density gradients as a single peak of low buoyant density consistent with the compartment being an endocytotic vesicular compartment. The magnitude, but not density, of this peak varies with the extent of catecholamine secretion and HRP uptake. Associated with this HRP peak is the chromaffin granule marker, dopamine β-hydroxylase, whose magnitude parallels that of HRP uptake. One interpretation to these data is that membrane recapture includes chromaffin granule membranes (Phillips et al., 1983; Patzak & Winkler, 1986; von Grafenstein & Knight, 1992). A similar low density HRP peak and an associated DβH peak are found with triggered HRP uptake into intact cells (von Grafenstein & Knight, 1992). Third, the amounts of triggered HRP uptake into permeabilized cells are very similar to amounts taken up into intact cells. In both cases the HRP space taken up is similar to the volume that is released from secretory granules. Taken together the data suggest that the underlying features of triggered endocytosis in leaky cells are similar to those observed in intact cells.

Stimulation-dependent HRP uptake shows the same calcium and MgATP dependence as catecholamine secretion. This is interpreted as endocytosis being driven by exocytosis. An alternative explanation could be that endocytosis is triggered by calcium in the presence of MgATP in a similar manner to, but independently from, exocytosis. Several lines of evidence support the former suggestion and argue against the latter.

First, incubating the cells with botulinum toxin not only renders the cells incapable of exocytosis, but also prevents HRP uptake even though optimal Ca^{2+} and MgATP are present. These data are consistent with the results of earlier studies of triggered endocytosis in intact chromaffin cells (von Grafenstein et al., 1992). Although it cannot be excluded that botulinum toxin blocks endocytosis independently of exocytosis, this seems unlikely as most biological toxins which are active at very low concentrations are also very specific in their site of action.

Second, once exocytosis has occurred, endocytosis can proceed in the nominal absence of Ca^{2+} and MgATP—conditions that do not support exocytosis.

Ca^{2+}-dependent or Ca^{2+}-independent forms of endocytosis have been reported in other systems (Koike & Meldolesi, 1981; Lew et al., 1985; Di Virgilio et al., 1988; Maruyama, 1989) and there seems to be no general rule as to whether endocytosis is Ca^{2+} dependent or not. As continuous endocytosis requires the plasma membrane area to be balanced by exocytosis, any apparent Ca^{2+}-dependent endocytosis may, in fact, simply reflect a previous calcium-dependent exocytotic step.

The experiments carried out here to investigate the nucleotide dependence of membrane recapture following triggered exocytosis also support the idea that exocytosis drives endocytosis. If the concentration of ATP is varied before triggering exocytosis with calcium, the HRP uptake measured afterwards faithfully reflects the ATP-dependence of catecholamine secretion. Once exocytosis has been triggered, however, endocytosis seems to proceed independently of MgATP, or for that matter, independently of many other nucleotides tested. This apparent independence on MgATP and other nucleotides of endocytosis following exocytosis is surprising. Chromaffin cells contain large amounts of MgATP in secretory vesicles and so it is difficult to exclude the possibility that some MgATP leaks out and becomes available in the cytoplasm. Endogenous ATP production may provide an alternative source of ATP. However, the use of the metabolic inhibitors KCN and 2-deoxyglucose did not prevent membrane retrieval and the presence of the nonhydrolyzable ATP analogues were not inhibitory. In contrast to membrane retrieval, exocytosis was found to be strongly dependent on MgATP in samples that were run in parallel to those in which endocytosis was studied. We can therefore conclude that

if any ATP is needed to support membrane retrieval, the concentration required is much lower than that required for exocytosis. Membrane recapture is somewhat slower in permeabilized cells than in intact cells. At present, we cannot exclude the possibility that in permeabilized cells a cytoplasmic factor is lost that accelerates membrane recapture and requires ATP, although we believe that in this case we would have detected at least some effect of ATP or some inhibition by nonhydrolyzable ATP analogues. The metabolic requirements of other endocytotic systems have been studied in some detail. For example receptor-mediated endocytosis of transferrin occurs via coated pits and has recently been found to be ATP dependent (Smythe et al., 1989). Internalization of insulin, on the other hand, although receptor mediated, can occur via uncoated invaginations and seems not to be ATP dependent (Smith & Jarett, 1990). Membrane recapture following exocytosis may be a third, distinct form of endocytosis. Here, the energy-requiring step may be the insertion of secretory vesicle membrane or priming of secretory vesicle membrane by an ATP-dependent phosphorylation step prior to exocytosis and endocytosis. It has been suggested that secretory vesicle membrane does not mix with the plasma membrane after exocytotic membrane fusion and that post exocytosis membrane recapture is specific for secretory vesicle membrane (Dowd et al., 1983; Phillips et al., 1983; Patzak & Winkler, 1986; Bonzelius & Zimmermann, 1990). In this arrangement there is no need to move proteins into coated pits and this may explain the lack of ATP-dependence in post-secretory membrane recapture. The energy-requiring step may be the insertion of secretory vesicle membrane into the plasma membrane or priming of secretory vesicle membrane by an ATP-dependent phosphorylation step prior to exocytosis and endocytosis.

Some exergonic dephosphorylation may occur during exocytosis and some during membrane recapture. It seems unlikely, however, that a second, Ca^{2+}-triggered and ATP-dependent phosphorylation step is required once exocytosis has occurred.

The data presented here shed light not only on the regulation of endocytosis, but are also relevant for the understanding of secretory exocytosis. As a complete secretory cycle may involve both exocytosis and endocytosis, any factors that are needed for secretion to proceed may reflect the requirements of exocytosis, endocytosis or both. Our data show rather clearly that the requirements for exocytosis and coupled endocytosis are distinct in chromaffin cells and that the Ca^{2+} and ATP requirements for the secretory cycle found in earlier studies are most likely those for exocytosis rather than for endocytosis.

We are grateful to Mr. John Gibbs for excellent technical assistance, and to the Medical Research Council (UK) for financial support.

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