A multicompartmental model of fluid-phase endocytosis in rabbit liver parenchymal cells

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Fluid-phase endocytosis was studied in isolated rabbit liver parenchymal cells by using ^{125}I -poly(vinylpyrrolidone) (PVP) as a marker. First, uptake of ^{125}I -PVP by cells was determined. Also, cells were loaded with ^{125}I -PVP for 20, 60 and 120 min, and release of marker was monitored for 120–220 min. Then we used the Simulation, Analysis and Modeling (SAAM) computer program and the technique of model-based compartmental analysis to develop a mechanistic model for fluid-phase endocytosis in these cells. To fit all data simultaneously, a model with three cellular compartments and one extracellular compartment was required. The three kinetically distinct cellular compartments are interpreted to represent (1) early endosomes, (2) a prelysosomal compartment equivalent to the compartment for uncoupling of receptor and ligand (CURL) and/or multivesicular bodies (MVB), and (3) lysosomes. The model predicts that approx. 80 % of the internalized ^{125}I -PVP was recycled to the medium from the early-endosome compartment. The apparent first-order rate constant for this recycling was 0.094 min^{-1} , thus indicating that an average ^{125}I -PVP molecule is recycled in 11 min. The model also predicts that recycling to the medium occurs from all three intracellular compartments. From the prelysosomal compartment, 40 % of the ^{125}I -PVP molecules are predicted to recycle to the medium and 60 % are transferred to the lysosomal compartment. The average time for recycling from the prelysosomal compartment to the medium was estimated to be 66 min. For ^{125}I -PVP in the lysosomal compartment, 0.3 %/min was transferred back to the medium. These results, and the model developed to interpret the data, predict that there is extensive recycling of material endocytosed by fluid-phase endocytosis to the extracellular environment in rabbit liver parenchymal cells.

INTRODUCTION

Most cells have the ability to internalize extracellular molecules by endocytosis. Hormones, nutrients and enzymes, as well as toxins and pathogens, are internalized by receptor-mediated endocytosis [1,2]. Concurrent with receptor-mediated endocytosis, a part of the extracellular fluid is also internalized into the endosome. Furthermore, fluid-phase endocytosis may take place independently of receptor-mediated endocytosis [1]. Uptake of molecules by fluid-phase endocytosis is proportional to the concentration of molecules in the extracellular fluid. Fluid-phase endocytosis is a constitutive process in most cells. However, during mitosis it seems to cease, and agents such as phorbol myristate acetate, antidiuretic hormone and some growth factors may enhance accumulation of fluid-phase markers [1,3,4].

Most often, the uptake of a molecule by receptor-mediated endocytosis will far exceed the uptake by fluid-phase endocytosis. It has, however, been speculated that fluid-phase endocytosis may be quantitatively important when the concentration of the macromolecule in the extracellular fluid is much higher than the dissociation constant of the receptor, when the receptor is missing, or when the number of receptors is very low [1,2].

Some newly formed endosomes will rapidly (within minutes) fuse with the plasma membrane [5,6]. The rest will probably fuse with each other to form larger

endosomes, the so-called CURL (compartment for uncoupling of receptor and ligand) [7]. These vesicles are acidified, so that most receptor–ligand interactions dissociate, and the unoccupied receptors may be recycled to the cell surface in small vesicles that bud off from the CURL [7]. The CURL is gradually transformed into multivesicular bodies (MVB). Small primary lysosomes transport lysosomal enzymes to the MVB, and the degradation of endocytosed material may therefore start [8,9]. The fate of the MVB is not clear. They may gradually develop into secondary lysosomes, or fuse with secondary lysosomes, or transport their contents via smaller vesicles to the secondary lysosomes.

In order to gain more insight into the kinetics of fluid-phase endocytosis, we studied uptake of the fluid-phase marker poly(vinylpyrrolidone) (PVP) by rabbit liver parenchymal cells. It has been shown previously that this molecule is taken up into cells without being adsorbed to the membrane, and that it is not degraded in the lysosomes [1]. We applied model-based compartmental analysis to develop a working-hypothesis mechanistic model to describe and quantify the dynamics of the process.

MATERIALS AND METHODS

Radioisotopes and chemicals

^{125}I -PVP (40–50 $\mu\text{Ci}/\text{mg}$) was obtained from New

Abbreviations used: PVP, poly(vinylpyrrolidone); CURL, compartment for uncoupling of receptor and ligand; MVB, multivesicular bodies; SAAM, Simulation, Analysis and Modeling; CONSAM, conversational SAAM; FSD, fractional standard deviation; AIC, Akaike's Information Criterion.

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England Nuclear, Boston, MA, U.S.A. Unlabelled PVP and collagenase (Type I) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of cells

Male Chinchilla rabbits, weighing 2–3 kg, were purchased from G.m.b.H. Versuchstierkunde und -zucht, 7950 Biberach 1, West Germany. The animals were given food (Maintenance Feed; Ewos, Oslo, Norway) and water *ad libitum*. The rabbits were anaesthetized by intraperitoneal injection of Dormicum (0.5 ml/kg; Roche, Basel, Switzerland) and 5 min later by intramuscular injection of Hypnorm (0.4 ml/kg; Janssen Chimica, Beerse, Belgium).

Liver cells were prepared by a collagenase perfusion method as described previously [10]. The crude parenchymal cell pellet was resuspended in 20 mM-Hepes buffer containing 1% albumin [11], and pure parenchymal cells were isolated by centrifugal elutriation (model JE-6 rotor and J-21 centrifuge; Beckman Instruments, Palo Alto, CA, U.S.A.) as described in ref. [12]. Viability as determined by exclusion of Trypan Blue dye averaged 95%, and contamination by liver non-parenchymal cells was always less than 1%.

Uptake and release experiments

Liver parenchymal cells (10^7 cells/ml) were incubated in 20 mM-Hepes buffer containing 1% albumin [11] in a shaking water bath at 37 °C. For uptake experiments, cells were incubated with $1 \mu\text{M}$ - ^{125}I -PVP, and samples of cells were withdrawn at various times up to 4 h. The cells were sedimented by centrifugation at 50 g for 2 min and washed three times by resuspension and centrifugation (50 g, 2 min) in the incubation buffer. Cell-associated radioactivity was then determined in the cell pellet. Total radioactivity in the cell suspension was determined by direct counting of samples of the suspension.

For the release experiments, isolated parenchymal cells (10^7 cells/ml) were incubated with $1 \mu\text{M}$ -PVP at 37 °C for 20, 60 or 120 min, and washed three times by centrifugation (50 g, 2 min). The cells were then resuspended in incubation buffer, and release of radioactivity was measured after various times by determining cell-associated radioactivity and total radioactivity in the cell suspension. Viability of cells at the final time point in each experiment was never less than 85%. To test whether cell death contributed to the observed release of radioactivity, we measured the release of lactate dehydrogenase from control cell suspensions. The release after 220 min did not exceed 1% of the total activity in the cells, suggesting that cell death did not contribute significantly to the release of radioactivity from the cells.

Statistical and kinetic analysis

Three separate experiments were done to measure PVP uptake, and three each to measure release from cells loaded for 20, 60 or 120 min. Data for each of four data sets are presented as arithmetic means of the three separate experiments. The variation between individual results in an experiment was less than 10%. Individual data on cell-associated radioactivity and radioactivity in the medium were normalized to 100% [cell-associated c.p.m. (%) + medium c.p.m. (%) = 100%]. Non-specific radioactivity in the cell pellet was determined at zero time in the uptake experiments and subtracted from the cell-associated radioactivity.

Tracer data were analysed by compartmental analysis by using the SAAM29 (Simulation, Analysis and Modeling [13]) and CONSAM29 (Conversational SAAM [14]) computer programs run on a VAX 11/780 computer (Digital Equipment Corp., Marlboro, MA, U.S.A.). The group variance for cell-associated radioactivity was determined in separate uptake experiments ($n = 6$), and a fractional standard deviation (FSD) of 0.068 was used as a weighting factor in the non-linear-regression analysis. Limits for the adjustable parameters were set by literature values [15].

After initial modelling of each data set separately, the four data sets were combined and co-modelled to ascertain whether all data would fit the same structural model. A simple starting model was formulated (see the Results section); initial estimates of the model parameters and model structure were then adjusted by using the interactive CONSAM program to obtain a consistent best fit of the mathematically simulated medium and cell-associated data to the observed values. Final parameter values for each adjustable parameter were then generated by using SAAM's iterative non-linear least-squares routine.

As a method of estimating maximum likelihood, the weighted version of Akaike's Information Criterion (AIC) [16,17] was used (a) to identify the 'best' model when compatible alternative models with the same number of adjustable parameters were being evaluated and (b) to test statistically whether increases in model complexity were justified (i.e. significantly decreased the residual sum of squares in spite of an increased number of adjustable parameters):

$$\text{AIC}_p = L + 2p$$

where p = number of adjustable parameters, $L = n \ln \text{RSS} - \sum_{i=1}^n \ln W_i$, n = number of data points, RSS = weighted sum of squares of residuals, $W_i \approx 1/(\text{SD}_i)^2$, $\sum_{i=1}^n W_i = n$. For a model to be considered statistically 'better' than others, it must decrease AIC_p by more than 1–2 units.

The nomenclature and method of calculation for kinetic parameters are similar to those published previously [17]. The parameters reported include:

(a) rate constants, $L(I, J)$'s, or the fraction of compartment J 's tracer transferred to compartment I per unit time;

(b) mean transit times, $\bar{t}(I)$, or the time an average molecule spends in compartment I during a single transit,

where $\bar{t}(I) = [1/L(I, I)]$ and $L(I, I) = \sum_{\substack{J=0 \\ J \neq I}}^n L(I, J)$.

RESULTS

Uptake and release experiments

For the uptake experiments, liver parenchymal cells (10×10^6 cells/ml) were incubated with $1 \mu\text{M}$ - ^{125}I -PVP, and cell-associated radioactivity and radioactivity in the medium were measured after various times (Fig. 1). The accumulation of radioactivity was most rapid during the first minutes. About 0.08% and 0.16% of the added radioactivity were cell-associated after 60 min and 240 min respectively.

Fig. 1. Uptake of ^{125}I -PVP by rabbit liver parenchymal cells

Data are means of three separate experiments (Δ); —, model-predicted fraction of radioactivity in cells versus time. For details, see the Materials and methods section.

For the release experiments, isolated parenchymal cells were loaded by incubation with $1\ \mu\text{M}$ - ^{125}I -PVP at 37°C for 20, 60 or 120 min, and release of radioactivity was measured after various times (Figs. 2a–2c). The release of radioactivity was much faster when cells were loaded with PVP for 20 min, compared with 60 or 120 min. The release was highest during the first minutes, then it gradually declined.

Model development

To establish a starting model for compartmental analysis, we assumed that tracer first entered an endosomal compartment when taken up by cells, and that it subsequently could either be recycled back to the medium or be transferred irreversibly to lysosomes (Fig. 3). It was immediately evident that our experimental data would not fit this simple three-compartment model.

We then gradually increased the complexity of the model, with respect to both recycling and number of compartments. Several different models with a similar number of adjustable parameters, but with recycling between various compartments, were tested. More specifically, we tested (a) models with two, three or four intracellular compartments, (b) recycling between the various intracellular compartments, and (c) recycling of tracer from the various intracellular compartments directly to the medium.

By increasing the complexity of the model, we observed that the value of AIC_p could be decreased substantially. To get a good fit to the experimental data, a substantial portion of the internalized tracer had to recycle to the medium. Furthermore, in all models, a better fit was obtained when radioactivity in presumed lysosomes recycled directly back to the medium rather than to other intracellular compartments.

The model developed to fit these data on fluid-phase endocytosis (Fig. 4) gave a consistent fit to data from both the uptake experiment (Fig. 1) and the three release experiments (Fig. 2). This working-hypothesis model consists of one extracellular compartment and three intracellular compartments (Fig. 4). The model predicts that tracer recycles to the medium from all the intra-

Fig. 2. Release of radioactivity from rabbit liver parenchymal cells loaded for 20 min (a), 60 min (b) or 120 min (c) with ^{125}I -PVP

Data are means of three separate experiments. Cell-associated ($\square$) and released (Δ) radioactivity was determined after various times. The continuous line represents the model-predicted fraction of radioactivity in cells and medium versus time. For details, see the Materials and methods section.

cellular compartments. By increasing the complexity of the model further, we were not able to decrease AIC_p by more than 2 units, suggesting that increased complexity was not needed to describe the present data sets.

Rate constants defining the model of fluid-phase endocytosis are shown in Fig. 4. The fluid-phase endocytotic uptake can be derived from $L(2,1)$, and

Fig. 3. A simple three-compartment model where tracer first enters an endosomal compartment, and is subsequently either recycled back to the medium or transferred irreversibly to the lysosomes

The summer compartment 10 represents cell-associated radioactivity.

equals $0.27 \mu\text{l/h}$ per 10^6 cells. Of the internalized tracer, 82 % was rapidly recycled to the medium, whereas 18 % was transferred to a second intracellular compartment. From this compartment, 28 % was recycled to the

medium, whereas 72 % was transferred to a third compartment. About 0.3 % of the tracer in this compartment was released to the medium per h. The mean transit time for PVP in compartment 2 was 10 min, whereas mean transit times for PVP in the second and third intracellular compartments were 19 and 333 min respectively.

DISCUSSION

In this study, we used model-based compartmental analysis to describe and quantify data on fluid-phase endocytosis by rabbit liver parenchymal cells. Such an approach has been previously applied to some other systems (e.g. various plasma lipoproteins [18], minerals [19], ketone bodies [20], glucose [21] and non-esterified fatty acids [21]). By applying it to data on uptake and release of ^{125}I -PVP, we generated several new hypotheses about the dynamics of this process. To use this form of kinetic analysis, the investigator conceptualizes a physical model which defines the minimum number of inter-changing compartments needed to describe the system and which is compatible with knowledge or assumptions

Fig. 4. Proposed working-hypothesis compartmental model for fluid-phase endocytosis

The summer compartments 13, 14, 15 and 16 represent cell-associated radioactivity. Compartments 1, 4, 7 and 10 represent radioactivity in the medium. Values are rate constants, $L(I, J)$ (min^{-1}). The initial conditions (IC) are given as percentage distribution of tracer. The models simulate the data shown in Figs. 1 and 2. For further details see under 'Statistical and kinetic analysis'.

about the system. Initial estimates for model parameters and their limits are also postulated. Data from an appropriate study are then analysed by using the Simulation, Analysis and Modeling computer programs SAAM [13] and CONSAM [14]. With CONSAM, the temporal response of the physical model after introduction of a tracer into the system is simulated as the solution to a set of differential equations, and the observed data are compared with simulated values. The investigator alters parameter estimates and the model itself to obtain a consistent best fit of the model to the data. Final parameter estimates and their statistical uncertainties are then generated by an iterative non-linear least-squares procedure by using the SAAM or CONSAM program. Through this process, a model is developed which is compatible with both experimental observations and previous knowledge and assumptions about the system. This technique has several other powerful assets, including its utilization of information about previous studies for conceptualization of the initial model and for constraining parameters, its capacity to consider simultaneously data from multiple experiments, and its ability to solve systems which are not in a steady state.

We studied the uptake of the fluid-phase marker PVP by rabbit liver parenchymal cells and applied model-based compartmental analysis to develop the best model that would fit our experimental data. Such an approach has not been previously applied to study fluid-phase endocytosis. PVP has previously been shown to be taken up exclusively by fluid-phase endocytosis in rat liver parenchymal cells [15]. Based on the current data, the uptake of extracellular fluid was $0.27 \mu\text{l/h}$ per 10^6 cells. This is in agreement with earlier reports on fluid-phase endocytosis *in vivo* [6,22], as well as *in vitro* [5,6,15]. Assuming that the initial vesicles formed during fluid-phase endocytosis are $0.2 \mu\text{m}$ in diameter [1] and the diameter of the parenchymal cells is $20 \mu\text{m}$ [1], this rate of uptake reflects an internalization of membrane corresponding to the whole cell surface every 10 min. Yet both the cell and the intracellular organelle volumes change very little with time. It is therefore evident that a high proportion of the internalized membrane and a portion of the endocytosed components must be returned to the plasma membrane and extracellular space, respectively, by exocytosis. Our estimate of membrane internalization agrees closely with that calculated by Scharschmidt *et al.* [6] for cultivated rat hepatocytes.

Previous studies on isolated hepatocytes, macrophages, fibroblasts, L cells and adipocytes have shown that a marker internalized by fluid-phase endocytosis first enters a compartment that rapidly exchanges its contents with the extracellular medium [1,23,24]. In the present model, the rate constant for recycling of tracer from early endosomes was predicted to be 0.08 min^{-1} , corresponding to a transit time of 12 min or a $t_{1/2}$ of 9 min, which is within the range of values published previously ($t_{1/2}$ between 1 and 20 min) [6,23–26]. The current model predicts that more than 80% of the internalized PVP was recycled from the initial intracellular compartment to the medium. Our model suggests that recycling to the extracellular space occurs not only from early endosomes, but from all three intracellular compartments. From compartment 3, which most likely is equivalent to CURL and/or MVB, the model predicts that one-third of the tracer was released to the medium, whereas the rest was transferred to compartment 4. The transit time for recycling from

compartment 3 to the medium was estimated to be 67 min. In the present study, recycling of marker from compartment 3 to compartment 2 did not improve the fit of the model to the experimental data. CURL functions to deliver some receptors to the apical or basolateral plasma membrane of the hepatocyte [7]. The recycling of fluid phase could be a consequence of this receptor recycling.

The lysosomes continuously receive membrane and solute from endosomes and autophagosomes. In the steady state a mechanism must therefore exist for removing and recycling lysosomal membranes. The transfer of fluid-phase marker from lysosomes to the extracellular medium suggested by the present compartmental analysis could be a result of such a recycling. Analysis of the kinetics of exocytosis of fluid-phase markers has previously suggested release from at least two compartments. A compartment with slow turnover similar to our compartment 3 was demonstrated by Besterman *et al.* [23] in macrophages and fibroblasts. Entry of a fluid-phase marker into a slowly turning-over compartment has also been demonstrated in cultured rat hepatocytes [6]. Direct evidence for transfer of endocytosed protein from lysosomes to the medium has been obtained with mouse peritoneal macrophages. It was found that degradation products and undegraded protein were released from the same degradative compartment, presumably the lysosomes [27].

In summary, our use of model-based compartmental analysis has generated a more precise estimate of the kinetics of fluid-phase endocytosis than has previously been possible. The model suggests that more than 80% of the internalized PVP was recycled from early endosomes to the medium. Furthermore, recycling to the extracellular space was not restricted to early endosomes, but took place from all three intracellular compartments. One-third of the tracer turned over from the prelysosomal compartment was released to the medium. A small but significant release of tracer from the lysosomes to the medium was also observed.

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