Regulation of transferrin receptor recycling by protein phosphorylation

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The effect of the protein phosphatase inhibitor okadaic acid on transferrin receptor internalization and recycling was examined in HeLa and K562 cells. Okadaic acid inhibited receptor uptake by more than 85% in both cell lines, whereas it affected transferrin recycling to differing degrees: recycling in HeLa cells was inhibited by greater than 90%, compared with only 65% in K562 cells. Okadaic acid also caused a marked redistribution of receptors in each cell line, which was accounted for by the difference in the extent to which transferrin uptake and recycling

were inhibited. These effects were most likely mediated by a protein kinase, as they were delayed by 10–15 min and could be suppressed by prior incubation with certain protein kinase inhibitors. In addition, it was found that specific kinase inhibitors affected basal rates of transferrin uptake and recycling, although the extent of these effects differed between cell lines. Together, these results suggest that a complex pattern of protein phosphorylation influences the flux of the endocytic pathway in interphase cells.

INTRODUCTION

The mechanism of clathrin-mediated endocytosis has become increasingly well defined over recent years by the combination of cytological studies and biochemical manipulations. Receptors destined to be internalized are localized into coated pits, which comprise 'adaptor' complexes and clathrin heavy and light chains [1]. New coated pits are initiated by the specific interaction between the adaptor complexes and a conserved β -turn structure located within the cytoplasmic domain of the receptor [2,3]. Adaptors, themselves each comprising four subunits (see ref. [4] for review), also mediate the recruitment of clathrin triskelia from a soluble pool, in a process that requires energy and other cytosolic factor(s) [5,6]. Invagination of the mature coated pit proceeds in an ATP-independent fashion [7], and is followed by scission of the membrane and release of the coated endocytic vesicle into the cytosol.

Receptors are delivered from the coated vesicle to the early endosome by a specific membrane fusion event which is driven by ATP and mediated by cytosolic and membrane-bound proteins [8,9]. In addition, as membrane fusion must proceed via the close apposition of the opposing membranes, it is essential that at least part of the clathrin coat of the endocytic vesicle is removed. This release is promoted, at least *in vitro*, by the heat-shock cognate protein, hsc70 [10,11].

The organization of the early endosome has been a subject of some debate. The recent morphological studies would suggest that it is a dynamic system of tubules and vesicles, the extent of interconnection of which is probably determined by an equilibrium between membrane fission and fusion [12,13]. The early endosome is the site where the endocytic pathways of different receptors diverge. Some, such as the epidermal growth factor receptor, are delivered to later more acidic endosomal compartments and ultimately to the lysosome [14]. Others, for example receptors for transferrin and asialoglycoprotein, are segregated from the bulk of the endosomal compartment and return to the cell surface to participate in further rounds of ligand uptake [15,16].

Receptor-mediated endocytosis enables cells to internalize a variety of ligands. Of the many different receptors that have been

identified, most appear to share the same pathway of internalization via clathrin-coated pits (see ref. [17] for review). As individual receptor types may require to be internalized at specific rates or only under certain conditions, mechanisms must be available that regulate the entry of these receptors into the endocytic pathway. Many receptors, for example that for transferrin [18], enter this pathway constitutively. Alternatively, receptors can remain on the cell surface until ligand binding induces receptor internalization [19]. Receptor internalization might be triggered by phosphorylation, as is the case for the epidermal growth factor receptor [20], or might be a consequence of receptor clustering, as occurs for the Shiga toxin receptor [21]. A novel mechanism for regulating receptor internalization is suppression of a negative selection signal. Thus the CD4 receptor is prevented from entering coated pits by an interaction between its cytoplasmic domain and the protein kinase p56^{lck}; dissociation of p56^{lck} allows the CD4 receptor to be internalized efficiently [22].

In contrast with these several examples for which the uptake of individual receptors is modulated, there have been few studies that have examined whether the volume or rate of the endocytic pathway itself is regulated. However, circumstantial evidence suggests that this does occur, as it is known that the extent of endocytic traffic varies markedly between different cell types [23]. Furthermore, there are occasions when the endocytic rate must be increased significantly, such as during the extremely rapid retrieval and reutilization of the presynaptic membrane in stimulated nerve cells [24]. Conversely, in mitotic cells both receptor internalization and recycling are depressed [25,26].

Entry of cells into mitosis is controlled by activation of cdc2 kinase [27], which has been shown to inhibit both coated-vesicle formation [28] and endocytic vesicle fusion *in vitro* [29,30]. Therefore there is now substantial evidence that modulation of protein phosphorylation underlies the inhibition of the endocytic pathway in mitotic cells. There is also evidence that protein phosphorylation might regulate the endocytic pathway in interphase cells, as stimulation by phorbol esters induces partial down-regulation of transferrin receptors in some cell types [31]. However, further studies have been limited by the availability of specific modulators of protein phosphorylation. The extent of

Abbreviation used: DMSO, dimethyl sulphoxide.

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phosphorylation of any given protein is the product of a balance between a kinase and opposing phosphatase [32], and the recent discovery of highly specific inhibitors of protein phosphatases has allowed some progress to be made. Thus the phosphatase inhibitor okadaic acid [33] has been shown to inhibit fluid-phase uptake of horseradish peroxidase in interphase cells [34], as well as fusion between endocytic vesicles in cell-free extracts [30]. We now extend these observations to show that okadaic acid inhibits uptake and recycling of the transferrin receptor in HeLa and K562 cells. We also demonstrate that specific kinase inhibitors modulate both the response to okadaic acid and the control levels of receptor recycling in each cell line.

EXPERIMENTAL

Materials

Desferroxamine (trade name Desferal) was bought from Ciba-Geigy. Okadaic acid (obtained either as free acid or as an ammonium salt) and methyl okadoate were purchased from Scientific Marketing Associates, and stored in liquid nitrogen as 1 mM stocks in dimethyl sulphoxide (DMSO). Samples from this stock were diluted ten times in water and stored for up to a month at -20°C . Staurosporine (Boehringer-Mannheim) was stored as 5 mM and 1 mM stocks in DMSO at -20°C . All other protein kinase inhibitors were bought from Calbiochem, and stored as 1 mM and 2 mM stocks in DMSO at $+4^{\circ}\text{C}$. ATP was dissolved in water and the 40 mM solution neutralized with NaOH before storage at -20°C . Salmon-sperm DNA (Sigma) was stored as 100 mM stock in 5 mM NaOH at -20°C .

Cells

K562 cells were propagated in RPMI 1640 medium supplemented with penicillin, streptomycin and 5% (v/v) fetal calf serum. HeLa cells were propagated either on plastic flasks or in shaker flasks in minimum essential medium modified for suspension cultures (Gibco; Paisley, U.K.; cat. no. 041-04380) containing penicillin, streptomycin, non-essential amino acids and 5% (v/v) fetal calf serum. Cells used for all experiments were in mid-exponential phase (4×10^5 – 7×10^5 cell/ml).

Radiolabelling

^{125}I -transferrin was prepared by the Iodogen method, as described previously [35]. Typically, 400 μg of holotransferrin (Sigma) was labelled with 4 μl of Na^{125}I (100 Ci/ml; Amersham International) to achieve a specific radioactivity of approx. 2×10^5 c.p.m./ μg . All measurements were performed using a Canberra Packard γ counter (model A800C) and samples were measured until 10^5 counts had been detected.

Binding assays

Cells were harvested by centrifugation and washed once in warm Dulbecco's PBS, then once in warm binding medium [BM; CO_2 -independent medium (Gibco; cat no. 041-02045) containing L-glutamine, non-essential amino acids, penicillin, streptomycin and 0.2% (w/v) BSA]. Washed cells were resuspended in warm BM to a concentration of 2×10^7 cells/ml. Okadaic acid (1.5 μM) was added to half the cells and DMSO (0.15%, v/v) to the remainder. After 1 h at 37°C , cells were chilled on ice. After a further 20 min ^{125}I -transferrin was added to a concentration of

10 $\mu\text{g}/\text{ml}$ and the mix was incubated for 90 min at 4°C . Unbound transferrin was removed by washing the cells twice in ice-cold PBS/0.2% BSA by centrifugation (30 s at 16000 g), and the remaining cell pellet was counted for radioactivity.

Internalization and recycling assays

Internalization of ^{125}I -transferrin was determined by incubating cells with radiolabelled transferrin at 37°C , then stripping any ligand bound to the cell surface with a pH 5/pH 7 treatment. Cells were harvested, washed and resuspended in warm BM as for binding assays. After 1 h with 1.5 μM okadaic acid or 0.15% DMSO (control) added, ^{125}I -transferrin (10 $\mu\text{g}/\text{ml}$) was then also added. Transferrin internalization was stopped at various times by removing duplicate samples (10^6 cells) from the incubation and diluting them into $20 \times$ the volume of ice-cold pH 5 saline [100 mM NaCl, 50 mM citric acid, pH 5.0, 0.2% (w/v) BSA] containing 50 μM desferroxamine, and incubating for 10–30 min. After pelleting again, cells were resuspended in PBS containing 50 μM desferroxamine and 0.2% BSA, then incubated for a further 15 min. The cells, now stripped of surface transferrin, were pelleted and counted for radioactivity.

To measure recycling rates, cells were washed in PBS and BM as for binding assays, then incubated with ^{125}I -transferrin (10 $\mu\text{g}/\text{ml}$ in BM) for 30 min at 37°C in order to label the internal pool of transferrin receptors. Either okadaic acid or DMSO (to 1.5 μM or 0.15% respectively) was then added. After 1 h, unlabelled holotransferrin (4 mg/ml final concentration) was also added to the incubation. At various times duplicate samples of 10^6 cells were stripped of surface-bound transferrin by the pH 5/pH 7 treatment described above, and ^{125}I -transferrin still associated with the cells was measured.

Cell extracts

Cells (about 2×10^6 per sample) were centrifuged and the pellets resuspended in cold perchloric acid (2.5%). After 15 min at 4°C , samples were centrifuged for 5 min and the resultant pellets kept for DNA measurement. Universal indicator (2 μl) was added to the supernatants (170 μl), which were then neutralized with buffered KOH solution (2 M KOH, 0.75 M triethanolamine). Neutralized extracts were stored at -80°C before ATP measurement.

ATP measurement

Firefly extract was prepared by homogenizing 50 mg of firefly lanterns (Sigma FLE-50) in 10 ml of water, incubating the extract in the dark for 1 h at 4°C , centrifuging (17000 g, 10 min, 4°C), then storing the supernatant at -20°C . ATP standards were prepared by diluting ATP stock (40 mM) into perchloric acid (2.5%) which had been neutralized as described above.

Each ATP assay mixture [36] comprised 5–10 μl of neutralized cell extract or ATP standard in 180 μl of ATP buffer (prepared immediately before use by mixing equal volumes of the following stock solutions: 10 mM KH_2PO_4 , 80 mM MgSO_4 , 100 mM Na_2HAsO_4). Firefly extract (10 μl) was added immediately before the measurement of luminescence for 10 s using a Berthold Microumat LB 96 P luminometer.

DNA measurement

Cell pellets from the initial perchloric acid extraction (described above) were resuspended in 333 μl of perchloric acid (5%) and acid-hydrolysed for 30 min at 80°C [37]. Standards (10–150 pmol

of salmon sperm DNA) were treated similarly. Freshly prepared DNA buffer (100 ml of acetic acid, 1.5 ml conc. H_2SO_4 , 2% acetaldehyde, 1.5 g of diphenylamine) was then added to all samples to give a final volume of 1 ml and the mixture left overnight at room temperature. DNA concentration was then calculated from the A_{265} using an S&M Cecil Series 2 spectrophotometer.

RESULTS

Internalization of transferrin

Uptake of ^{125}I -transferrin into K562 and HeLa cells was followed by measuring the increasing resistance to a surface-stripping procedure designed to remove transferrin bound to receptors

Figure 1 Okadaic acid inhibits transferrin endocytosis

HeLa (■) or K562 (●) cells were incubated at 37 °C in control binding medium (□) or with medium containing 1.5 μM okadaic acid (■). After 1 h, ^{125}I -transferrin was added. At the times indicated, duplicate samples of 10^6 cells were surface-stripped using an ice-cold pH 5/pH 7 wash cycle designed to remove non-associated and surface-bound transferrin. Surface-stripped cells were counted for radioactivity to measure the amount of internalized transferrin. The number of internalized transferrin molecules was calculated assuming M_r (transferrin) = 75 000. Values are expressed as means $\pm$ S.E.M. ($n = 6$).

only on the cell surface. The cycle of washes at pH 5 and pH 7.4 (in the presence of an iron chelator) mimicked the changes in environment that occur during a normal cycle of transferrin uptake, recycling and release, and resulted in the removal of 80–90% of radiolabelled transferrin bound to cells at 4 °C (not shown). Uptake of ^{125}I -transferrin at 37 °C was rapid in control cells, half-maximal internalization occurring after 3 and 2.5 min in HeLa and K562 cells respectively (Figures 1a and 1b). There was little apparent increase in transferrin internalized in either cell line between 15 min and 1 h, indicating that between these times the rate of transferrin recycling matched the rate of transferrin uptake. The amount of ^{125}I -transferrin internalized after 1 h therefore provided an estimate for the pool of internal transferrin receptors (4.3×10^5 /cell for HeLa, 2.5×10^5 /cell for K562). Preincubation of both cell lines for 1 h at 37 °C with 1.5 μM okadaic acid caused a marked reduction in the rate of ^{125}I -transferrin uptake (Figures 1a and 1b). Calculation of initial rates from three experiments showed that uptake was blocked by 86% in HeLa cells and by 94% in K562 cells. Inhibition by okadaic acid was specific, as methyl okadoate, an inactive analogue, did not affect transferrin uptake (not shown).

To assess more specifically possible cytotoxic effects of okadaic acid immediately after a 1 h exposure, its effect on cellular ATP levels was measured. The total amount of ATP in samples of cells treated with okadaic acid was, on average, 72% and 63% that of control HeLa and K562 cells respectively (see Table 3). This decrease in total ATP could be due to a significant proportion of the cells becoming inviable or to an average fall in ATP in all cells even though they remained viable. To distinguish between these possibilities we assessed the percentage of viable cells by their ability to accumulate fluorescein after the addition of fluorescein diacetate, a membrane-permeable fluorogenic ester. The ester is enzymically hydrolysed by intracellular esterases and the hydrolysed product is lost from cells that are non-viable [38]. In both cell lines, exposure to okadaic acid did not significantly affect the number of cells that accumulated fluorescein (see Table 3). Similarly, there was no significant difference between control and treated cells that excluded 0.1% Trypan Blue (not shown), suggesting that the measured ATP levels reflect a fall within all the cells. Furthermore, the inhibition was partially reversible; when K562 cells were incubated with okadaic acid, followed by a washout in fresh medium over an extended period, the rate of transferrin uptake recovered towards that of control cells (Figure 2). Although the recovery was not complete, this was not due to cell death as over 85% of the cells accumulated fluorescein. In addition, there was no difference in ATP levels detected between control cells and those previously exposed to okadaic acid (not shown).

Transferrin receptor recycling

Endocytosis of transferrin in A431 cells is blocked during mitosis, and examination of the receptor population at the cell surface has indirectly shown that receptor recycling is also arrested. We decided to investigate whether receptor recycling was affected in okadaic acid-treated cells. In this study, we measured receptor recycling directly, by labelling the internal pool of receptors with ^{125}I -transferrin, then incubating the cells with a large excess of unlabelled holotransferrin. A previous study [39] has shown that at 37 °C, pH 7.3, the dissociation of apotransferrin from its receptor has a half-time of 6 s. Unlabelled holotransferrin will therefore exchange rapidly with recycling ^{125}I -apotransferrin before re-internalization can occur. Hence the recycling of radiolabelled transferrin to the cell surface can be measured by the decrease in radioactivity inside the cells.

Figure 2 Inhibition of endocytosis by okadaic acid is reversible

K562 cells were incubated with (■) or without (□) 1.5 μ M okadaic acid for 1 h in BM. Cells were pelleted for 30 s in a Microfuge, then resuspended to 3×10^5 cells/ml in culture medium without okadaic acid. After 10 h the cells were pelleted, washed once in PBS then resuspended in BM at 37 °C. 125 I-transferrin was added and duplicate samples of cells were removed from the incubation mixture at the indicated times. Cells were surface-stripped to remove non-associated and surface-bound transferrin, and then counted for radioactivity. Results from three experiments were converted into molecules/cell and are shown as means $\pm$ S.E.M. ($n = 6$).

Studies of control cells showed that about 80% of 125 I-transferrin was released efficiently from the internal pool in HeLa cells (Figure 3a), and about 90% was released from K562 cells (Figure 3b). The times taken for 50% to be released from HeLa and K562 control cells were 12 min and 7 min respectively. Okadaic acid-treated HeLa cells, however, released little 125 I-transferrin, cell-associated radioactivity falling by only about 15%, even after 45 min. Although the rate of transferrin release was reduced substantially in K562 cells treated with okadaic acid, they appeared somewhat more resistant than HeLa cells (Figure 3b). A titration of okadaic acid from 0.1 to 10 μ M (not shown) indicated that 1.5 μ M gave the optimum inhibition of recycling in K562 cells.

Receptor distribution

The effect of okadaic acid on transferrin endocytosis and recycling is explained most simply by an inhibition of the membrane transport pathways. However, the effect might also be caused by a dramatic reduction in the population of receptors located on the pathway, as the result of, for example, their removal to a degradative pathway or to a cryptic pool of transferrin receptors not available for transferrin binding. To exclude these possibilities, we measured the number of functional transferrin receptors both on the surface of and inside cells treated for 1 h at 37 °C with or without okadaic acid. Surface receptors were quantified by measuring binding of 125 I-transferrin to cells at 4 °C after the treatment. The intracellular receptor pool was measured by pre-equilibrating 125 I-transferrin with cells at 37 °C, and stripping ligand from the cell surface at 4 °C after the okadaic acid treatment.

The majority of receptors (about 77% and 86% in HeLa and K562 cells respectively) were intracellular in both cell lines under control conditions (Table 1). Okadaic acid disturbed the equi-

Figure 3 Okadaic acid inhibits transferrin recycling

HeLa (a) or K562 (b) cells were incubated with 125 I-transferrin for 30 min at 37 °C to label the internal pool of receptors, then incubated for a further 1 h with (■) or without (□) 1.5 μ M okadaic acid also included. Excess unlabelled holotransferrin was then added to the incubation mixture and, at the indicated times after this addition, duplicate samples of cells were surface-stripped. Cells were counted for radioactivity to measure the amount of transferrin retained. (The amount of transferrin present in cells immediately before the chase was estimated by extrapolating the curves back from $t = 1$ min to $t = 0$.) Results were converted into molecules/cell and are shown as means $\pm$ S.E.M. ($n = 6$ for HeLa cells; $n = 12$ for K562 cells).

librium between surface and internal receptors, although in opposite directions in the two cell lines. In K562 cells, 1 h exposure to okadaic acid caused a change in surface receptor number from 46000 to about 96000 receptors/cell, a rise of 108%, which was accompanied by a corresponding fall in internal receptors of about 26%, from 278000 to 206000 per cell. In contrast, the number of surface receptors decreased in HeLa cells by about 46% and the number of internal receptors increased by an average of 13%. Scatchard analysis (not shown) confirmed that the effects on surface transferrin binding were the result of changes in receptor number rather than ligand affinity. Furthermore, the total number of receptors available for transferrin binding was not significantly altered by okadaic acid in either cell line; the sum of internal and external receptors remained at

Table 1 Okadaic acid causes redistribution of transferrin receptors

Cells were incubated with or without okadaic acid ($1.5 \mu\text{M}$) for 1 h at 37°C and the cell surface and intracellular pools of transferrin receptors were measured. Cell surface receptors were measured by incubating cells with ^{125}I -transferrin for 2 h at 4°C , then washing in PBS to remove non-associated transferrin. Internal receptors were measured by preincubating cells for 30 min at 37°C with ^{125}I -transferrin, then adding okadaic acid for a further 1 h, before surface-stripping to remove non-associated and surface-bound transferrin. Measurements were made on triplicate samples of cells each time. Values are means $\pm$ S.E.M. ($n = 9$, internal receptors; $n = 15$, surface receptors).

Cell line	$10^{-3} \times$ Internal receptors (molecules/cell)		Change caused by okadaic acid	$10^{-3} \times$ Surface receptors (molecules/cell)		Change caused by okadaic acid (%)	$10^{-3} \times$ Total receptors (molecules/cell)	
	— Okadaic acid	+ Okadaic acid		— Okadaic acid	+ Okadaic acid		— Okadaic acid	+ Okadaic acid
HeLa	793 ± 25	900 ± 32	+ 13	239 ± 4	129 ± 17	— 46	1032 ± 26	1049 ± 36
K562	278 ± 16	206 ± 14	— 26	46 ± 2	96 ± 7	+ 108	324 ± 16	302 ± 16

Figure 4 Effect of okadaic acid on transferrin uptake is delayed

HeLa (Δ , $\blacktriangle$) or K562 ($\square$, $\blacksquare$) cells were resuspended in BM and incubated at 37°C with ($\blacktriangle$, $\blacksquare$) or without (Δ , $\square$) okadaic acid ($1.5 \mu\text{M}$). At the indicated times ^{125}I -transferrin was added and the incubation continued for a further 4 min. Triplicate samples of cells were chilled and surface-stripped and the internalized radioactivity was measured. Results were normalized, 100% being the amount of ^{125}I -transferrin internalized in 4 min by cells not exposed to okadaic acid ($190\,000$ and $152\,000$ molecules/cell in HeLa and K562 cells respectively). Values are means $\pm$ S.E.M. from three experiments ($n = 9$).

about 3×10^5 and 1×10^6 per K562 and HeLa cell respectively. It should be noted that the number of internal receptors in HeLa cells was about double the number measured in the previously described uptake and recycling experiments. Variation of this magnitude in HeLa cells has been described before [40] and the extent of the effect of okadaic acid on uptake and recycling was the same regardless of total receptor number (not shown).

Okadaic acid induced a marked change in transferrin receptor distribution, even though both receptor uptake and recycling were inhibited. This might be accounted for if the two steps in the recycling pathway were inhibited at different times after addition of okadaic acid, or may reflect differences in the extents of inhibition: even a small change in the ratio of internalization/recycling rates would lead to a substantial imbalance in receptor distribution after 1 h. To distinguish between these possibilities, we measured the lag times between okadaic acid addition and the inhibition of uptake and recycling in both cell lines. Uptake rates

Figure 5 Effect of okadaic acid on transferrin recycling is delayed

HeLa cells were incubated with ^{125}I -transferrin to saturate internal receptors, then with okadaic acid also added (to $1.5 \mu\text{M}$) for the indicated times. Unlabelled transferrin (4 mg/ml) was then added and the incubation continued for a further 8 min. Triplicate samples of cells were surface-stripped and the amount of ^{125}I -transferrin remaining inside the cells was then measured. The relative amount of transferrin released (T_r) after Δt preincubation with okadaic acid was calculated from the following formula: $T_r = [(c.p.m._{T-60} - c.p.m._{\Delta t}) / (c.p.m._{T-60} - c.p.m._{T-0})] \times 100$. Values are means $\pm$ S.E.M. from four experiments ($n = 12$).

were estimated by incubating cells for 4 min with labelled transferrin. As shown in Figure 4, okadaic acid inhibited ^{125}I -transferrin uptake by approx. 85% using this criterion, consistent with our earlier estimate. Inhibition by okadaic acid occurred quickly, but was not immediate. In both cell lines there was a short lag time before any effect was noticed, and thereafter inhibition increased with time. In both cell lines it took about 10 min to reach half-maximal inhibition.

Recycling rates were estimated by loading cells with transferrin and measuring the reduction in cell-associated radioactivity 8 min after addition of unlabelled ligand to the medium. It was difficult to obtain accurate data for the rate at which okadaic acid blocked recycling in K562 cells, as inhibition was only partial, even after 1 h (see Figure 3). In HeLa cells, in which inhibition was more complete, the rate could be followed with more confidence (Figure 5). As with internalization, a lag occurred before any effect on receptor recycling was observed. Inhibition increased thereafter, although it took approx. 15 min

Figure 6 Kinase inhibitors suppress the effect of okadaic acid on transferrin uptake

HeLa (Δ, ▲) or K562 (□, ■) cells were incubated with different concentrations of staurosporine (a) or K252a (b) for 15 min at 37 °C. Incubations were continued for a further 45 min with (□, Δ) or without (■, ▲) 1.5 μM okadaic acid. ¹²⁵I-transferrin was added and, after a further 4 min, triplicate samples of cells were chilled and surface-stripped to remove non-associated and surface-bound transferrin. The amount of internalized radioactivity was then measured and converted into molecules/cell. Values are means ± S.E.M. (n = 12).

to reach half-maximal values. This time is somewhat longer than that for inhibition of uptake, even allowing for the difference in measurement times.

Protein kinase(s) mediate the effects of okadaic acid

Okadaic acid is a lipophilic agent which should enter cells readily and which binds to type 1 and type 2A phosphatases within

seconds and with high affinity [41]. The most likely reason therefore, for the relatively slow effect of this compound on the transferrin cycle is that inhibition is caused by hyperphosphorylation of component(s) of the pathway by protein kinase(s), rather than inhibition of dephosphorylation *per se*. The extent of inhibition should therefore reflect the activity of these kinase(s). To confirm this we examined the ability of different protein kinase inhibitors to suppress the effect of okadaic acid. Staurosporine [42], a broad-spectrum kinase inhibitor, reduced the inhibitory effect of okadaic acid on ¹²⁵I-transferrin uptake in a concentration-dependent manner, but to different extents in the two cell lines (Figure 6a). In HeLa cells treated with both staurosporine and okadaic acid, transferrin uptake reverted to the level found in cells exposed to staurosporine alone; however, in K562 cells the reversal was only partial. Surprisingly, the effect of staurosporine was more complex, as it affected the rate of ¹²⁵I-transferrin uptake significantly by itself. Uptake was stimulated by low concentrations of staurosporine in K562 cells, although it returned to basal levels at higher concentrations. In HeLa cells 10 μM staurosporine reduced transferrin uptake by over 50%; the extent of inhibition did not increase further at higher concentrations of the drug (not shown). The related kinase inhibitor K252a [42] partially reversed the inhibitory effect of okadaic acid on transferrin uptake (Figure 6b) in both cell lines and also caused a significant reduction in basal levels of transferrin uptake in HeLa cells. The effects of these kinase inhibitors are specific, as KT5720 and KT5926, structurally related kinase inhibitors [43,44] with different specificities, did not substantially affect either basal transferrin uptake or the inhibition by okadaic acid (not shown).

As staurosporine and K252a influenced the basal rate of transferrin uptake and the inhibition of uptake caused by okadaic acid, we extended our studies to include the effects of kinase inhibitors on transferrin recycling. The results are summarized in Table 2. In K562 cells, and to a lesser extent in HeLa cells, both of these inhibitors caused a substantial reduction in the inhibition of transferrin recycling caused by okadaic acid. In contrast, the related inhibitors KT5926 and KT5720 had slight, if any, effect. Staurosporine and K252a also affected the basal rates of transferrin recycling in both cell lines, causing a small increase in recycling in K562 cells and a reduction in the rate of recycling in HeLa cells.

Some of the results seen with kinase inhibitors could have been the influence of non-specific cytotoxic effects, so cell viability was assessed. The ability of cells to accumulate fluorescein after addition of fluorescein diacetate was measured. Staurosporine and K252a did not affect viability using this criterion (Table 3). We also measured ATP levels in cells incubated for 1 h with the kinase inhibitors alone and together with okadaic acid (Table 3). The cellular ATP levels in all the conditions tested were more than 60% the level found in cells not exposed to any inhibitors. In addition, the effects of staurosporine on transferrin uptake could be partially reversed in K562 cells (Table 4). Control cells and cells previously exposed to staurosporine were subjected to a prolonged incubation in staurosporine-free culture medium. Whereas staurosporine significantly reduced the inhibitory effect of okadaic acid before the wash-out procedure, when cells were exposed to okadaic acid after staurosporine washout there was no such recovery in transferrin uptake even though 81% of cells were still viable. However, prior exposure to staurosporine itself significantly reduced transferrin uptake after the washout and reduced cell viability to about 60%. These effects could not be seen in K562 cells before the washout (Figure 6a), suggesting that they were additional effects manifested only some time after the initial 1 h exposure.

Table 2 Effects of kinase inhibitors on transferrin recycling and its inhibition by okadaic acid

Cells were incubated at 37 °C with ^{125}I -transferrin (10 $\mu\text{g}/\text{ml}$) for 30 min, then with various protein kinase inhibitors as indicated for a further 15 min. Okadaic acid was added to 1.5 μM , then these samples and parallel samples not exposed to okadaic acid were incubated for a further 45 min. The amount of cell-associated transferrin resistant to surface-stripping was then measured in triplicate samples, and also in samples chased for a further 10 min at 37 °C with 4 mg/ml unlabelled holotransferrin. From these values, the percentage of the intracellular pool of transferrin released after 10 min under each condition was calculated. Values are means $\pm$ S.E.M. from the indicated number of experiments.

Inhibitor (concn.)	Transferrin released after 10 min in HeLa cells (%)		Transferrin released after 10 min in K562 cells (%)	
	— Okadaic acid	+ Okadaic acid	— Okadaic acid	+ Okadaic acid
None	48 $\pm$ 3 (<i>n</i> = 9)	10 $\pm$ 2 (<i>n</i> = 9)	59 $\pm$ 2 (<i>n</i> = 8)	24 $\pm$ 5 (<i>n</i> = 8)
Staurosporine (10 μM)	38 $\pm$ 2 (<i>n</i> = 3)	30 $\pm$ 4 (<i>n</i> = 3)	66 $\pm$ 6 (<i>n</i> = 3)	47 $\pm$ 4 (<i>n</i> = 3)
K252a (10 μM)	33 $\pm$ 8 (<i>n</i> = 3)	21 $\pm$ 3 (<i>n</i> = 3)	63 $\pm$ 4 (<i>n</i> = 5)	40 $\pm$ 4 (<i>n</i> = 5)
KT5926 (20 μM)	36 $\pm$ 11 (<i>n</i> = 3)	11 $\pm$ 5 (<i>n</i> = 3)	62 $\pm$ 12 (<i>n</i> = 3)	29 $\pm$ 6 (<i>n</i> = 3)
KT5720 (20 μM)	46 $\pm$ 7 (<i>n</i> = 3)	15 $\pm$ 1 (<i>n</i> = 3)	59 $\pm$ 6 (<i>n</i> = 3)	30 $\pm$ 12 (<i>n</i> = 3)

Table 3 Effects of kinase inhibitors and okadaic acid on cellular ATP levels and cell viability

Samples of HeLa and K562 cells were incubated at 37 °C with kinase inhibitors as indicated for 15 min. The incubation was then continued for a further 45 min with half the cells exposed to okadaic acid (OA). Samples of cells were then tested to assess any changes caused by the different incubations. (a) The ratio of ATP/DNA: cells were treated with 2.5% perchloric acid, centrifuged, and the resultant pellets assayed for DNA; the supernatants were neutralized and assayed for ATP. Results are means $\pm$ S.E.M. from three experiments (*n* = 3). (b) Cell viability assessed by the percentage of cells that exhibited fluorescence after 10 min exposure at room temperature to 0.1 $\mu\text{g}/\text{ml}$ fluorescein diacetate in PBS. Results are means from two experiments, in which 150–250 cells were counted from each incubation. nd, Not determined.

Cell line	Test	Control		Staurosporine		K252a		KT5720		KT5926	
		— OA	+ OA	— OA	+ OA	— OA	+ OA	— OA	+ OA	— OA	+ OA
HeLa	ATP/DNA (pmol/ μg)	32 $\pm$ 4	23 $\pm$ 4	34 $\pm$ 6	39 $\pm$ 6	28 $\pm$ 8	25 $\pm$ 6	19 $\pm$ 6	21 $\pm$ 8	22 $\pm$ 5	26 $\pm$ 5
	Viable cells (%)	88	90	92	91	90	93	nd	nd	nd	nd
K562	ATP/DNA (pmol/ μg)	35 $\pm$ 4	22 $\pm$ 4	47 $\pm$ 20	40 $\pm$ 3	50 $\pm$ 10	42 $\pm$ 3	46 $\pm$ 11	30 $\pm$ 2	38 $\pm$ 6	28 $\pm$ 1
	Viable cells (%)	93	92	91	90	90	92	nd	nd	nd	nd

Table 4 Effects of staurosporine on transferrin uptake are reversible

K562 cells were incubated with or without staurosporine for 1 h in BM. Cells were then pelleted and resuspended in culture medium without staurosporine. After 12 h, cells were washed by centrifugation in BM and then resuspended in either control BM or BM containing okadaic acid. After 1 h at 37 °C, ^{125}I -transferrin was added to the incubation medium and the amount internalized by cells in 4 min measured. Results from two experiments (which did not differ by more than 20%) were pooled and are shown as the mean number of transferrin molecules internalized per cell both before and after the 12 h wash-out.

	Transferrin internalized (molecules/cell)			
	Control		Exposed to staurosporine	
	— Okadaic acid	+ Okadaic acid	— Okadaic acid	+ Okadaic acid
Before washout	157 000	23 000	168 000	71 500
After washout	144 000	25 000	77 500	27 000

In summary therefore, inhibition of the transferrin cycle by okadaic acid was reduced substantially by inclusion of certain protein kinase inhibitors. The same kinase inhibitors added after incubation with okadaic acid, however, did not affect the level of inhibition (not shown). These data suggest that okadaic acid acts by causing hyperphosphorylation of key components of the endocytic apparatus. In addition, however, the spectrum of effects of protein kinase inhibitors on basal rates of recycling

reveals a more complex role for protein phosphorylation in the regulation of endocytic traffic.

DISCUSSION

In this report we describe the perturbation of the transferrin cycle in two human cell lines by the specific Ser/Thr phosphatase inhibitor okadaic acid. In both HeLa and K562 cells uptake of radiolabelled transferrin from the medium was inhibited by more than 85% (Figure 1), confirming and extending a previous observation that fluid-phase endocytosis is inhibited in cells exposed to okadaic acid [34]. By direct measurements we have shown that recycling of transferrin to the cell surface from intracellular compartments is also affected by okadaic acid. In contrast with transferrin uptake, the effect of okadaic acid on receptor recycling differed somewhat between cell lines (Figure 3). Inhibition in HeLa cells approached 90%, whereas in K562 cells an optimum of only about 60% inhibition was observed. The significance of this is not clear, but may reflect differences in the distribution of the transferrin receptor throughout the recycling pathway between each cell line, coupled with variation in the extent to which okadaic acid blocks each stage on the recycling pathway.

Various observations suggest that these results were caused by specific effects rather than non-specific cytotoxic effects. Despite the extremely tight binding of okadaic acid to phosphatases [41], a considerable degree of reversion to normal levels of transferrin uptake in K562 cells was observed on prolonged washout of the drug (Figure 2). The reversion was not complete, but we suggest

that this is due to incomplete removal of okadaic acid. However, we cannot rule out the possibility that incomplete recovery is caused by prolonged exposure to nanomolar concentrations of okadaic acid or to effects that occur sometime after the initial exposure. In our studies we were primarily concerned with any toxic effects manifested shortly after a 1 h incubation. Cell integrity, assessed by Trypan Blue exclusion and the ability to accumulate fluorescein after exposure to fluorescein diacetate [38], was unchanged by okadaic acid after 1 h (Table 3). The ATP levels in cells treated with okadaic acid fell to about 72% and 63% in K562 and HeLa cells respectively and, as there was no corresponding decrease in cell integrity, we suggest that this represents an average decrease in ATP concentration across all of the cells. A previous study with K562 and HeLa cells [45] shows that transferrin uptake is unaffected when cellular ATP levels are depleted to 60% of the control value. It is therefore unlikely that the inhibition of transferrin uptake is due to the fall in ATP concentration *per se*.

The effects of okadaic acid on the endocytic pathway could be explained by the need for active phosphatases to promote essential steps on the pathway. Alternatively, inhibition of phosphatases might act indirectly, by altering the balance of phosphorylation of components of the transport machinery. We favour the latter explanation, for two reasons. First, the action of okadaic acid is delayed for 10–15 min (Figure 4), despite its binding to phosphatases within seconds [41]. It is unlikely that this delay is a consequence of delayed entry of okadaic acid into cells, as it is a lipophilic drug and should enter cells readily. Indeed, a previous study has shown that okadaic acid has no immediate effect on coated-pit invagination in broken cells, where access of the drug to its target should occur without delay [28]. Similarly, okadaic acid was delayed in its ability to inhibit endoplasmic reticulum–Golgi transport [46] and endosomal vesicle fusion [30] in cell-free systems. Secondly, the action of okadaic acid on both receptor uptake and recycling was reversed to a substantial extent by prior exposure of cells to the protein kinase inhibitors staurosporine and K252a (Figures 6a and 6b).

The protein kinase inhibitors used in this study were originally identified as inhibitors of protein kinase C [42–44]. Although it is possible that protein kinase C plays a role in the inhibition of transport by okadaic acid, we cannot confirm that this is the case, given the relatively high concentrations of inhibitor that we have used compared with those that normally indicate a protein kinase C-mediated response [42–44]. However, we note that a recent report [47] has shown that protein kinase C may influence the binding of ADP ribosylation factor to Golgi membranes, thus implicating this kinase in the regulation of the secretory pathway. Another candidate kinase would be cdc2, as previous reports have shown that both coated-pit invagination [28] and endosomal vesicle fusion [29,30] are inhibited by this kinase. The effects described here, however, are not mediated by cdc2 kinase as its activity is tightly controlled during the cell cycle. In both cell lines described, about 95% of cells are in interphase, when cdc2 kinase activity is low, and no evidence of cdc2 activation by okadaic acid has been found in intact [34] or broken [30] mammalian cells. Furthermore, no evidence of other mitotic effects mediated by cdc2 kinase, such as chromosome condensation or nuclear envelope breakdown, has been found in this (not shown) or other [34] studies.

As we have followed the effect of okadaic acid on a receptor-mediated transport pathway, it was important to distinguish between effects on the mechanism of transport itself and effects on receptor function. We were particularly concerned to establish that okadaic acid did not influence the total number of accessible transferrin receptors. Therefore we measured the number of

receptors expressed on the surface and inside control and okadaic acid-treated cells. Although marked changes in receptor distribution were noted, we calculated that the total number of receptors was not altered significantly in either cell line (Table 1).

Okadaic acid treatment led to dramatic changes in transferrin receptor distribution in both cell lines. A previous report described an alteration in transferrin receptor distribution in A431 cells during mitosis [26], and concluded that this redistribution was a consequence of a temporal difference between the inhibition of receptor internalization and receptor recycling to the surface. We therefore examined whether this might also explain the dramatic reduction in the population of surface receptors in HeLa cells on exposure to okadaic acid. Surprisingly, we found that inhibition of receptor recycling was delayed for at least as long as inhibition of receptor internalization after application of okadaic acid. The most likely explanation therefore for the change in receptor distribution induced by okadaic acid treatment of HeLa cells is that recycling is blocked to a greater extent than uptake. Although this difference would appear small, under normal conditions about 15% of the surface pool of receptors are replaced every minute [17], so even a slight alteration in the balance of entry/exit would lead to significant changes over the course of the experiment. Conversely, treatment of K562 cells with okadaic acid led to a net redistribution of receptors to the cell surface. This could be accounted for by the greater inhibition of receptor uptake relative to recycling.

A number of potential targets for the action of okadaic acid have been described. The transferrin receptor itself is hyperphosphorylated on Ser-24 after treatment of cells with phorbol esters [48]. However, it is unlikely that phosphorylation of the receptor plays a role in its recycling kinetics, as replacement of Ser-24 by alanine does not affect receptor distribution [48]. More likely targets are those proteins involved in endocytic vesicle production and vesicle consumption. For example, it has recently been shown that dynamin function is essential for coated-vesicle production and transferrin uptake [49,50]. Moreover, dynamin function appears to be regulated by a complex pattern of phosphorylation, involving protein kinase C and casein kinase II [51]. Components of the coated vesicle are also phosphorylated, most notably the assembly polypeptides and clathrin light chains [52]. The functional significance of these modifications is not clear, but some may affect coated-vesicle acidification [53]. The results described here are also consistent with inhibition of vesicle-fusion events [31] by okadaic acid. Candidate substrates involved in this event have not been identified, although syntaxin, a component of the presynaptic membrane implicated in vesicle targeting, has been identified as a substrate for casein kinase II [54].

The effects of protein kinase and phosphatase inhibitors on the endocytic pathway described in this report indicate that the volume of endocytic transport might be tightly regulated in the interphase cell. Such a conclusion fits well with the recent observations that multiple types of GTP-binding proteins, including heterotrimeric G-proteins, are involved in the production [55] and fusion [56] of endocytic vesicles, and with the identification of a lipid kinase [57] as an essential component in the vacuolar targeting pathway in yeast.

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