

Kinetic Analysis of the Triggered Exocytosis/Endocytosis Secretory Cycle in Cultured Bovine Adrenal Medullary Cells

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Abstract. Cultured bovine adrenal medullary cells are an excellent preparation for quantitative analysis of the secretory exocytosis/endocytosis cycle. In this paper we examine the kinetics of endocytosis after stimulation of secretion. Membrane retrieval was monitored by uptake of the fluid phase marker horseradish peroxidase. Horseradish peroxidase was found to be suitable because it can be washed off completely, assayed quantitatively, and its uptake increases linearly with concentration. If this marker is present during stimulation, the rate of uptake is initially slower than catecholamine secretion but faster at a later time, suggesting that the formation of endocytotic vesicles follows exocytosis. To monitor the time-dependent concentration of secretory vesicle-plasma membrane fusion product (Ω -profiles), secretion was halted at various

time intervals after stimulation and the excess membrane allowed to transform into endocytotic vesicles in the presence of horseradish peroxidase. By adding horseradish peroxidase at various times after inhibition of secretion, the time course of membrane retrieval could be measured directly.

All our results are consistent with a two-step kinetic model in which exocytosis and membrane retrieval are consecutive events. The estimated volumes of the compartments involved are roughly equal. The rate of endocytosis is strongly temperature-dependent but unaffected by extracellular calcium in the range of 10^{-8} – 2.5×10^{-3} M, suggesting that calcium is not required at the site of endocytotic membrane fusion. Membrane retrieval is also unaffected by Lanthanum (1 mM) but is slowed by hypertonic media.

CHROMAFFIN cells secrete catecholamines by exocytosis, during which secretory vesicle membrane is inserted into the plasma membrane permitting discharge into the extracellular space of the contents of the secretory vesicles. Since exocytosis increases the cell surface, it must in the long term be balanced by endocytosis of an equivalent membrane area (1, 17). A complete secretory cycle, therefore, involves both exocytosis and membrane retrieval. Valuable information on secretion has been obtained by assaying the appearance of secretory product in the extracellular space. An important feature of exocytosis triggered by physiological stimuli is its absolute requirement for extracellular calcium (6–8). Stimulation permits calcium to enter the cell (5, 29) and, in the presence of MgATP, a rise in the cytoplasmic free Ca concentration seems to be a sufficient stimulus to activate the secretory cycle (2, 11, 13). However, the details of coupling between exocytosis and endocytosis are not known and it has proved difficult to measure endocytosis under conditions where it can be separated from exocytosis.

Several groups have characterized the exocytosis/endocytosis cycle morphologically using fluorescence immunocytochemical methods (9, 24) or immunogold labeling tech-

niques (22) based on antigens of the chromaffin granule membrane which become exposed at the cell surface during exocytotic secretion. These studies are not quantitative but clearly show that secretory vesicle membrane does not mix freely with the plasma membrane (22, 24). Lingg et al. (15) and Patzak et al. (21) have made an attempt to characterize membrane retrieval quantitatively. They were able to demonstrate that, in agreement with morphological results, antigens of the secretory vesicle inner membrane become exposed to the cell surface during secretion and largely disappear within 30 min. These studies set an upper limit to the time required for retrieval of specific proteins, but so far, no truly quantitative information is available on the kinetics of the exocytosis/endocytosis cycle. Here we use the fluid phase marker horseradish peroxidase (HRP)¹ to monitor the time course of endocytosis and bulk membrane retrieval quantitatively. Our results can be explained by a kinetic model which assumes that exocytotic membrane fusion and endocytotic membrane retrieval are consecutive events. Extracellular calcium is not essential for membrane retrieval.

1. *Abbreviation used in this paper:* HRP, horseradish peroxidase.

Materials and Methods

Cell Culture

The isolation of chromaffin cells was carried out essentially as described previously (12). Aseptic conditions were maintained as much as possible. Briefly, the glands were dissected, sliced, and the tissue slices dissociated by three subsequent enzyme digestions. Care was taken that none of the cortical material that frequently surrounds blood vessels was carried into the digestion steps. We found that any residual cortical tissue dissociated more readily than chromaffin tissue. Therefore, the supernatant fluid after the first enzyme digestion was discarded and the tissue slices washed with Ca-free Locke's solution to further increase the final purity. The cell purity was assessed by staining with neutral red (25) and was found to be routinely >70%. The cells were finally suspended in DME supplemented with 10% FCS, 5 mM Hepes, 2.5 mM NaOH, 5.3 g/l glutamine, 5 μM cytosine arabinoside, 5 μM fluorodeoxyuridine, and 44 mg/l gentamycin sulfate, and plated at a density of $2-5 \times 10^5$ cells/well in multiwell plates obtained from Flow Laboratories, Inc. (McLean, VA). Maintenance of the culture was carried out as described (16). Cells were used between 2 d and 2 wk after plating. For control experiments, cortical cells were cultured using a similar protocol.

Stimulation and Arrest of Secretion

Before the cells were used for experiments, the culture medium was removed and replaced by 0.5 ml Locke's solution containing 0.1% BSA, 10 mM Hepes, and 5 mM NaOH, pH 7.4; 22°C (BSA-Locke's). After 10 min, this solution was removed and replaced by 0.3 ml fresh BSA-Locke's. The cells were stimulated by adding carbamylcholine at a final concentration of 10^{-4} M. Secretion was stopped by either adding hexamethonium to a final concentration of 1 mM or by simply washing off the supernatant fluid.

When solutions had to be changed during an experiment, the multiwell plate containing the cells was first inverted with an empty multiwell plate and subsequently with one containing the new solutions. In experiments on the calcium dependence of exocytosis we kept at least 1 mM of free divalent cation in all media. When EGTA was used, the pH was readjusted to 7.4 with NaOH.

HRP Uptake

HRP (type II; Sigma Chemical Co.) was dissolved in BSA-Locke's and added, unless otherwise indicated, at a final concentration of 1-4 mg/ml either (a) 2 min before the cells were stimulated, (b) at the time secretion was stopped, or (c) at various time intervals thereafter depending on the type of experiment. The time schedules were organized in such a way that it was possible to halt HRP uptake into all cells of a multiwell plate simultaneously. HRP uptake was halted by plunging the multiwell plate into a tray containing 2 liters of ice cold BSA-Locke's. The multiwell plate was moved gently for 5 min. This procedure was repeated twice with fresh ice cold BSA-Locke's. The plates were drained and the attached cells dissolved in a solution containing 10 mM sodium acetate, pH 6.5, and 0.1% Triton X-100. Samples of 0.3 ml were taken for the assay of HRP (26, 27) and 100 μl for fluorimetric assay of catecholamine (31). In addition to HRP type II, we also tested the isoenzymes VII, VIII, and IX, and a range of other fluid phase markers including Lucifer Yellow CH, [3 H]sucrose, alcohol dehydrogenase, [125 I]-albumin, [3 H]dextran, and fluorescently labeled dextrans of 20-120 kD. Although stimulation-dependent uptake of these markers could be demonstrated, we preferred to use HRP because it can be washed off easily, uptake into unstimulated cells is low, and the high sensitivity of the HRP-enzyme assay allows accurate measurements over four orders of magnitude and a large range of experimental conditions.

In some experiments, electron micrographs were prepared as described previously (12) in order to visualize uptake of HRP.

Assay of Other Fluid Phase Markers

The same assay was used for all HRP isoenzymes. The fluorescence of Lucifer Yellow was measured at a 425-nm excitation wavelength and a 520-nm emission wavelength after precipitating protein with 10% TCA. Alcohol dehydrogenase was assayed as described (3).

Data Analysis

BASIC programs were written to express catecholamine secretion as per

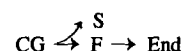
cent of totals (supernatant fluid plus cell monolayer) and to calculate HRP-enzyme activities from spectrophotometer readings. For curve fitting the "Patternsearch" program developed by Dr. R. J. Beynon (see Green et al. [10]) was run on an Apple II microcomputer. Standard errors of functions $f(x_1, \dots, x_i, \dots, x_n)$ of original data x_i were calculated using Gauss' error propagation formula:

$$SE(f[x_1, \dots, x_i, \dots, x_n]) = \left\{ \sum_{i=1}^n (\partial f[x_1, \dots, x_i, \dots, x_n] / \partial x_i \times SE[x_i])^2 \right\}^{1/2}$$

Theory

In analyzing our data, it was useful to have a mathematical model of the exocytosis/endocytosis cycle. We constructed a kinetic model which is based on the following assumptions. (a) During exocytosis, secretory vesicle membrane fuses with the plasma membrane to form excess surface membrane (fusion product [F]). At the same time secretion product (S) appears in the extracellular space. The rate of this reaction is proportional to the number of chromaffin granules left in the releasable pool. (b) The rate of membrane retrieval is proportional to the amount of additional membrane present on the cell surface at any time during the exocytosis/endocytosis cycle. This excess membrane area is the result of both formation by exocytosis and decay by membrane retrieval. In the simplest case, the same membrane patches which have been incorporated into the plasma membrane are retrieved by endocytosis. This is not, however, a necessary assumption in our model although it is suggested by the available experimental evidence (18, 24). During membrane retrieval, endocytotic vesicles (End) are formed.

This sequence of events can be described by the basic scheme:



where CG denotes the releasable pool of chromaffin granules. End can be estimated by measuring the uptake of a membrane marker or, if we assume that the average volume of the endocytotic vesicles is constant, by measuring the uptake of a fluid phase marker. The average size of these endocytotic vesicles introduces a scaling factor which cancels out if we calibrate theoretical and experimental results in terms of the total releasable pool of catecholamine and the corresponding total retrieved pool of marker. Any molecule either fluid phase or membrane bound may be a suitable marker provided its uptake increases linearly with membrane retrieval.

According to our assumptions both exocytosis and subsequent membrane retrieval are first order reactions and can therefore be described by rate constants λ_1 (exocytosis) and λ_2 (membrane retrieval).

Eq. 1-4 describe the respective time courses of the concentrations of the species S, CG, F, and End.

$$S(t) = CG_0 - CG(t), \quad (1)$$

$$\text{End}(t) = CG_0 - CG(t) - F(t) = S(t) - F(t), \quad (2)$$

$$dCG(t)/dt = -\lambda_1 CG(t), \quad (3)$$

$$dF(t)/dt = \lambda_1 CG(t) - \lambda_2 F(t), \quad (4)$$

where CG_0 is the releasable pool of chromaffin granules. The solutions of Eq. 1-4 are

$$S(t) = CG_0(1 - \exp[-\lambda_1 t]), \quad (5)$$

$$F(t) = CG_0 \lambda_1 / (\lambda_2 - \lambda_1) (\exp[-\lambda_1 t] - \exp[-\lambda_2 t]), \quad (6)$$

$$\text{End}(t) = CG_0(1 - \lambda_2 / [\lambda_2 - \lambda_1] \exp[-\lambda_1 t] + \lambda_1 / [\lambda_2 - \lambda_1] \exp[-\lambda_2 t]). \quad (7)$$

F(t) shows a transient time course. Its maximum F_{\max} is large if membrane retrieval is slow compared to exocytosis ($\lambda_2 < \lambda_1$) and small if the reverse is true. It is given by

$$F_{\max} = CG_0 (\lambda_1 / \lambda_2)^{\lambda_2 / (\lambda_2 - \lambda_1)}. \quad (8)$$

F_{\max} is reached at the time t' .

$$t' = \ln(\lambda_1 / \lambda_2) / (\lambda_1 - \lambda_2). \quad (9)$$

Examples of S(t), F(t), and End(t) are given in Fig. 1 for the cases $\lambda_2 = 2\lambda_1$ ($\tau_2 = \tau_1/2$; τ being $1/\lambda$) and $\lambda_2 = \lambda_1/2$ ($\tau_2 = 2\tau_1$).

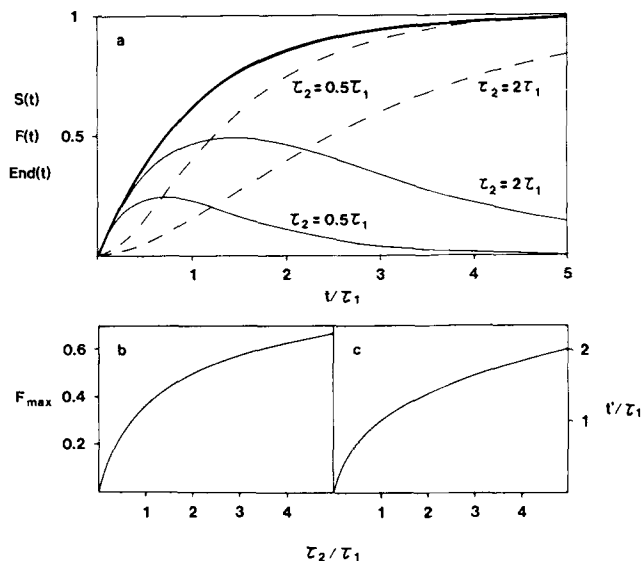


Figure 1. Mathematical model of the secretory exocytosis/endocytosis cycle. (a) (Thick line) Time course of appearance of secretion product in the extracellular space. (Thin lines) Time course of formation of plasma membrane-secretory vesicle membrane fusion product $F(t)$ as described by Eq. 6 for the cases $\tau_2 = 0.5\tau_1$ and $\tau_2 = 2\tau_1$. (Dashed lines) Time course of formation of endocytotic vesicles as described by Eq. 7 for $\tau_2 = 0.5\tau_1$ and $\tau_2 = 2\tau_1$. (b) Maximum F_{\max} of $F(t)$ as a function of τ_2/τ_1 . (c) Time t' when $F(t)$ reaches its maximum as a function of τ_2/τ_1 .

Results

Is HRP a Fluid Phase Marker?

HRP was added to cultured cells at concentrations ranging from 0.024 mg/ml to 10 mg/ml. After a 2-min preincubation, cells were stimulated with carbachol for 10 min. The HRP uptake evoked during this time is shown in Fig. 2. The data

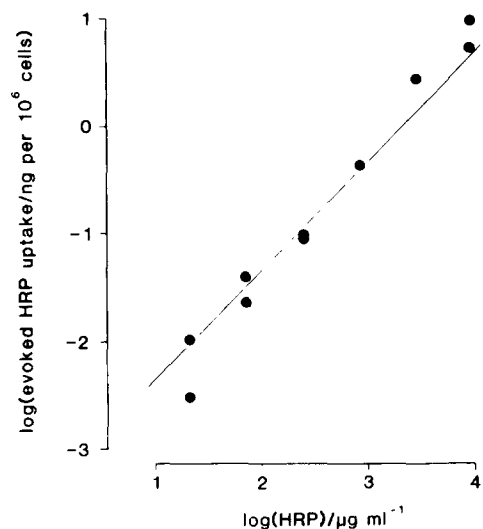


Figure 2. Evoked uptake of HRP as a function of the HRP concentration in the medium. Cells were preincubated with HRP for 3 min and stimulated with 10^{-4} M carbachol for 10 min at 23°C . The cells were washed, dissolved in Triton X-100-containing solution (0.1%), and the HRP concentration was measured enzymatically.

are plotted in a double logarithmic manner. A straight line with slope equal to +1 is expected if HRP is a fluid phase marker and any deviation from linearity must reflect complications due to HRP binding or other factors. The data is consistent with HRP uptake occurring predominantly by trapping of extracellular fluid.

The data can be used to calculate the volume which is taken up during membrane retrieval and this can be compared with the volume discharged during catecholamine secretion. If we assume a catecholamine concentration of ~ 0.6 M in chromaffin granules (23, 32), the calculated ratio of the HRP-space taken up/volume of chromaffin granules discharged is 1.11 in this experiment and 1.05 in another one (not shown). In some experiments we used [^3H]sucrose and Lucifer Yellow in addition to HRP. The calculated spaces for these relatively small molecules were similar to or somewhat larger than the secretory granule volume. Uptake of small molecules into unstimulated cells however was very high and increased by only $\sim 50\%$ on stimulation. In contrast, stimulation increased HRP uptake up to sixfold above control levels. Higher uptake of small molecules as compared to large molecules during secretion has been found previously (19). It seems likely that the plasma membrane is relatively permeable to small molecules which would explain the high background uptake. This permeability may be increased by the permeability of the secretory vesicle membrane incorporated into the plasma membrane during secretion thereby increasing the apparent uptake of fluid phase. The influence of size and charge was further investigated by examining the stimulated uptake of a number of other fluid phase markers. The results are summarized in Table I and suggest that neither molecular weight nor charge have any dramatic effect on uptake at the concentrations used.

Because of its convenience and the large increment in uptake due to stimulation, we used HRP to monitor membrane retrieval. Our results indicate that the volume of fluid retrieved is roughly equal to the vesicle volume depleted of catecholamine. This agrees well with the size of capacitance changes of the chromaffin cell membrane during stimulation

Table I. Comparison of Various Fluid Phase Markers with HRP II

Molecule	Evoked uptake/ evoked secretion*
[^3H]sucrose (mol wt 342.3)	~ 100
Lucifer Yellow (mol wt 550.4)	195 ± 93
HRP II (mol wt 40,000)	100 ± 4.4
HRP VII \ddagger	79 ± 14
HRP VIII \parallel	119 ± 14
HRP IX \S	129 ± 15
Alcohol dehydrogenase (mol wt 150,000)	61 ± 22

Data pooled from four different experiments with at least triplicate determinations of secretion and uptake for both stimulated and unstimulated cells. Lucifer Yellow was used at a concentration of 2.5 mg/ml and compared with HRP at a concentration of 1 mg/ml. HRP isoenzymes were compared with each other at a concentration of 0.5 mg/ml. Alcohol dehydrogenase was used at a concentration (3.7 mg/ml) equimolar to that of HRP II (1 mg/ml).

* Results are expressed as percent of the ratio obtained for HRP II which was included in each experiment as a standard.

\ddagger The sedimentation coefficients of HRP isoenzymes are identical (26).

\S Acidic molecule.

\parallel More acidic than HRP VII as estimated by electrophoretic mobility.

\S Basic molecule.

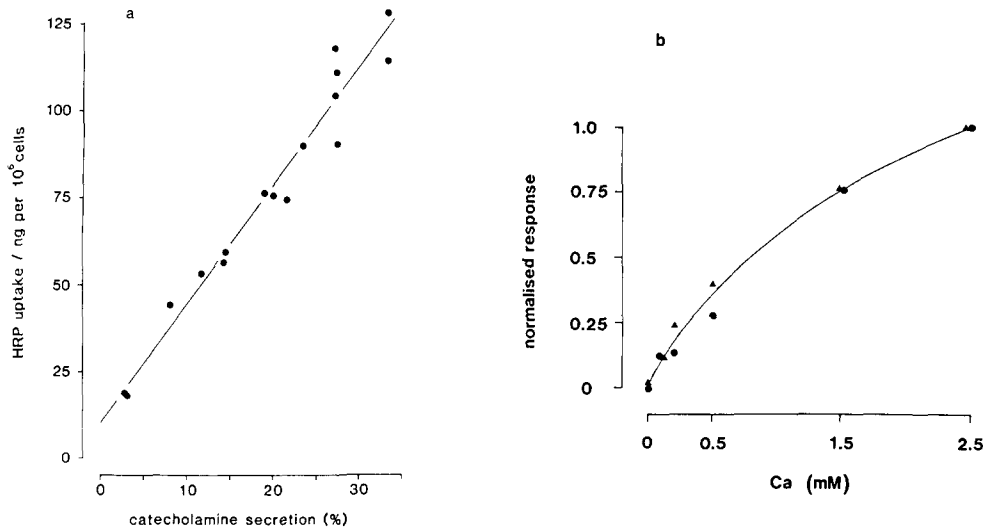


Figure 3. Relationship between catecholamine secretion and HRP uptake. (a) Cells were preincubated for 2 min with 4 mg/ml HRP and stimulated with 10^{-4} M carbamylcholine. At various time intervals after carbamylcholine addition, secretion was halted with 1 mM hexamethonium and after 32 min the cells were washed. Evoked uptake of HRP throughout the entire time period is plotted against evoked catecholamine secretion in the same time period. (b) Cells were exposed to BSA-Locke's containing 1 mg/ml HRP and calcium concentrations from 0–2.5 mM. At Ca levels below 2.5 mM calcium was replaced by magnesium to keep the concentration of divalent cations constant at 2.5 mM. Evoked catecholamine secretion (solid triangles) and HRP uptake (solid circles) are expressed as fractions of responses measured at 2.5 mM Ca^{2+} .

(18) and published morphological studies of the exocytosis/endocytosis secretory cycle (22).

Relationship between HRP Uptake and Secretion

An experiment was performed to study the relationship between membrane retrieval and HRP uptake. Cells were stimulated with carbachol for various time periods to induce addition of a varying amount of secretory vesicle membrane to the plasma membrane. Secretion was halted with the nicotinic antagonist hexamethonium and 10 min after the last hexamethonium addition the cells were washed. HRP was present in the bath throughout the entire time period. The time scale of the experiment was sufficiently long to allow all membrane addition that had occurred during exocytosis to be balanced by retrieval of an equivalent amount of membrane (see Fig. 6). The results of such an experiment are shown in Fig. 3 a. The amount of HRP taken up under these conditions is linearly related to catecholamine secretion. These results show that HRP can be used to monitor membrane retrieval associated with evoked secretion.

Another way to control the amount of secretory vesicle membrane added to the plasma membrane is to vary the extracellular concentration of Ca. The results of such an experiment are shown in Fig. 3 b. The cells were stimulated with 10^{-4} M carbachol for 15 min. Uptake of HRP correlates well with catecholamine release and in the absence of external Ca, carbachol alone is not able to trigger uptake of HRP.

Accumulation of Endocytotic Vesicles

The time-dependent accumulation of extracellular fluid in response to continuous exposure to carbachol was inves-

tigated using the protocol shown in Fig. 4 a. Cells were stimulated with carbachol and a variable time interval allowed before the cells were washed. To monitor fluid phase uptake HRP was added 2 min before the cells were stimulated. Controls were treated in the same way except that BSA-Locke's was added instead of carbamylcholine. Fig. 4 b shows the time-dependent HRP uptake for both cases. It is linear if cells are not stimulated. However upon stimulation, HRP uptake is increased more than twofold above controls.

The time courses of catecholamine secretion and HRP uptake are compared in Fig. 4 c. The linear HRP uptake into unstimulated cells was subtracted using a straight line obtained by linear regression analysis. The time courses of catecholamine secretion and HRP uptake are clearly different. Catecholamine secretion appears to be a first order reaction of the type described by equation 5. A theoretical curve has been fitted to the data by varying the total releasable pool CG_0 and the rate constant λ_1 . In contrast to secretion which decreases continuously, HRP uptake is initially slow and only later accelerates. The time course of HRP uptake into stimulated cells can be fitted very well on the basis of our model. The continuous line is a theoretical curve predicted by Eq. 7. In the fitting process CG_0 and λ_2 was allowed to vary. The rate constant for exocytosis λ_1 was taken from the secretion data. Out of four experiments, two were analyzed in detail by fitting theoretical curves. The time constants τ ($= 1/\lambda$) for exocytosis and membrane retrieval were 4.4 and 2.8 min, respectively, for the experiment shown in Fig. 4, b and c, and 5.7 and 4.7 min for a second experiment (not shown). The experiments suggest strongly that exocytosis and endocytosis are consecutive processes. If this interpretation is correct, the simplest arrangement would be that

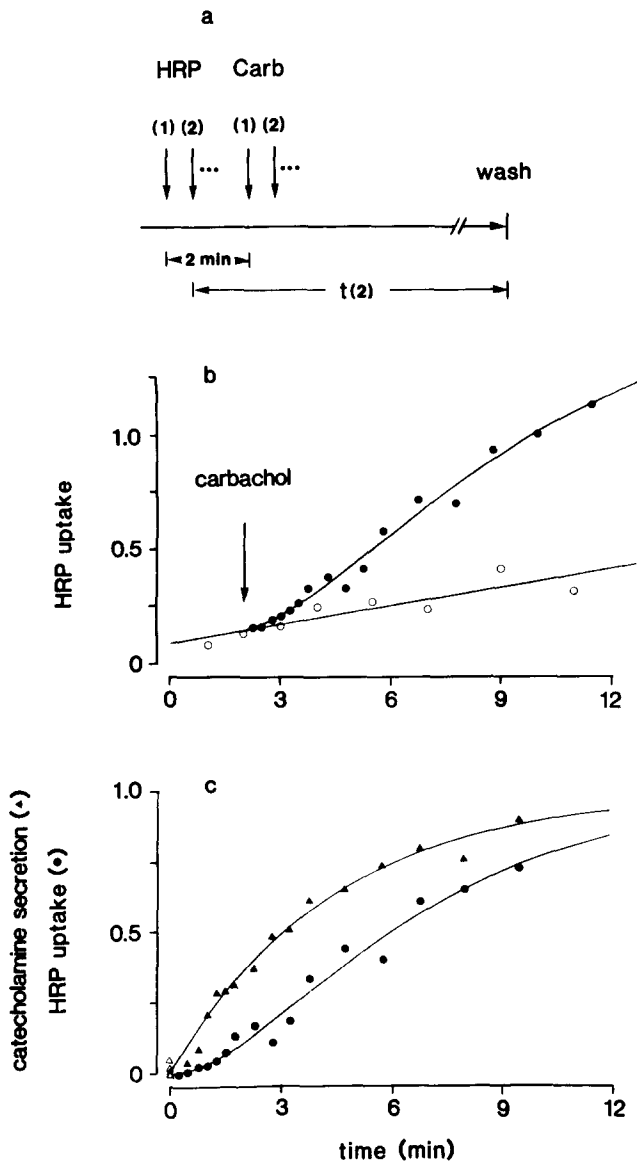


Figure 4. Uptake of HRP during stimulated secretion at 23°C. (a) Outline of the experimental protocol. At various time intervals before the cells were washed, HRP was added to the medium at a final concentration of 4 mg/ml. 2 min later cells were stimulated with 10^{-4} M carbachol. This is illustrated for samples labeled (1) and (2). $t(2)$ is the time interval for sample labeled (2). (b) Time-dependent HRP uptake into stimulated cells (solid circles) and unstimulated controls (open circles). A linear function was fitted to HRP uptake into unstimulated cells. The sum of this linear function and Eq. 7 was fitted to HRP uptake into stimulated cells. (c) Comparison of HRP uptake (solid circles) with catecholamine secretion (solid triangles). Linear HRP uptake into unstimulated cells is subtracted from HRP uptake into stimulated cells. All data are expressed as a fraction of the total releasable pool and the corresponding total retrievable pool.

exocytosis generates an intermediate product—the fusion product or Ω -profiles seen in transmission electron micrographs—from which endocytotic vesicles are formed at a similar rate; but our data provide no information about the source of the retrieved membrane.

Some experiments were performed in which HRP was added at different times after exposure to carbachol and the

cells were subsequently processed for electron microscopy. HRP was visible within the cells in endocytotic vesicles and the number of these HRP containing vesicles was maximal when HRP was added shortly after carbachol and declined if it was added at later times. This confirms the analytical data and provides direct evidence that HRP is internalized in endocytotic vacuoles. The size of the vacuoles was rather variable but on the average similar to that of chromaffin granules. As the cells were fixed 30 min after HRP addition, HRP may have been transferred to a different population of vesicles from the ones that effected membrane retrieval.

Accumulation of Fusion Product

The ability to halt secretion at any time should enable us to follow the appearance and decay of exocytotic fusion product independently of exocytosis. We found that carbachol-

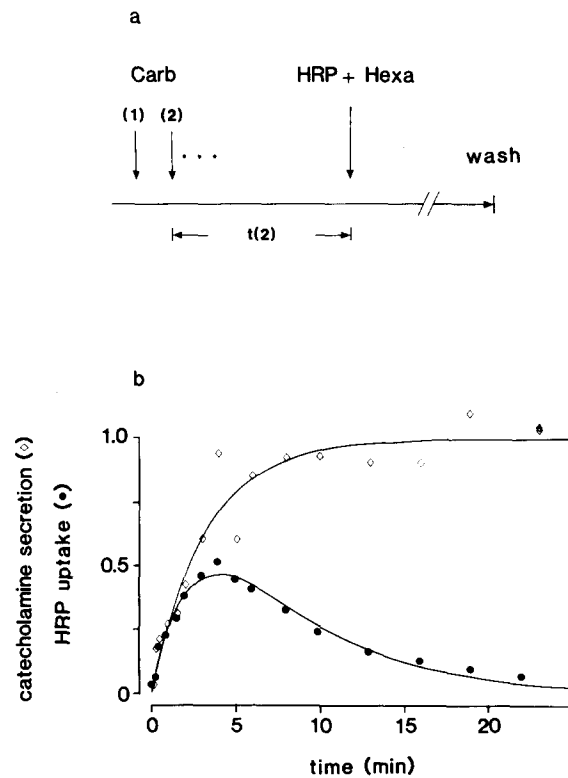


Figure 5. Time course of appearance of secretory vesicle-plasma membrane fusion product at 23°C. (a) Outline of the experimental protocol for samples (1), (2),... At various time intervals before the cells were washed [$t(2) + 10$ min for sample (2)], they were stimulated with carbamylcholine. To unstimulated controls an equal volume of BSA-Locke's was added. At 10 min before wash, secretion was halted with hexamethonium. At the same time HRP was added. (b) HRP uptake during the constant time interval after halting secretion is plotted over the variable time interval between stimulation and end of secretion (solid circles). Catecholamine secretion throughout the entire time period (open diamonds). Background secretion and HRP uptake were constant and subtracted from all data. To determine the total releasable pool and the corresponding uptake of HRP, cells in three wells were stimulated for 33 min. All results are expressed as a fraction of HRP taken up and catecholamine released during this time. The continuous lines are theoretical curves. Eq. 5 was used to fit the secretion data by varying the rate constant of exocytosis and Eq. 6 was fitted to the HRP uptake data by varying the rate constant of endocytosis.

induced secretion and, presumably therefore the formation of any new fusion product, can be stopped completely by the nicotinic antagonist hexamethonium (1 mM). If we add HRP at the same time and include a sufficiently long time interval before wash, all fusion product formed between stimulation and stop should be converted to endocytotic vesicles. Therefore we should be able to obtain quantitative information on the amount of fusion product at the time of stop. By varying the time interval between stimulation and stop we should reveal the time course of the formation of fusion product. Fig. 5 *a* shows an outline of our experimental protocol. The results of such an experiment are shown in Fig. 5 *b*. All results are calibrated and expressed as fractions of the total pool which was estimated by stimulating two wells for 25 min in the presence of HRP. The data show a transient time course as expected for a species which undergoes continuous formation and decay.

The continuous line in a theoretical curve of the type predicted by Eq. 6 and was fitted to the data by varying only one parameter, the rate of endocytosis λ_2 . The time constant of exocytosis was obtained by fitting equation 5 to the secretion data from the same experiment. The background was not time-dependent because all samples, stimulated or unstimulated, were exposed to HRP for the same length of time. The background data were simply averaged and added as a constant term to Eq. 6 describing the time course of the formation of fusion product $F(t)$, which was then used for fitting. Under these conditions, the calculated time constants for exocytosis/endocytosis were 3.2 min/5.2 min for the experiment shown in Fig. 5, and 3.0 min/4.8 min for a second experiment (not shown).

Time Course of Membrane Retrieval after Halting Secretion

The experiments described so far provide information on the time constant of endocytosis under conditions which are able to support secretion as well. Complementary information can be obtained if secretion is halted and membrane retrieval is studied independently of exocytosis. Fig. 6 *a* shows an outline of the experimental protocol. Cells were stimulated with carbachol and after 4 min, secretion was halted with hexamethonium. This sequence was reversed for the controls. HRP was added at various time intervals after stopping secretion. A sufficiently long time interval was included before the final wash to allow all fusion product still present at the time of HRP addition to be transformed to endocytotic vesicles. This protocol therefore measures the disappearance of fusion product after stopping secretion. The results of one such experiment are shown in Fig. 6 *b*. The background decreases linearly with time because the time interval between HRP addition and final wash decreases as the time interval between stopping secretion and addition of HRP increases. A linear function was fitted to the background data and added to a function describing exponential decay. The average time constant for four experiments at 23°C was 3.7 ± 0.9 min (SEM). At 37°C the time constant was 1.5 min (see also Fig. 7).

Temperature Dependence of Membrane Retrieval

The experimental protocol described in the previous section allows us to change the conditions during membrane retrieval. An outline of the experimental protocols for examin-

ing the effect of temperature on membrane retrieval is shown in Fig. 7, *a* and *b*. After a constant stimulation period at room temperature, secretion was stopped with hexamethonium and the temperature changed from room temperature (23°C) to either 37°C or to 4°C. At various time intervals afterwards, HRP was added. After 6.5 min, the 4°C-samples were also brought to 37°C. In both cases, a sufficiently long time interval at 37°C was allowed before the final wash for complete transformation of the fusion product into endocytotic vesicles. The results are shown in Fig. 7 *c*. There is a rapid decrease at 37°C which was fitted by an exponential decay curve. ($\tau_2 = 1/\lambda_2 = 1.5$ min at 37°C compared to 3.7 min at 23°C). No such decay can be observed at 4°C. However, if HRP is added during the subsequent 37°C period, the excess membrane rapidly disappears. These results demonstrate that membrane retrieval is impaired at

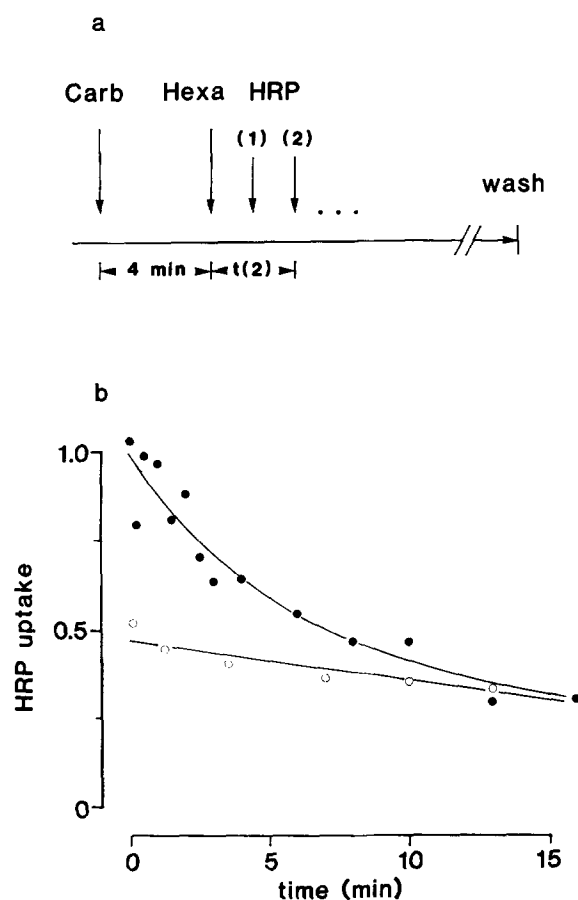


Figure 6. Time course of membrane retrieval at 23°C after stopping secretion. (a) Outline of the experimental protocol. Cells were stimulated with 10^{-4} M carbamylcholine. 4 min later, secretion was halted with 1 mM hexamethonium. To controls hexamethonium was added before carbamylcholine. HRP was added to individual wells [samples (1), (2), ...] at various time intervals [t(2) for sample (2)] after hexamethonium addition. 30 min after carbamylcholine addition cells were washed. (b) HRP uptake is plotted over the variable time interval between stop of secretion and addition of HRP. A straight line was fitted to HRP uptake into unstimulated cells (open circles) and an exponential decay curve to HRP uptake plus the straight line to HRP uptake into stimulated cells (closed circles). Data were normalized such that the theoretical curve for stimulated cells is 1 at $t = 0$. This is equivalent to 45% of the measured total retrievable pool.

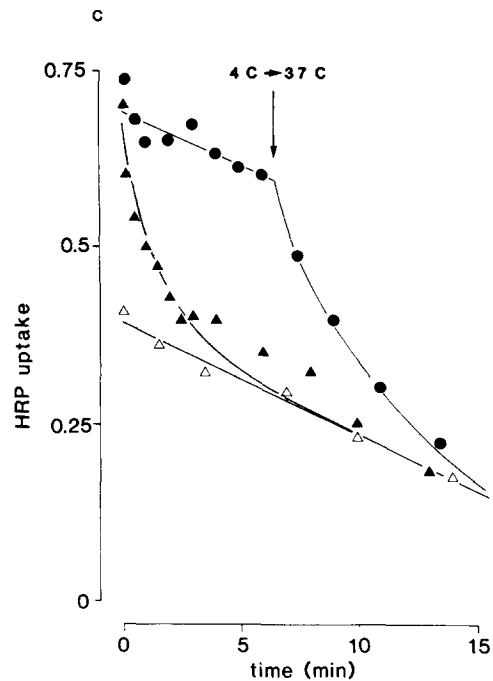
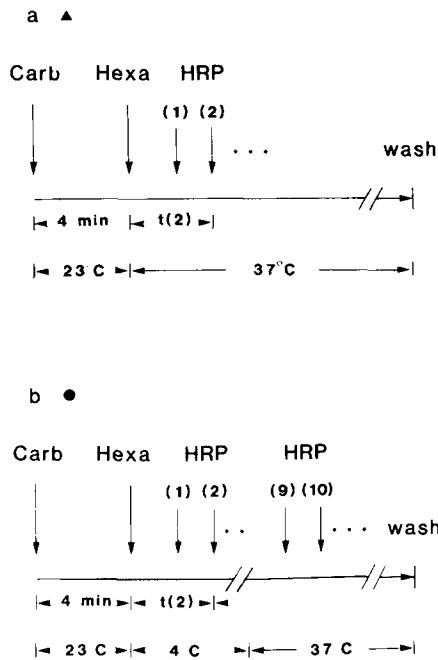


Figure 7. Temperature dependence of membrane retrieval. (a and b) Experimental protocols (a for triangles in c, b for circles in c). (a-c) Cells in two multiwell plates (a and b) were stimulated with 10^{-4} M carbamylcholine for 4 min at 23°C . Secretion was halted with 1 mM hexamethonium. To obtain unstimulated controls, the order of addition of carbamylcholine and hexamethonium was reversed (open triangles in c). The supernatant fluid was removed by inversion with an empty multiwell plate. The plates to which the cells were attached were inverted with multiwell plates both containing BSA-Locke's and hexamethonium but one had been equilibrated at 37°C (a and solid triangles in c) and the other one at 0°C (b and solid circles in c). Incubation of the cells was continued at

the new temperatures. HRP was added to individual wells [samples (1), (2) in a and b] at various time intervals [t(2) for sample (2)] after the temperature change. 6.5 min after addition of hexamethonium the 4° plate (b and solid circles in c) was warmed up to 37°C and addition of HRP to individual wells was continued [samples (9), (10), ... in b]. Finally the supernatant fluid was removed as before and cells were washed. (c) Uptake of HRP is plotted over the time interval between stop of secretion and addition of HRP. Uptake is expressed as slope on the spectrophotometer recorder during the HRP enzyme assay.

4°C , but resumes on rewarming to 37°C and the amount of HRP taken up seems largely independent of the time spent at 4°C . We did not try to fit a theoretical curve to the 37°C tail of the $4^{\circ}\text{C} \rightarrow 37^{\circ}\text{C}$ experiment, because the temperature change was not fast enough compared to the time constant of membrane retrieval.

Calcium Dependence of Membrane Retrieval

The dependence of triggered endocytosis on external Ca was examined using a protocol similar to that described in the previous section. The cells were stimulated at room temperature in the presence of 2.5 mM Ca. After 4 min, secretion was stopped with hexamethonium and the bathing solutions

Table II. Calcium Dependence of Membrane Retrieval

Stimulation	Ca in medium after halting secretion	HRP uptake immediately after halting secretion	HRP uptake 4 min after halting secretion	Decrease of HRP uptake after 4 min
		% of total pool		
	mM			
Plate 1				
+	2.5	29.8 ± 0.0	18.2 ± 1.1	38.9 ± 4.6
+	0.25	29.2 ± 1.2	18.2 ± 1.6	37.8 ± 7.0
-	2.5	9.4 ± 1.2	8.9 ± 0.3	
-	0.25	10.8 ± 0.0	14.5 ± 0.7	
Plate 2				
+	2.5	43.8 ± 9.8	19.0 ± 1.9	56.6 ± 11.5
+	10^{-5}	33.7 ± 5.7	13.4 ± 1.4	60.1 ± 8.3
-	2.5	10.0 ± 0.5	9.2 ± 1.9	
-	10^{-5}	8.7 ± 0.7	8.0 ± 1.0	

Stimulation and end of secretion as in Fig. 7. To obtain unstimulated controls, the order of addition of carbamylcholine and hexamethonium was reversed. The plates to which the cells were attached were first inverted with empty multiwell plates and then with plates containing BSA-Locke's + 1 mM hexamethonium and free Ca concentrations from 10^{-8} M to 2.5×10^{-3} M. HRP was added to triplicate (stimulated samples) or duplicate (controls) wells immediately after the change of solutions and 4 min later. 16 min after the change of solutions the cells were washed. Uptake of HRP is expressed as a fraction of HRP uptake during the entire time period (total pool). For each concentration of free Ca, a separate plate was used and a control in the presence of 2.5 mM Ca was run to correct for any variation between plates.

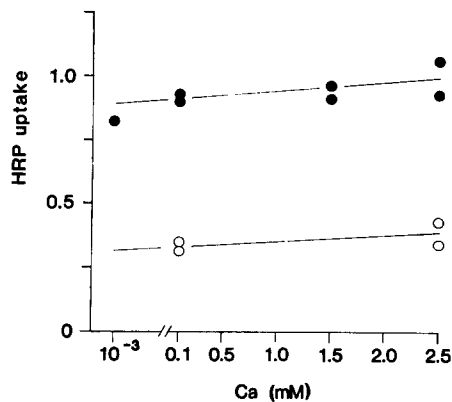


Figure 8. Calcium dependence of membrane retrieval. Cells were stimulated with 10^{-4} M carbamylcholine for 4 min at room temperature. Secretion was halted with 1 mM hexamethonium and the supernatant fluid removed by inversion with an empty multiwell plate. The plates to which the cells were attached were inverted with a multiwell plate containing BSA-Locke's and hexamethonium with a free Ca concentration from 10^{-6} M to 2.5×10^{-3} M. 1 min after inversion, HRP was added to all wells and 3 min later the supernatant fluid was removed again and the cells were washed (*solid circles*). No carbamylcholine was added to unstimulated controls (*open circles*). Results are expressed as a fraction of HRP uptake into stimulated cells at Ca = 2.5 mM.

were changed. To monitor the disappearance of fusion product, HRP was added immediately after the change of solutions and 3 min later. The percent decay during this time period is shown in Table II. Even if the free Ca concentration in the medium is buffered to $<10^{-8}$ M, the rate of membrane retrieval is not reduced compared to the rates measured at a free Ca level of 2.5 mM. We also examined the influence of extracellular Ca under slightly different conditions. We stimulated the cells and changed the solutions as described, but waited for 1 min before HRP was added to be sure that all contaminating extracellular Ca had been complexed by the calcium buffer. Fluid phase uptake was monitored by including HRP for 3 min. The total uptake obtained by this method for various extracellular Ca concentrations is shown in Fig. 8. Again, membrane retrieval seems to be largely unaffected by removing Ca or complexing it with EGTA.

Other Factors Affecting Membrane Retrieval

The effect of Lanthanum on membrane retrieval was studied at concentrations of 0, 0.2, and 1 mM. The results are very similar with calculated time constants of 4.9 ± 0.8 min, 6.8 ± 2.8 min, and 6.4 ± 1.6 min, respectively. Increasing the osmotic pressure, however, markedly increased this time constant (>10 -fold at 300 mM added sucrose) showing that the time constant of endocytosis can be altered experimentally.

Discussion

Monitoring Membrane Retrieval with HRP and Other Fluid Phase Markers

HRP is a widely used fluid phase marker (20, 27). Swanson et al. however found that in macrophages it stimulates pinocytosis and redirects the flow of pinocytosed fluid (28). This

is mediated by binding of the HRP glycoprotein to mannose receptors on the surface of macrophages. There is no evidence for this type of behavior in adrenal chromaffin cells where uptake is linearly related to HRP concentration. One explanation for the different behavior of macrophages and chromaffin cells may be a lack of mannose receptor-mediated endocytosis in chromaffin cells. The presence of HRP does not significantly influence the rate of membrane retrieval for the following reason: the type of experiment shown in Fig. 4 measures the time constant of endocytosis when HRP is present and the type of experiment shown in Fig. 6 shows the decay of fusion product in the absence of HRP which is added to measure the residual amount of fusion product after some time has elapsed. The average time constants are 3.75 min for the experiment in Fig. 4 and 3.7 ± 0.9 for the experiment in Fig. 6.

The experimental data on membrane retrieval after stimulation of exocytosis by carbachol are well fitted by a single exponential, suggesting that retrieval uses a relatively homogeneous population of vesicles. Our data provide some information on the size of these vesicles. We have calculated the ratio of the HRP space taken up per volume of chromaffin granules discharged for nine different experiments assuming an average catecholamine concentration of 0.6 M within chromaffin granules. The average was 1.32 ± 0.21 (SEM). This value suggests that the volume of HRP retrieved is on the average similar to the volume of chromaffin granules discharged. Electron microscopy has provided data in support of a range of vesicle sizes including the size of chromaffin granules and larger sizes (12). In a recent study, chromaffin granule membranes were immunostained for a short period of time (1 min) during which the cells were stimulated followed by brief reincubation (5–15 min) to allow secretory vesicle membrane exposed to the cell surface to be internalized (22). The observed vesicle sizes agree well with our data. Capacitance measurements have revealed steps—presumed to reflect endocytosis (18)—with an average size identical to the average size of the steps associated with exocytosis. Although further quantitative electron microscopy seems desirable, the data strongly suggest that membrane is retrieved into vesicles comparable in size to the chromaffin granules. The conspicuously larger values sometimes detected by capacitance measurements as with EM after extensive stimulation may reflect membrane retrieval after compound exocytosis. Provided such vesicles do not flatten completely at the cell surface, the existence of compound exocytosis would not be detected by HRP uptake.

The Mechanism of Membrane Retrieval

We have estimated the rate of membrane retrieval in various ways. All our data are well fitted by a model which assumes that endocytotic vesicles are generated from an intermediary product formed from chromaffin granules during exocytosis. Our data permit a number of possibilities to be excluded: (a) Endocytosis does not precede exocytosis as might be the case if membrane retrieval is necessary to make available exocytotic sites at the plasma membrane because in this case we should see a fast rate of endocytosis first and a slower rate later. (b) During exocytosis, a precisely equivalent membrane area is not retrieved simultaneously to maintain a constant surface all the time because in this case exocytosis and endocytosis should have the same kinetics. (c) Membrane re-

trieval does not occur in a synchronized way after exocytosis is complete as in this case we should have observed a clear lag and a time course inconsistent with our kinetic model.

The time for half complete membrane retrieval in our experiments is considerably faster than that measured by Lingg et al. (15), Patzak et al. (21), and Phillips et al. (24) (3–5 min compared to 15–30 min) even though most of our data were obtained at 23°C rather than at 37°C. It is possible that the mechanism for bulk membrane retrieval is different from that for retrieval of specific membrane proteins. However, such a conclusion is premature before truly quantitative methods are used for monitoring the time course of retrieval of specific secretory vesicle membrane proteins. Our data and model calculations suggest that exposure of secretory granule membrane at the cell surface is greatest at earlier time points rather than 10 min after stimulation, the time interval after which Lingg et al. (15) and Patzak et al. (21) used to stimulate the cells.

Our kinetic data do not unequivocally exclude the possibility that during exocytosis a factor is produced with an exactly proportional concentration to that of plasma membrane—secretory vesicle membrane fusion product, which in turn triggers retrieval of a proportional amount of membrane area different from that of secretory vesicle membranes. Phillips et al. (24) however report that there is no increased endocytosis of plasma membrane glycoproteins assayed with concanavalin A binding and Thilo (30) has convincingly demonstrated that in mast cells no plasma membrane glycoproteins or glycolipids are retrieved during exocytosis. It seems likely, therefore, that membrane retrieval in chromaffin cells may also be specific to secretory vesicle membrane.

If membrane retrieval is specific for secretory vesicle membrane, our observation that exocytosis and membrane retrieval occur continuously at the same time raises interesting questions on the cellular signals involved. Obviously, the cell has to distinguish between secretory vesicle membrane surrounding intact chromaffin granules and vesicle membrane exposed to the extracellular space. The signal could be a combination of the one that is used to initiate exocytosis and the changes in transmembrane gradient of solutes, electric field, and pH that occur during the fusion process. Variation of the extracellular and intracellular milieu should provide further answers to these questions. A particularly interesting feature of our results is the finding that endocytosis can be blocked in the cold—but once rewarmed continues as if the cold exposure had not happened. It will be interesting to identify the substrate of the “memory” seen in these experiments.

The Site of Calcium Requirement within the Secretory Cycle

We have estimated the rate of membrane retrieval during continuous secretion in two different ways. (a) By monitoring the time course of the formation of endocytotic vesicles. (b) By monitoring the time-dependent concentration of secretory vesicle—plasma membrane fusion product. We know that in adrenal chromaffin cells cholinergic agonists stimulate Ca entry into the cell and give rise to an elevated cytoplasmic level of free Ca. We can therefore assume that these two experiments measure the rate of endocytosis during elevated cytoplasmic calcium concentrations. In a third type of ex-

periment we measured the rate of membrane retrieval after stopping secretion with hexamethonium. Hexamethonium blocks the sequence of events that triggers calcium entry into the cytoplasmic space of chromaffin cells (29). We know that an elevated cytoplasmic free Ca is a sufficient stimulus for secretion. Since we measure membrane retrieval in this experiment in the absence of secretion, we can assume that the cytoplasmic free Ca is lower than any level which is able to support secretion. Furthermore, in separate experiments we have stopped secretion and reduced the extracellular Ca concentration to $<10^{-8}$ M which is very likely to reduce the cytoplasmic Ca concentration as well. We find a similar time constant for membrane retrieval under all conditions. It seems very likely therefore that, in sharp contrast to exocytosis, continuous Ca entry or a continuously elevated cytoplasmic Ca are not necessary to support membrane retrieval. As endocytosis involves membrane fusion at an extracellular site, our results on adrenal medullary cells exposed to levels of free Ca $<10^{-8}$ M show rather clearly that in this tissue very little or no calcium is required at the site of endocytotic membrane fusion.

This conclusion stands in sharp contrast to a number of studies on other systems. Ceccarelli and Hurlbut (4) find that membrane retrieval at the neuromuscular junction is Ca_o dependent, and Koike and Meldolesi report that membrane retrieval in parotid acinar cells is Ca_o dependent (14). However, these studies are on rather different secretory systems. One possibility may be that Ca is required to prime secretory vesicles for instance by phosphorylation so that they can undergo endocytosis after fusion with the plasma membrane. In our case this step could occur during activation of exocytosis and once the vesicles are primed for endocytosis, continuously high levels of Ca may not be necessary for membrane retrieval. Ceccarelli and Hurlbut, however, used α -latrotoxin, a toxin which stimulates secretion in a Ca-independent manner, and Koike and Meldolesi stimulated secretion in the parotid gland with isoprenaline, a β -adrenergic agonist. In both cases, a Ca-dependent phosphorylation step may not occur during activation of secretion, explaining the Ca requirement during membrane retrieval. Experiments using α -latrotoxin or other Ca-independent secretagogues in our system might help to clarify this point.

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