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Calmodulin dependence of transferrin receptor recycling in rat reticulocytes

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Kinetic analysis of transferrin receptor properties in 6–8 day rat reticulocytes showed the existence of a single class of high-affinity receptors (K_d 3–10 nM), of which 20–25% were located at the cell surface and the remainder within an intracellular pool. Total transferrin receptor cycling time was 3.9 min. These studies examined the effects of various inhibitors on receptor-mediated transferrin iron delivery in order to define critical steps and events necessary to maintain the functional integrity of the pathway. Dansylcadaverine inhibited iron uptake by blocking exocytic release of transferrin and return of receptors to the cell surface, but did not affect transferrin endocytosis; this action served to deplete the surface pool of transferrin receptors, leading to shutdown of iron uptake. Calmidazolium and other putative calmodulin antagonists exerted an identical action on iron uptake and receptor recycling. The inhibitory effects of these agents on receptor recycling were overcome by the timely addition of Ca^{2+}/ionomycin. From correlative analyses of the effects of these and other inhibitors, it was concluded that: (1) dansylcadaverine and calmodulin antagonists inhibit iron uptake by suppression of receptor recycling and exocytic transferrin release, (2) protein kinase C, transglutaminase, protein synthesis and release of transferrin-bound iron are not necessary for the functional integrity of the iron delivery pathway, (3) exocytic transferrin release and concomitant receptor recycling in rat reticulocytes is dependent upon Ca^{2+}/calmodulin, (4) dansylcadaverine, dimethyldansylcadaverine and calmidazolium act on iron uptake by interfering with calmodulin function, and (5) the endocytotic and exocytotic arms of the iron delivery pathway are under separate regulatory control.

INTRODUCTION

Iron delivery to erythroid cells for haem synthesis requires the functional integrity of the receptor-mediated transferrin iron pathway. Intracellular levels of free haem [1] or of chelatable iron [2,3] have been described as signals which modulate the receptor-driven pathway in various cells, either through stimulation of transcriptional and translational expression [4,5] or through post-translational modifications [6–9]. The mechanisms through which these signals are translated into modulation of the receptor-mediated iron delivery pathway are being actively investigated. Phosphorylation of the transferrin receptor was considered a potential effector of receptor flux [10–12] but subsequent studies showed that receptor phosphorylation was not required for endocytosis [13,14] or recycling of transferrin receptor to the cell surface [6–9]. Relatively little is known regarding phosphorylation of membrane, vesicular and/or cytoskeletal proteins as regulatory factors in receptor–transferrin flux. Likewise, the precise role(s) of kinases mediating protein phosphorylation [15], of which protein kinase C (PKC), cyclic AMP-dependent kinase and a multifunctional Ca^{2+}/calmodulin-dependent kinase [16–18] appear particularly important in several other cell types, remain(s) to be elucidated.

Various inhibitors used for analysis of receptor-mediated events have been employed by us in an effort to delineate the nature of regulatory events affecting the receptor-mediated transferrin iron delivery pathway in rat reticulocytes. These studies were begun to analyse the effects of dansylcadaverine, an inhibitor of transglutaminase [19], on receptor-mediated iron delivery. Subsequent studies with related inhibitors endeavoured to define the mechanism(s) by which dansylcadaverine inhibited the iron delivery pathway in an effort to identify critical factors involved in the functional integrity of the pathway. The results of these studies show that: (1) dansylcadaverine is a potent inhibitor of iron delivery, acting to suppress receptor recycling and to deplete the surface transferrin receptor pool, (2) calmidazolium and other calmodulin antagonists exert an inhibition of transferrin-mediated iron delivery that is identical in all respects to the effects of dansylcadaverine, (3) PKC, transglutaminase and protein synthesis are not involved in transferrin-mediated iron delivery in rat reticulocytes and (4) the inhibitory effects of dansylcadaverine and calmidazolium on receptor recycling can be overcome by the timely addition of exogenous Ca^{2+} and calcium ionophore. The results lead us to conclude that exocytic release of transferrin and receptor recycling in rat reticulocytes is a Ca^{2+}/calmodulin-dependent event.

MATERIALS AND METHODS

Preparation of reticulocytes

Sprague–Dawley white rats were made anaemic by three daily intraperitoneal injections of 2.5%

Abbreviations used: PKC, protein kinase C; PBS, phosphate-buffered saline; IC_{50}, concn. causing 50% inhibition; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride; W13, *N*-4-(6-aminobutyl)-5-chloro-1-naphthalene sulphonamide.

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acetylphenylhydrazine in phosphate-buffered saline (PBS). At 3–5 days after the final injection, blood was aspirated from the heart or abdominal aorta into a heparinized syringe and centrifuged at 1000 *g* for 5 min. Reticulocytes were enriched to 80–95 % by density centrifugation on Ficoll or Percoll [20].

Preparation of transferrin

Rat transferrin, purified from serum or plasma [21,22], was depleted of iron by sequential dialyses against 0.1 M-citrate/citric acid/sodium acetate (pH 4.5), distilled water and 0.9% NaCl, and iron-loaded to 35–100 % Fe saturation with Fe^{3+}-nitrilotriacetate as described previously [21]. The degree of iron saturation was calculated from the specific activity of ^{59}Fe, using an absorption coefficient at 280 nm of 14.1 [22] for diferric transferrin, or from the A_{470}/A_{280} ratio (100 % saturation = 0.040–0.045). Transferrin was iodinated with Enzymobead (Bio-Rad); unbound ^{125}I was decreased to 1–4 % by chromatography on Sephadex G25 and dialysis against 0.9% NaCl for 2–3 days. Recovery of ^{125}I-labelled transferrin was measured by its absorbance at 280 nm or by colorimetric assay for total protein.

Cell incubations

Reticulocytes were suspended in medium containing 5 vol. of complete amino acids prepared from a stock solution [23]; 92 vol. of phosphate- or Hepes-buffered saline containing 0.4 mM-$MgCl_2$, 0.7–1.0 mM-$CaCl_2$, 1–2 % defatted iron-free BSA, 5–10 mM-glucose; 1 vol. of 55 mM-glutamine; 1 vol. of minimal essential medium vitamins (Gibco); 1 vol. of penicillin/streptomycin (5000 μunits/ml); and 0.2% Phenol Red (pH 7.2–7.4). This medium supported the linear uptake of ^{59}Fe into haemoglobin for more than 6 h and maintained cell viability for at least 24 h, based upon demonstrable persistence of iron uptake and Trypan Blue exclusion. (Mono)dansylcadaverine (Sigma) was dissolved directly in medium at a final concentration of 100–350 μM with heating, and the pH was adjusted to pH 7.2 with NaOH. Calmidazolium (CalBiochem) dissolved in 95 % ethanol at 10 mM was added to cell suspensions to final concentrations of 3–12 μM. Other agents (chloroquine, cycloheximide) were either added directly to medium or from stock solutions prepared in distilled water {10 mM-H-7 [1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride]; Seikagaku America, Inc.}, or in dimethyl sulphoxide [8 mM-phorbol 12-myristate 13-acetate (PMA); Sigma].

Iron uptake. Iron uptake was measured in reticulocytes incubated at 37 °C in medium containing 0.2–0.5 μM ^{59}Fe–transferrin (35–50 % Fe-saturated) and, where pertinent, the agent to be tested. Aliquots were removed at various times of incubation and washed in ice-cold PBS to remove unbound radioactivity. Total cell radioactivity was measured in a Packard AutoMinaxi gamma counter; cells were then haemolysed in hypo-osmotic buffer (5 mM-phosphate, pH 7.2, or 20 mM-Hepes, pH 8.0) and centrifuged at 1500 *g* for 20 min to separate stroma from cytosol. Haem was extracted from various cell fractions with cyclohexanone [21].

Transferrin binding activity and receptor kinetics. The effects of various agents on transferrin binding activities and receptor kinetics were analysed using a commercially available program (Combicept; Packard Instruments) in which binding data are simultaneously processed by Scatchard and saturation-curve analysis. Reticulocytes were incubated at 0–4 °C or 37 °C in medium containing 0.005–0.5 μM-^{125}I-labelled diferric transferrin and the agent to be tested. Non-specific binding was obtained from a parallel set of incubations containing a 50–200-fold excess of non-radioactive transferrin. Incubations were terminated by withdrawal of duplicate aliquots of each sample into ice-cold PBS and centrifugation at 1000 *g* to pellet the cells. After several washes with PBS, the pellets were placed in a Packard AutoMinaxi gamma counter connected to an IBM PC for analysis of ^{125}I-transferrin binding.

Endocytic uptake of transferrin was measured in cells incubated with ^{125}I-labelled diferric transferrin at 37 °C in the presence or absence of various agents. Aliquots taken at various times were washed in ice-cold PBS and total cell-associated radioactivity was determined. Each cell suspension was then divided into two aliquots, one of which was digested with 0.25% Pronase in PBS for 2–3 h at 0–4 °C, whereas the other was kept in ice-cold PBS.

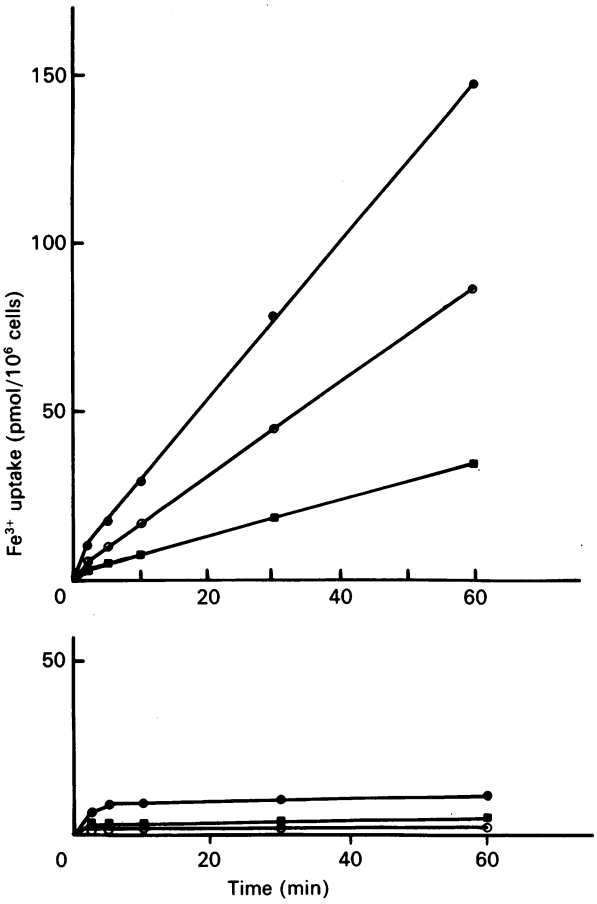

Fig. 1. Effects of dansylcadaverine on iron uptake

Reticulocytes were incubated in medium supplemented with 20% (v/v) ^{56}Fe-saturated rat plasma, and aliquots were removed at the indicated times for measurement of ^{59}Fe uptake in intact whole cells (●), cytosol (○) and stroma (■). Stroma and cytosol were prepared by lysis in 20 mM-Hepes/Cl, pH 8.0, followed by centrifugation at 1500 *g* for 20 min. (*a*) Control, untreated cells, (*b*) same conditions as in (*a*) except for the addition of 200 μM-dansylcadaverine to medium.

Cells in each aliquot were pelleted by centrifugation, and radioactivity in the cell pellet was determined. The amount of cell-associated radioactivity resistant to mild proteolytic digestion was a measure of the degree of transferrin endocytosis.

Exocytic release of transferrin and receptor recycling was determined in cells which were pulse-labelled with 0.2–0.5 μM-^{125}I-transferrin for 35–50 min and then chased in non-radioactive medium supplemented with ^{56}Fe–transferrin in the presence or absence of various agents. Aliquots were removed at 0–25 min to determine the amount of ^{125}I-transferrin released into the medium and the radioactivity remaining cell-associated at the various time points. Endocytotic and exocytotic rates were calculated by linear regression analysis of the data.

RESULTS

Dansylcadaverine was a strong inhibitor of iron uptake by rat reticulocytes [concn. causing 50 % inhibition (IC_{50}) 105 μM] and, at 125–350 μM, it decreased iron uptake by more than 90 % (Figs. 1*a* and 1*b*). The decreased amount of iron that was taken up in the presence of the amine remained principally in the stroma (Fig. 1*b*), with minimal entry into the cytosol. Dansylcadaverine did not prevent utilization of intracellular iron for haem synthesis. For example, when haem synthesis is inhibited by succinylacetone, incoming ^{59}Fe is accumulated in an intramitochondrial precursor pool which serves as a primary iron source for renewed haem synthesis when succinylacetone is removed [21]. Dansylcadaverine did not affect the utilization of intramitochondrial iron for renewed haem synthesis; in 6 day reticulocytes, the rates of ^{59}Fe incorporation into haem were 1.95 and 2.12 pmol/min per 10^7 cells in control and dansylcadaverine-treated cells respectively; in 8 day reticulocytes, the respective rates were 0.47 and 0.42 pmol/min per 10^7 cells. These results confirmed that the primary action of dansylcadaverine was to restrict uptake and entry of iron into the cell.

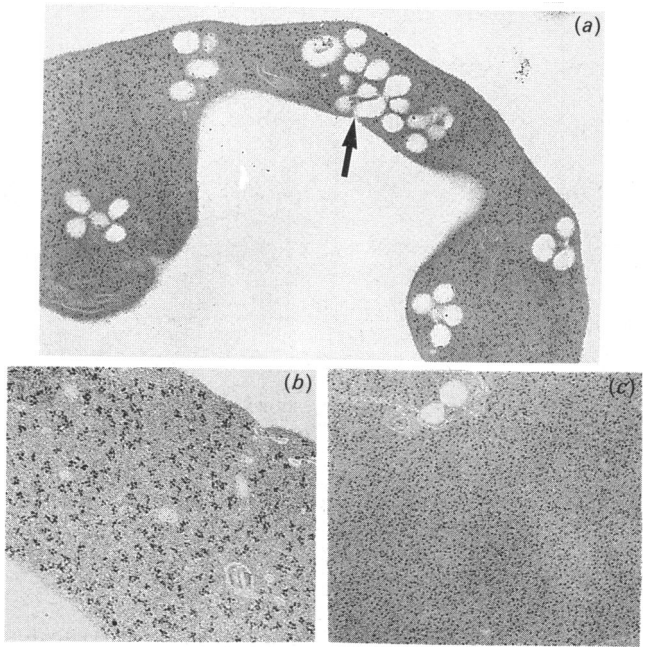

Fig. 3. Electron micrographs depicting the effects of 200 μM-dansylcadaverine on reticulocytes

(*a*) Reticulocyte taken after 45 min of incubation with dansylcadaverine showing focal aggregates of vesicles (endosomes) accumulated in the cytoplasm. Some vesicles appear to be in direct contact with the cell exterior (arrow), whereas others are fused or fusing. Note the conspicuous presence of monosomes in the cytoplasm. Magnification × 7780. (*b*) Control reticulocyte showing polysomal clusters typical of untreated cells. Magnification × 17300. (*c*) Cell incubated in dansylcadaverine showing diffuse distribution of monosomes throughout the cytoplasm. Magnification × 14700.

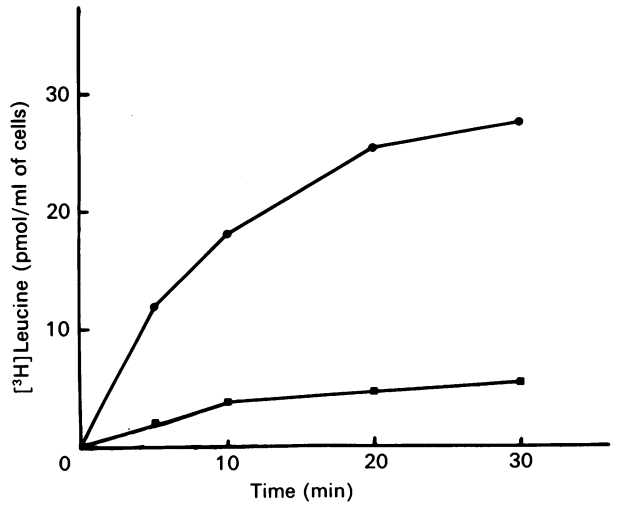

Fig. 2. Effect of dansylcadaverine on protein synthesis

Cells were incubated in leucine-free medium supplemented with 20 % (v/v) iron-saturated rat plasma and containing 1.25 μCi of [^{3}H]leucine/ml (sp. radioactivity 52.2 Ci/mol). Aliquots were removed at the indicated times for determination of trichloroacetic-acid-precipitable radioactivity in cytosol prepared by hypo-osmotic lysis. Each point represents the average of two separate experiments. ●, Control cells; ■, cells incubated with 350 μM-dansylcadaverine.

At concentrations which inhibited iron uptake, dansylcadaverine decreased the incorporation of [^{3}H]leucine into protein by more than 80 % (Fig. 2). Electron microscopic examination of dansylcadaverine-treated cells revealed extensive polysomal disaggregation (Figs. 3*b* and 3*c*), visual evidence that dansylcadaverine acted directly at the translational level to inhibit protein synthesis. The direct action of dansylcadaverine on translational activity was further supported by experiments which showed that dansylcadaverine did not decrease the availability of [^{3}H]leucine in precursor pool(s) used for protein synthesis; the specific radioactivity of soluble intracellular [^{3}H]leucine was 7–20 % higher in dansylcadaverine-treated (sp. radioactivity 576400 ± 96000 d.p.m./nmol; $n = 3$) than in untreated (sp. radioactivity 507100 ± 61000 d.p.m./nmol; $n = 3$) cells. Furthermore, depletion of cellular iron by chelation with desferrioxamine or by incubation in transferrin-free medium decreased protein synthesis by only 8–18 %, or 4–5-fold less inhibition than observed with dansylcadaverine, excluding iron availability or feedback inhibition through iron deprivation and consequent haem deficiency

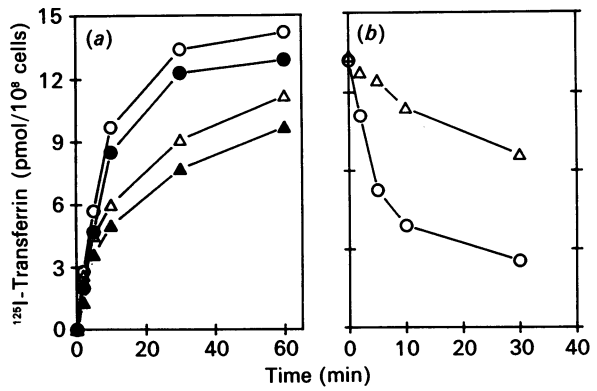

Fig. 4. Representative experiment showing the effects of dansylcadaverine on transferrin binding, endocytosis and release

(*a*) Reticulocytes (1×10^8/ml) were incubated at 37 °C in control medium or in medium containing 300 μM-dansylcadaverine; each suspension was supplemented with 0.2 μM-^{125}I-transferrin (sp. radioactivity 2406 Ci/mol). Aliquots were removed at the indicated times for determination of total transferrin binding (○, control; △, 300 μM-dansylcadaverine) and then divided into two equal aliquots; one aliquot was digested with 0.25% Pronase in iso-osmotic buffer for 3 h at 0–4 °C, whereas the other aliquot was incubated in iso-osmotic buffer alone. The amount of radioactivity that was resistant to proteolysis represented the amount of ^{125}I-transferrin endocytosis (●, ▲). (*b*) Release of internalized ^{125}I-transferrin. Reticulocytes (1×10^8) were pulse-labelled in control medium containing 0.2 μM-^{125}I-transferrin for 50 min and then transferred to fresh medium or medium containing 300 μM-dansylcadaverine to which 0.5 μM-^{56}Fe-transferrin was added. Aliquots were removed to measure the amount of ^{125}I-transferrin remaining in the cells at the indicated times of chase incubation at 37 °C. ○, Control; △, 300 μM-dansylcadaverine.

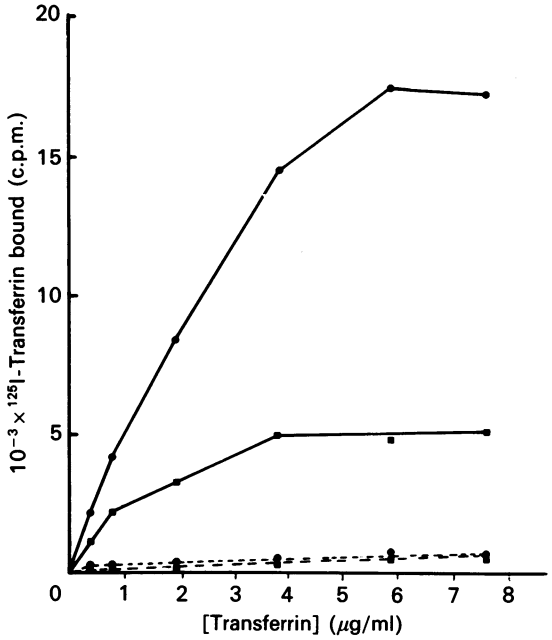

Fig. 5. Effects of dansylcadaverine on surface receptor binding activity remaining after preincubation of cells with inhibitor

Reticulocytes (1×10^8/ml) were incubated at 37 °C for 45 min in medium (■) or without (●) 300 μM-dansylcadaverine and supplemented with 0.5 μM-^{56}Fe–transferrin. The respective cell suspensions were then transferred into fresh medium containing various concentrations of ^{125}I-transferrin (sp. radioactivity 4506 Ci/mol) and incubated in a shaking ice bath at 0–4 °C for 3 h. Cells were then washed in ice-cold saline before measurement of ^{125}I-transferrin binding. Non-specific binding (broken lines) was determined under identical conditions except for the addition of a 50-fold excess of non-radioactive iron-saturated transferrin.

as factors contributing to dansylcadaverine-induced reduction of protein synthesis.

Effects of dansylcadaverine on transferrin receptor cycling

At 0–4 °C, the average binding affinities (K_d) of receptor for diferric transferrin in control and dansylcadaverine-treated cells were 8.5 ± 3.0 and 7.4 ± 2.9 nM respectively. The number of surface transferrin receptors measured at 0–4 °C in 6–8 day reticulocytes ranged from 12000 to 35000/cell, with no significant differences between control and dansylcadaverine-treated cells. Kinetic analyses of ^{125}I-transferrin binding to control cells at 37 °C revealed a total of 95000–125000 transferrin receptors. The difference between the numbers of receptors detected at 0–4 °C (surface receptor only) and at 37 °C (total complement of cell receptors) indicated that approx. 80% of transferrin receptors in 6–8 day reticulocytes were intracellular under steady-state conditions.

In untreated reticulocytes, approx. half of receptor-bound ^{125}I-transferrin was internalized within 2 min of incubation at 37 °C, rising to a plateau level of 70–85% at 10 min (Fig. 4). Although dansylcadaverine decreased the amount of cell-associated ^{125}I-transferrin by approx. 30% (Fig. 4), the proportion of transferrin internalized was essentially unchanged; at 2 min, approx. 45% of receptor-bound ^{125}I-transferrin was internalized, increasing to a plateau level of 80–85% at 10–60 min (Fig. 4). Thus dansylcadaverine did not prevent endocytosis or alter the relative rate of transferrin internalization.

Dansylcadaverine, however, did inhibit exocytotic release of transferrin, as shown by pulse–chase analyses of ^{125}I-transferrin release. By 5 min of chase in control medium, approx. 50% of the ^{125}I-transferrin accumulated during the pulse was released into the medium (Fig. 4); continued chase led to the release of more than 75% of cell-associated radioligand by 30 min (Fig. 4). In the presence of dansylcadaverine, only 15% of the ^{125}I-transferrin taken up during the pulse was released after 5 min of incubation, increasing to only 30–35% release at 30 min (Fig. 4).

To confirm that dansylcadaverine inhibited receptor recycling, the concentration of surface transferrin receptors after preincubation with dansylcadaverine was determined. Preincubation of cells with dansylcadaverine for 45 min decreased subsequent ^{125}I-transferrin binding at 0–4 °C by more than 75% (Fig. 5); Scatchard analysis revealed no change in receptor binding affinity ($K_{d,control}$ 6.2 nM; $K_{d,dansyl.}$ 5.4 nM), but there was a nearly 3-fold decrease in surface transferrin receptor density (Fig. 5). Thus the principal effect of dansylcadaverine on iron uptake resulted from its inhibition of receptor recycling which depleted the surface population of transferrin

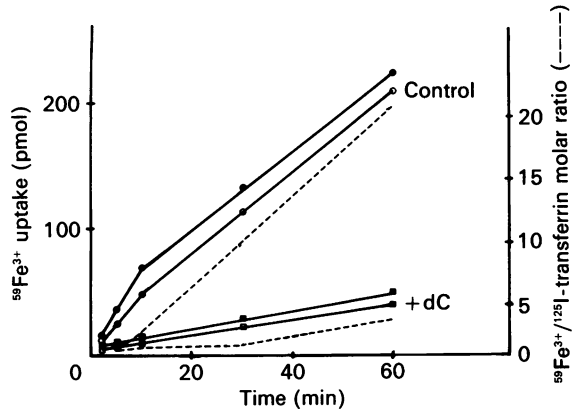

Fig. 6. Recovery of cells from dansylcadaverine treatment

Reticulocytes (2×10^8/ml) were incubated for 40 min at 37 °C with (+dC) or without (control) 300 μM-dansylcadaverine in medium containing 0.3 μM-^{56}Fe–transferrin; each of the respective cell suspensions was then resuspended and incubated in fresh medium devoid of inhibitor but containing 0.35 μM-^{59}Fe–transferrin. Cell aliquots were removed at the indicated times for measurement of ^{59}Fe uptake by whole intact cells (●, ■) and in cytosol (○, □) prepared by hypo-osmotic lysis. Broken lines represent the ^{59}Fe/^{125}I-transferrin molar ratios calculated from these data and from results of recovery of transferrin binding and uptake determined in cell suspensions incubated under identical conditions with 0.35 μM-^{125}I-transferrin.

receptors, thereby decreasing cellular access to exogenous transferrin. The intracellular arrest of receptor–transferrin recycling was revealed as conspicuous vesicular aggregates in the cytoplasm of dansylcadaverine-treated cells (Fig. 3*a*). Ultrastructural examination showed the majority of vesicles accumulating within the cytoplasm of dansylcadaverine-treated cells to contain ferritin-conjugated transferrin, indicating their probable identity as endosomes (results not shown).

The stromal localization of ^{59}Fe taken up by dansylcadaverine-treated cells (Fig. 1*a*) was consistent with fluorescence microscopic examination showing impaired endosomal acidification (results not shown) and intracellular accumulation of endosomes (Fig. 3). Upon removal of dansylcadaverine, this stromal ^{59}Fe was mobilized for use in haem synthesis and was nearly consumed for this activity within 30 min (results not shown). This result indicated rapid clearance of the inhibitor from the cell and elimination of its lysosomotropic or alkalinizing inhibition of endosomal acidification and release of transferrin iron. Despite immediate recovery of transferrin iron release following removal of dansylcadaverine, iron uptake from exogenous transferrin and receptor–transferrin flux remained impaired. Uptake of ^{59}Fe was decreased to 25–45% of control levels (Fig. 6) and, although binding of ^{125}I-transferrin was restored to control levels within 30–60 min after removal of inhibitor, endocytosis of transferrin was adversely affected, especially during the initial period of recovery. At 2–10 min after removal of the amine, only 30–60% of receptor-bound transferrin was internalized, eventually rising to 65–75% by 30–60 min. Calculation of the ^{59}Fe/^{125}I-transferrin molar ratio as a measure of the integrity of receptor–transferrin flux during iron delivery indicated that cells pretreated with the amine completed only two cycles of transferrin-mediated iron delivery during the 1 h interval after removal of the inhibitor, compared with 13 or 14 cycles in untreated cells (Fig. 6). These results indicated that receptor-mediated uptake and cycling of transferrin were not fully restored by removal of the inhibitor.

Mechanism of action of dansylcadaverine: comparative effects of other inhibitory agents

The inhibitory actions of dansylcadaverine on receptor recycling could reflect its lysosomotropic effects on endosomal acidification, preventing the dissociation of

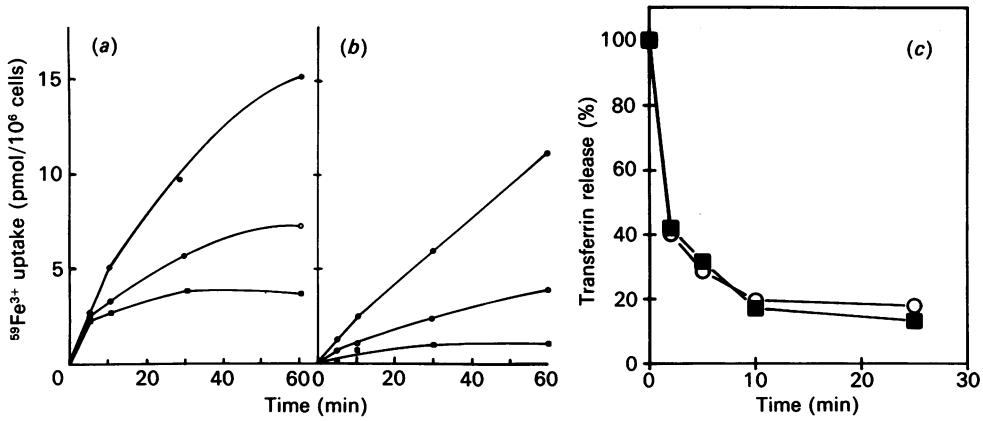

Fig. 7. Effects of chloroquine on iron uptake and exocytic release of transferrin (receptor recycling)

(*a*), (*b*) Iron uptake. Reticulocytes (10^7/ml) were incubated in control medium (●) or in medium containing 75 μM- (○) or 190 μM- (■) chloroquine. ^{59}Fe–transferrin (4.5 μM) was added to each cell suspension and aliquots were removed at the indicated times. Following measurement of total iron uptake (*a*), cells were lysed and cytosol was separated from stroma by centrifugation at 1500 *g* to measure the amount of ^{59}Fe recoverable in cytosol (*b*). Each point represents the mean of two separate experiments. (*c*) Exocytotic release of transferrin. Reticulocytes (10^7/ml) were pulse-labelled over 45 min in control medium containing 0.5 μM-^{125}I-transferrin; cells were washed and chased in control medium (○) or medium containing 190 μM-chloroquine (■). Aliquots were removed at the indicated times to determine the amounts of ^{125}I-transferrin remaining associated with cells. Each point represents the mean of two experiments.

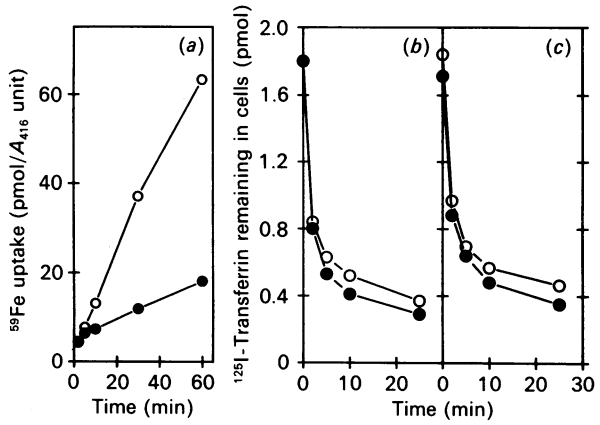

Fig. 8. Effects of methylamine on iron uptake (*a*) and exocytotic release of transferrin (*b*), (*c*)

(*a*) Reticulocytes (2×10^8/ml) were incubated in control medium (○) or in the presence of 15 mM-methylamine (●); uptake of ^{59}Fe was measured by addition of 0.2 μM-^{59}Fe–transferrin. (*b*), (*c*) Exocytotic release of transferrin or receptor recycling. Reticulocytes were pulse-labelled for 50 min in control medium containing 0.2 μM-^{125}I-transferrin and then chased in control medium or medium containing methylamine at 15 mM (*b*) or 38 mM (*c*).

transferrin-bound iron, or could be coupled to its inhibition of protein synthesis if continued synthesis of receptor protein or other proteins were essential for maintenance of the iron delivery pathway. To assess the relationship between these events and receptor recycling, other agents whose primary effects are expressed on these events were tested. The lysosomotropic amine, chloroquine [24], decreased both total cell and cytosolic uptake of iron, but inhibited cytosolic uptake, or iron entry into the cytosol, by more than it inhibited total cell uptake of iron. Chloroquine at 75 μM decreased total ^{59}Fe uptake by only 20–44 % during 60 min of incubation but, by 30 min, decreased cytosolic uptake of ^{59}Fe by 64–70 % (Figs. 7*a* and 7*b*). At 190 μM-chloroquine, total iron uptake decreased by 46–80 % over 60 min, whereas entry of iron into the cytosol was virtually shut down; after 30 min, only 8 % of total cellular ^{59}Fe was recovered in the cytosol (Figs. 7*a* and 7*b*), a result consistent with the lysosomotropic action of this agent on endosomal acidification and transferrin iron release [25]. Nonetheless, chloroquine exerted minimal effects on cellular binding and exocytic release of transferrin (Fig. 7*c*), confirming previous reports [25,26] that chloroquine inhibits iron uptake in reticulocytes primarily by interfering with endosomal acidification. Identical results were obtained with methylamine, an agent reported to inhibit receptor-mediated endocytosis of various ligands by interference with transglutaminase [27]. Methylamine at 15 mM virtually shut down iron uptake in reticulocytes (Fig. 8*a*) but had no effect on exocytic transferrin release and receptor recycling (Fig. 8*b*). Even on increasing the concentration of methylamine to nearly 40 mM, conditions under which the intracellular pH measured with the pH fluorochrome, BCECF, was approx. 7.9 (results not shown), there was no significant effect of cytoplasmic alkalinization on receptor recycling and exocytic release of transferrin (Fig. 8*c*). Thus dissociation of iron from internalized transferrin was not required to maintain receptor recycling and exocytic transferrin release, nor did the failure to acidify the endosomal compartment in dansylcadavarine-treated cells account for the inhibitory actions of this agent on receptor recycling.

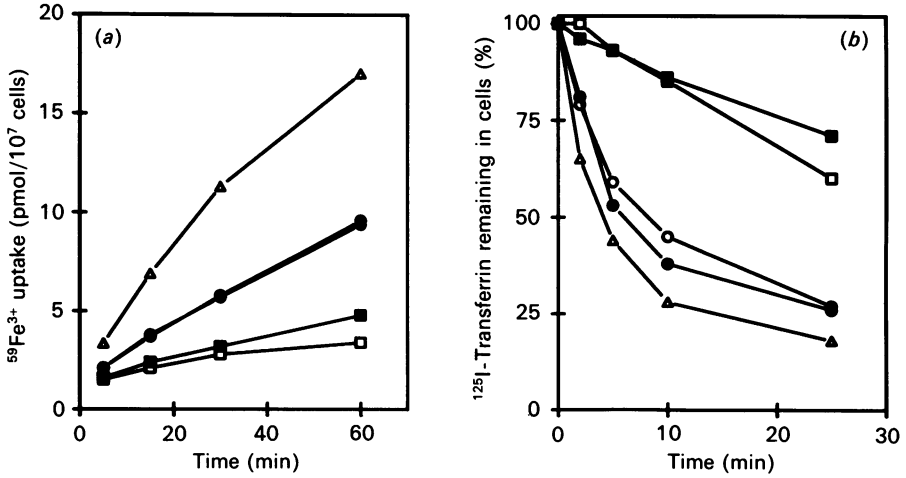

Fig. 9. Comparison of the effects of dansylcadaverine and dimethyldansylcadaverine on iron uptake (*a*) and exocytotic release of transferrin (*b*)

(*a*) Reticulocytes (1.7×10^8/ml) were suspended in control medium or in medium containing 100 μM- or 350 μM-dansylcadaverine or dimethylcadaverine. Cells were incubated at 37 °C under the respective conditions with 0.3 μM-^{59}Fe–transferrin, and aliquots were removed at the indicated times for measurement of total iron uptake. (*b*) Reticulocytes were pulse-labelled for 50 min in control medium containing 0.3 μM-^{125}I-transferrin, washed and chased in fresh non-radioactive medium with or without inhibitor as described above. Aliquots were removed at the indicated time points to determine the amount of ^{125}I-transferrin remaining cell-associated. Results are expressed as the percentage of radioactivity recovered in the cells at each time point relative to cell-associated radioactivity present at 0 min or at the onset of the chase. △, Control cells; ○, 100 μM-dansylcadaverine; ●, 100 μM-dimethyldansylcadaverine; □, 350 μM-dansylcadaverine; ■, 350 μM-dimethyldansylcadaverine.

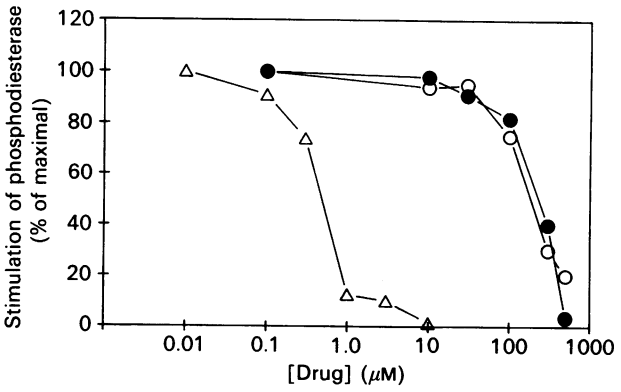

Fig. 10. Effects of dansylcadaverine (○), dimethyldansylcadaverine (●), and calmidazolium (△), on calmodulin stimulation of rat brain cyclic nucleotide phosphodiesterase

Phosphodiesterase was isolated from rat brain [59] and purified on a calmodulin–agarose affinity column. Its activity was assayed in the presence and absence of Ca^{2+}/calmodulin at the indicated drug concentrations. In the absence of drug, calmodulin stimulated enzyme activity by approx. 3-fold. Calmodulin stimulation was determined at each drug concentration and plotted as a percentage of maximal stimulation in the absence of drug. Reaction mixture (0.4 ml) contained 40 mM-Tris/HCl (pH 8.0), 10 mM-$MgCl_2$, 2.5 mM-mercaptoethanol, 1 μM (10^5 c.p.m.) [^{3}H]cyclic AMP, 50 μg of BSA, 1.4 μg of phosphodiesterase and either 1 mM-EGTA for basal activity or 0.2 mM-$CaCl_2$ and 30 nM purified bovine brain calmodulin for calmodulin-stimulated activity. Reactions were run for 10 min at 30 °C. Phosphodiesterase activity was assayed by the two-step radioisotope procedure [60], using Dowex anion-exchange columns to separate product from unreacted substrate.

At 1 μM, cycloheximide shut down protein synthesis in rat reticulocytes, but had no effect on iron uptake (rate of uptake = 0.5 and 0.49 pmol/min in control and cycloheximide-treated cells respectively). Accordingly, cycloheximide had minimal effects on transferrin binding activity or exocytotic release of transferrin (rate constants of 0.012 and 0.015 min^{-1} for control and 1 μM-cycloheximide-treated cells respectively; $n = 2$) at concentrations as high as 100 μM. These results indicated that the functional integrity of the iron delivery pathway in rat reticulocytes was not dependent on protein synthesis *de novo*.

Dansylcadaverine is a competitive inhibitor of transglutaminase(s), an action that is exerted via its primary amino group [19]. If the inhibitory effects of dansylcadaverine on iron uptake and receptor recycling were transglutaminase-mediated, modification of the primary amino group, which decreases the potency of the reagent as a transglutaminase inhibitor [19], should reduce or abolish the inhibitory effects of dansylcadaverine on iron delivery. However, dimethyldansylcadaverine, the *N*-tertiary amino analogue of dansylcadaverine [19], was as effective an inhibitor of iron uptake and exocytotic release of transferrin as dansylcadaverine (Fig. 9). Methylamine, a known transglutaminase inhibitor [19,27] had no effect on exocytic transferrin release at concentrations as high as 38 mM (Figs. 8*b* and 8*c*), despite its strong inhibition of iron uptake (Fig. 8*a*). These results excluded the possibility that inhibition of iron uptake and receptor recycling by dansylcadaverine was transglutaminase-mediated.

Neither H-7, a putative inhibitor of PKC (inhibition constant [K_i] = 6 μM; [28,29]), nor PMA, which activates PKC, had any effects on iron uptake, ^{125}I-transferrin endocytosis, or exocytic transferrin release. These results indicate that maintenance of receptor-mediated transferrin iron delivery in rat reticulocytes was not dependent upon PKC activity.

Calmodulin inhibitors

Since the chemical structure of dansylcadaverine is similar to the model structure proposed for calmodulin inhibitors [30,31], studies were undertaken to determine the effect of dansylcadaverine on calmodulin activity *in vitro* as well as the effects of putative calmodulin antagonists on the iron delivery process. Dansylcadaverine inhibited calmodulin stimulation of rat brain phosphodiesterase *in vitro* over a concentration range of 100–500 μM (IC_{50} = 180 μM; Fig. 10). Its dimethylated derivative, dimethyldansylcadaverine, exhibited a similar dose-dependent inhibition of calmodulin activation (IC_{50} = 230 μM; Fig. 10). Thus at concentrations of dansylcadaverine and dimethyldansylcadaverine which inhibited iron uptake and receptor recycling *in vivo*, both agents were effective antagonists of calmodulin activity *in vitro*.

Calmidazolium was approx. 400-fold more potent an antagonist of calmodulin activation of phosphodiesterase activity (IC_{50} 0.5 μM) than were the dansylcadaverine compounds (Fig. 10). Calmidazolium exerted a concentration-dependent inhibition of ^{59}Fe uptake (IC_{50} 6 μM) and, at 10–12 μM, maximally blocked iron uptake by rat reticulocytes (Fig. 11*a*). Although calmidazolium decreased binding and uptake of ^{125}I-transferrin by 35–50% (Fig. 11*b*), the relative rate of transferrin endocytosis was minimally affected. Thus, as in untreated reticulocytes, nearly 90% of receptor-bound ^{125}I-transferrin was resistant to mild proteolytic digestion within 5–10 min of incubation at 37 °C, indicating its internalization and inaccessibility to enzymic degradation by pronase (Fig. 11*b*). Calmidazolium strongly inhibited exocytotic release of ^{125}I-transferrin; the amount and relative rate of internalized ^{125}I-transferrin released during a 25 min chase were decreased to 35–50% of control levels (Fig. 11). Thus, like dansylcadaverine, the primary action of calmidazolium on iron uptake was expressed on receptor recycling and exocytic release of transferrin. Like dansylcadaverine, the inhibitory effects of calmidazolium on iron uptake and receptor–transferrin flux were not fully corrected by its removal.

W13 [*N*-4-(6-aminobutyl)-5-chloro-1-naphthalene sulphonamide], an inhibitor of calmodulin [24,32,33], produced a concentration-dependent decrease in iron uptake and exocytotic release of transferrin that was virtually identical to the effects of dansylcadaverine and calmidazolium. Trifluoperazine, a phenothiazine antagonist of calmodulin [30,31], also inhibited the iron delivery process but caused severe haemolysis which prevented its use in these studies.

Rescue of receptor recycling by calcium ionophore

The identical effects of dansylcadaverine and the putative calmodulin antagonists suggested that the common action of these agents on receptor recycling was

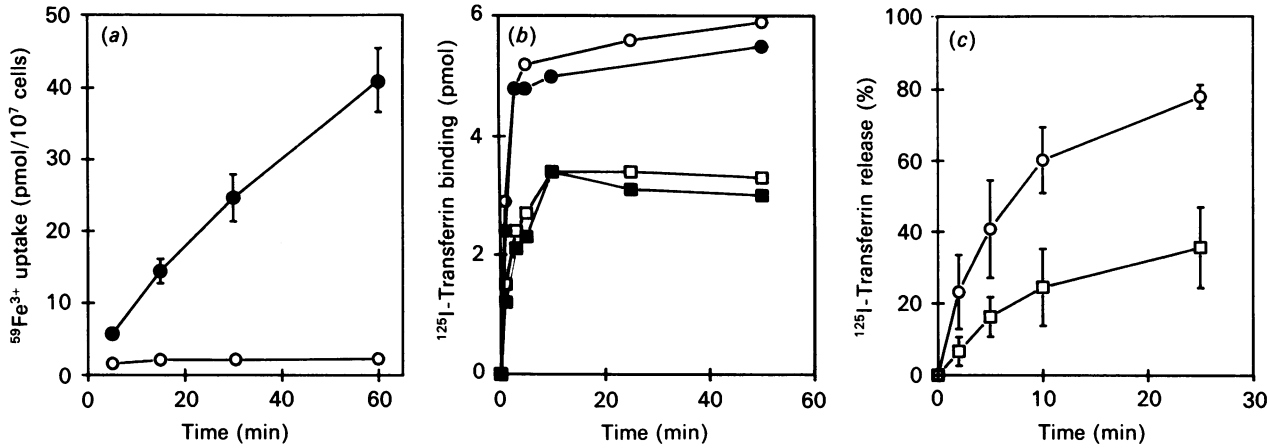

Fig. 11. Effects of calmidazolium (R24657) on iron uptake and transferrin receptor flux

(*a*) Iron uptake. Reticulocytes [(1–2) × 10^8/ml] were incubated in control medium (●) or medium containing 10 mM-calmidazolium (○); after addition of 0.2–0.5 μM-^{59}Fe–transferrin, cells were incubated at 37 °C and aliquots were removed at the indicated times for measurement of total cell ^{59}Fe uptake. Each point represents the mean of five experiments; vertical bars are S.E.M., where greater than size of symbol. (*b*) Binding and endocytotic internalization of ^{125}I-transferrin. Cells were incubated as described in (*a*) except that ^{59}Fe–transferrin was replaced by ^{125}I-transferrin for determination of total binding in control (○) and 10 μM-calmidazolium-treated (□) cells. Endocytotic internalization of ^{125}I-transferrin was determined in control (●) and calmidazolium-treated (■) cells using mild proteolysis with Pronase. Each point represents the mean of two separate experiments. (*c*) Exocytotic release of ^{125}I-transferrin from control cells (○) and cells incubated with 10 μM-calmidazolium (□). Cells were pulse-labelled with 0.2 μM-^{125}I-transferrin for 45 min and were then chased in fresh non-radioactive medium with or without calmidazolium and supplemented with 0.2–0.5 μM-Fe–transferrin. The amount of ^{125}I-transferrin remaining associated with cells at the indicated time points was measured and expressed as the percentage of radioligand released into the medium relative to total cell-associated radioactivity at 0 min or the onset of the chase.

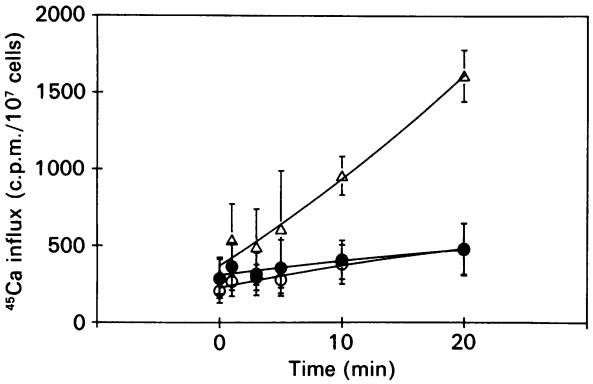

Fig. 12. Effect of dansylcadaverine and ionomycin on Ca^{2+} influx

Reticulocytes (7.5×10^7/ml) were incubated in control medium containing 1 mM-$CaCl_2$ (○) or in medium containing 200 μM-dansylcadaverine (●) or 200 μM-dansylcadaverine plus 2.5 μM-ionomycin (△). ^{45}Ca (sp. radioactivity 44.5 mCi/mg) was added to each cell suspension to a final concentration of 7 μCi/ml. Aliquots were taken at the indicated time points, washed twice in Hepes/NaCl/$LaCl_3$ and radioactivity was counted in a liquid scintillation counter. Each point represents the mean of three to five separate experiments, with S.D. values indicated by the vertical bars.

their interference with Ca^{2+}/calmodulin activation of the recycling process. Initial experiments assessing the effect of dansylcadaverine on Ca^{2+} influx indicated that this inhibitor did not alter the rate or amount of ^{45}Ca influx (Fig. 12). Although these experiments did not address the mechanism underlying Ca^{2+} flux in transferrin-mediated iron delivery, i.e. the result of Ca^{2+} mobilization from an internal compartment or influx of exogenous Ca^{2+} stimulated by receptor–transferrin interaction, it was evident that dansylcadaverine did not block Ca^{2+} uptake; moreover, since no significant difference was seen between control and dansylcadaverine-treated cells, it was unlikely that the inhibitor significantly altered intracellular Ca^{2+} levels. Evidence of a role for Ca^{2+} in the exocytic and recycling process was sought by analysing the effects of Ca^{2+} ionophores on receptor recycling. Cells pulse-labelled in control medium with ^{125}I-transferrin were chased in non-radioactive medium containing 1 mM-$CaCl_2$ and inhibitory concentrations of dansylcadaverine or calmidazolium; the extent to which added ionophore was able to overcome the inhibitory effects of either agent was then determined during the ensuing chase. Measurements of ^{45}Ca influx indicated a 2.5–3-fold increase in intracellular ^{45}Ca levels stimulated by ionomycin (Fig. 12). Simultaneous addition of Ca^{2+}/ionomycin and either inhibitor resulted in a concentration-dependent antagonism of the inhibitory actions of dansylcadaverine or calmidazolium on receptor recycling; at 1.0–1.5 μM, the amount of inhibition was decreased by 30–70% (Table 1), whereas inhibition of receptor recycling and exocytosis was totally overcome by addition of 2.5–3 μM-Ca^{2+}/ionomycin (Table 1). Omission of extracellular Ca^{2+} abolished the rescue effects of ionomycin, indicating that Ca^{2+} was required to overcome the inhibitory actions of dansylcadaverine and calmidazolium. However, if cells were pretreated for 35–50 min with either inhibitor, subsequent addition of Ca^{2+}/ionomycin failed to restore iron uptake or to counteract the inhibitory actions of these agents.

Contrary to the acceleration of receptor recycling

Table 1. Effect of ionomycin on inhibition of transferrin release

Reticulocytes were incubated for 50 min in control medium containing 0.1 μM-^{125}I-transferrin, washed to remove all exogenous radioligand and subdivided into equal aliquots. Cells in each aliquot were resuspended in fresh non-radioactive medium supplemented with 100 μM-$CaCl_2$ and 0.1 μM-^{56}Fe-transferrin and containing various additions as indicated. After removal of an aliquot to establish the amount of cell-associated radioligand at the onset of the chase, cells were chased and aliquots withdrawn at the indicated times to determine the amount of ^{125}I-transferrin released from the cells.

Time (min)	Transferrin release (%)		
	Control	+225 μM-Dansyl-cadaverine	+12 μM-Calmid-azolium
0	–	–	–
2	24.6±11.8	11.8±1.3	10.2±3.5
5	36.1±5.5	18.2±5.1	21.4±6.0
10	47.8±0.9	22.9±8.9	27.7±8.9
25	61.8±1.8	35.0±12.2	43.0±11.7
		+0.7 μM-Ionomycin	
2		11.3	16.5
5		15.7	27.8
10		25.7	39.8
25		42.3	50.6
		+1.5 μM-Ionomycin	
2		20.2	19.2
5		26.4	28.5
10		44.2	39.2
25		66.5	52.3
		+3 μM-Ionomycin	
2		34.1	20
5		59.8	30
10		65.1	51
25		71.3	70

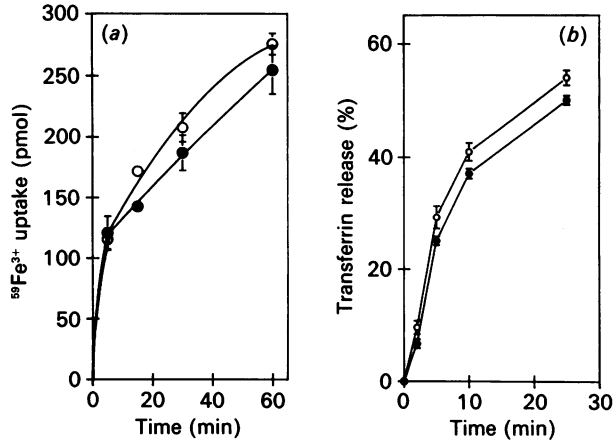

Fig. 13. Effects of ionophore on iron uptake (*a*) and exocytotic release of transferrin (*b*)

(*a*) Reticulocytes (10^8/ml) were incubated in control medium containing 2 mM-Ca^{2+} (○) or medium containing 2 mM-Ca^{2+} supplemented with 2.5 μM-ionomycin (●). Iron uptake was measured upon addition of 0.2 μM-^{59}Fe–transferrin. Each point represents the mean of three experiments. Vertical bars represent S.D.s. (*b*) Reticulocytes were pulse-labelled for 45 min in control medium containing 0.25 μM-^{125}I-transferrin and then chased in control medium or in medium supplemented with 2.5 μM-ionomycin as in (*a*).

associated with increased intracellular Ca^{2+} induced by ionophore in mouse tumour macrophages [6], incubation of reticulocytes with ionomycin in medium containing 1–2 mM-Ca^{2+} caused no significant change in either the rate of iron uptake (Fig. 13*a*) or the rate of exocytic transferrin release and receptor recycling (Fig. 13*b*). These results implied that, under the conditions existing in these experiments, i.e. the rate and amount of haem and haemoglobin synthesis supported under these conditions and, accordingly, the absence of signal(s) demanding accelerated iron delivery, the rate of receptor recycling is at or near maximal, and that increased intracellular Ca^{2+} alone is insufficient to alter the rate of transferrin-mediated iron delivery. Furthermore, the possibility that the rescue effects of Ca^{2+}/ionophore were due to independent stimulation of receptor recycling and not to its direct action on the Ca^{2+}/calmodulin complex was minimized by the failure of Ca^{2+}/ionomycin or ionomycin alone to enhance iron delivery and uptake in reticulocytes.

DISCUSSION

Kinetic analysis of transferrin binding properties in 6–8 day rat reticulocytes indicates the existence of a single class of high-affinity receptors (K_d = 3–10 nM). Under steady-state conditions, 20–25% of the total transferrin receptor population is located at the cell surface; the remaining receptors are contained in an intracellular pool which serves as a reservoir that permits the cell to respond to altered metabolic iron demand by post-translational modulation of exocytosis and receptor recycling [21]. The functional integrity of the iron delivery pathway is not dependent upon continued synthesis of receptor, as demonstrated by the insensitivity of iron uptake to cycloheximide, nor is release of transferrin-bound iron essential to maintain receptor–transferrin flux, as indicated by the failure of alkalinizing agents to affect receptor cycling in these cells. The total transferrin receptor cycling time calculated from data obtained with various inhibitors is 3.9 min or 13–14 cycles of transferrin-mediated iron delivery per hour, a value approximating transferrin receptor cycling times in other cells [34,35].

The primary action of dansylcadaverine and calmidazolium on receptor-mediated iron delivery in rat reticulocytes is to inhibit exocytotic release of transferrin and the concomitant recycling of transferrin receptors to the cell surface. Neither agent directly prevents endocytotic internalization of receptor-bound transferrin, although their inhibition of receptor recycling eventually decreases the amount and rate of transferrin uptake. This effect is demonstrated by calculation of transferrin (and transferrin receptor) fluxes in dansylcadaverine-treated cells (Fig. 4). In untreated 7 day reticulocytes, receptor-bound transferrin was internalized at a rate of approx. 4200 molecules/min per cell (r = 0.99), but this rate was only 2500 molecules/min per cell (r = 0.94) in dansylcadaverine-treated cells, despite other data indicating that endocytosis was relatively unaffected by the inhibitor. This discrepancy is resolved by the exocytotic (receptor recycling) rates calculated for dansylcadaverine-treated cells. In untreated cells,

transferrin was released at a rate of approx 3700 molecules/min per cell, or at a rate nearly equal to the rate of endocytosis [34]. In contrast, the rate of transferrin release, and therefore the restoration of surface transferrin receptors, in dansylcadaverine-treated cells was only 830 molecules/min per cell, or nearly a 4-fold decrease in the rate of receptor recycling. Thus dansylcadaverine (and calmidazolium), by inhibiting receptor recycling, impair receptor–transferrin flux, leading to intracellular accumulation of transferrin receptors and depletion of the surface receptor pool. It is probable that endocytosis and receptor recycling, i.e. influx and efflux of receptor, may affect each other through a feedback mechanism, but not until a significant redistribution of cellular transferrin receptors occurs, either at the cell surface or at the level of the intracellular transferrin receptor pool. Under steady-state conditions in freshly obtained reticulocytes, approx. 75–80% of the total receptor population is located within an intracellular pool. Under these conditions, neither dansylcadaverine nor calmidazolium had a significant effect on the influx of receptor-bound transferrin, that is, the relative rate of endocytosis was unaffected by the inhibitor (Fig. 4). However, with prolonged inhibition, there is an intracellular accumulation of receptor and depletion of the surface receptor population, an outcome which arises from a net imbalance between receptor influx and efflux. The redistribution of cellular transferrin receptors occurring in inhibitor-treated cells may account for the initial retardation of endocytotic transferrin uptake seen in cells pretreated with inhibitors: not until sufficient receptor is returned to the cell surface and a new steady state established upon withdrawal of the inhibitor does the relative rate of endocytotic influx approach control levels, i.e. at 30–60 min after inhibitor removal. It is not surprising that reticulocytes do not fully recover initial iron delivery capacity following traumatic pharmacological insult, since these cells lack both the transcriptional and translational plasticity [21] that is required to replace factors involved in mediation of the iron delivery pathway which may suffer irreparable damage from these experimental treatments.

The inhibitory effects of these agents appear to be related to the functions of intracellular Ca^{2+} and calmodulin as mediators of receptor recycling and exocytotic transferrin release in rat reticulocytes. Transferrin receptor recycling is functionally analogous to secretion in that it involves exocytosis and results in release of a cell-associated component into the extracellular medium. The Ca^{2+}/calmodulin-dependence of secretion and release of hormones [36–40], neurotransmitters [16,17] and other factors [41–44] provides ample experimental precedence for the involvement of calmodulin in the regulation of exocytosis. Our conclusion that receptor recycling in rat reticulocytes is a Ca^{2+}/calmodulin-dependent event is supported by the following data.

1. Both dansylcadaverine and calmidazolium were shown to inhibit calmodulin activity *in vitro* and exerted identical inhibition of receptor recycling in intact cells, including neutralization of their effects by the addition of calcium ionophore. Furthermore, other calmodulin antagonists, i.e. W13 and trifluoperazine, exhibited a similar inhibition of iron uptake and receptor recycling in rat reticulocytes. Although the effective dose of calmidazolium needed to affect iron delivery (approx. 3–6 μM) significantly exceeded its IC_{50} (0.5 μM) *in vitro*, this difference may reflect limited cellular permeability to calmidazolium. Furthermore, the concentration required to inhibit receptor recycling in reticulocytes was comparable with dosages reported elsewhere to inhibit calmodulin-dependent processes in intact cells [45–49].

2. Other actions of these agents on transglutaminases or PKC have been excluded. Dansylcadaverine was described initially to block receptor-mediated endocytosis of various ligands by its actions on transglutaminase [27]. Subsequent studies dispelled this interpretation, since dansylcadaverine was found to mainly block receptor recycling [34,50,51], an effect confirmed in this report. Moreover, blockade of the functional amino group responsible for its inhibition of transglutaminase [19] did not reduce its potency as an inhibitor of iron uptake and receptor recycling, arguing against the possibility that dansylcadaverine inhibits iron delivery by its action on transglutaminase. Whereas various calmodulin antagonists, including the naphthalene sulphonamides, have been found to inhibit activation of PKC by Ca^{2+} and phospholipids [2–4], any significant role for PKC in mediation of the iron delivery process in rat reticulocytes was minimized by the failure of agents postulated to act on this enzyme to exert any effect on the iron delivery pathway. Thus PMA, an active phorbol ester which stimulates PKC, had no effect on iron uptake, transferrin endocytosis or receptor recycling. Although it could be argued that receptor–transferrin flux in these cells may already be maximally stimulated, precluding further stimulation by enhanced PKC activity, this possibility was excluded by results obtained with a PKC inhibitor H-7, which had no discernible effects on iron delivery even at concentrations grossly exceeding its K_i for PKC. Previous studies concluded that calmodulin antagonists decreased iron uptake in reticulocytes and normoblasts by inhibiting transferrin endocytosis and interfering with PKC [49], although a role for calmodulin was not ruled out. Our results indicate that dansylcadaverine and calmodulin antagonists primarily suppress receptor recycling and are without significant effect on receptor-mediated endocytosis. Furthermore, no evidence for the involvement of PKC in maintaining the integrity of the receptor-mediated iron delivery pathway in rat reticulocytes was demonstrated by the use of phorbol ester or a selective PKC inhibitor. This finding is at odds with other studies showing that PKC is implicated in transferrin receptor cycling in K562 [10], HL60 [11,55] and other cell lines [6,8,9]. This discrepancy may reflect cell-specific variations as well as differing cell needs for iron; in K562 cells, for example, only 7% of incoming iron is used for haem synthesis [2], whereas > 90% is directed into haem in reticulocytes [21].

3. The chemical structures of dansylcadaverine [*N*-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulphonamide] and its dimethylated derivative are compatible with the idealized model proposed for calmodulin antagonists [30,31], and both agents are structurally similar to naphthalene sulphonamides such as W7 and W13 that are potent inhibitors of calmodulin [32,33]. Moreover, both dansylcadaverine and dimethyldansylcadaverine were effective inhibitors of calmodulin-activated phosphodiesterase activity *in vitro* over the same range of concentrations at which these agents blocked receptor recycling in intact cells. Having excluded their action on receptor-mediated iron delivery via transglutaminase or

PKC, these results point to their highly probable role as calmodulin antagonists, as previously predicted [19].

4. In other studies (J. A. Grasso, R. Shoup & K. Blake, unpublished work), several Ca^{2+}-dependent calmodulin-binding proteins have been identified in intracellular vesicles isolated from rat reticulocytes. Phosphorylation of these proteins *in vitro* is Ca^{2+}-dependent and is enhanced by calmodulin. Pretreatment of isolated vesicular peptides with dansylcadaverine does not block Ca^{2+}-dependent binding of ^{125}I-calmodulin to these proteins, indicating that dansylcadaverine does not bind to target peptides but, like other calmodulin antagonists, interacts directly with calmodulin. Dansylcadaverine inhibits metabolic phosphorylation of vesicular proteins *in vivo*, including several which bind calmodulin. Though preliminary, these data demonstrating Ca^{2+}-dependent calmodulin activation of vesicular peptides offer further support for the role of calmodulin in receptor–transferrin flux.

5. The concentration-dependent antagonism of the inhibitory actions of dansylcadaverine and calmidazolium by Ca^{2+}/ionophore strongly suggested the dependence of receptor recycling and exocytotic release of transferrin on Ca^{2+}/calmodulin. Ionophore alone was insufficient to counteract the inhibitory effects of these agents, but required and involved massive influx of extracellular Ca^{2+}. Simultaneous addition of Ca^{2+}/ionomycin and inhibitor prevented inhibition of receptor recycling, but addition of Ca^{2+}/ionophore after pretreatment with inhibitor failed to alleviate inhibition of the iron delivery pathway. These results are in accord with a direct action of these agents on calmodulin to prevent Ca^{2+}/calmodulin enzymic activation of target peptides involved in exocytosis and receptor recycling With simultaneous addition of Ca^{2+}/ionomycin and inhibitor, the massive influx of Ca^{2+} enhances formation of the active Ca^{2+}–calmodulin–enzyme complex and activation of target peptides. As the time interval between addition of inhibitor and Ca^{2+}/ionophore is increased, binding of the inhibitor to the Ca^{2+}/calmodulin complex prevents formation of the active enzyme complex, depleting the cell of functionally intact calmodulin and neutralizing the effect of subsequently added Ca^{2+}/ionophore. This interpretation assumes enhanced cellular permeability of Ca^{2+}/ionophore relative to permeability of inhibitor and/or higher affinity of Ca^{2+}/calmodulin for enzyme than for inhibitor.

The identity of the specific kinase implicated in mediation of receptor recycling in rat reticulocytes has not been determined. The data are consistent with the involvement of multifunctional Ca^{2+}/calmodulin-dependent protein kinase (multifunctional calmodulin kinase or calmodulin kinase II [16]) since (i) an influx of Ca^{2+} was associated with rescue of cells from calmodulin inhibitors, (2) the involvement of calmodulin is supported by the effects of selective inhibitors, and (3) no evidence was obtained to support a major role for PKC. The extent to which cyclic AMP and cyclic AMP-dependent kinase are involved or secondarily activated by Ca^{2+}/calmodulin in modulation of receptor recycling was not explored thoroughly. Evidence against a significant role for cyclic AMP- or cyclic nucleotide-dependent kinases was suggested by experiments using H-7 and HA1004, agents with a selective K_i for cyclic nucleotide-dependent kinases [28,29]. At concentrations up to 100 μM, neither agent exhibited any adverse effects on transferrin-mediated iron delivery. In the absence of more conclusive experiments, these data are only suggestive and do not allow us to definitively include or exclude a role for cyclic AMP-dependent kinase.

The various agents employed in these studies selectively inhibit exocytotic release of transferrin and receptor recycling, but do not limit or otherwise affect the endocytotic process, except indirectly by their eventual depletion of surface receptors. These results signify that Ca^{2+}/calmodulin activation is not implicated directly in mediation of endocytic internalization, and that the endocytotic and exocytotic arms of the iron delivery process are under separate regulatory control. Further evidence suggesting differential regulation of endocytosis and receptor recycling respectively may be seen in the divergent effects of agents acting on haem synthesis. Exogenous haemin inhibits receptor-mediated endocytosis of transferrin [56–58] but has no effect on receptor recycling [21]. Alternatively, inhibition of haem synthesis by succinylacetone stimulates receptor recycling to increase surface expression of transferrin receptors but is without effect on transferrin endocytosis [21]. The differential actions of those various agents on the iron delivery pathway strongly suggest that endocytotic uptake and exocytotic return of receptor–transferrin are interdependent events that are regulated through different mechanisms. The precise intracellular localization and morphological identity of the site of divergence of the endosomal and exosomal paths, and the basis for differential regulation of the two respective arms of the iron delivery process, remain to be clarified in rat reticulocytes.

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REFERENCES

1. Ward, J. J., Jordan, I., Kushner, J. P. & Kaplan, J. (1984) J. Biol. Chem. **259**, 13235–13240
2. Bottomley, S. S., Wolfe, L. C. & Bridges, K. R. (1985) J. Biol. Chem. **260**, 6811–6815
3. Roualt, T., Rao, K., Harford, J., Mattia, E. & Klausner, R. D. (1985) J. Biol. Chem. **260**, 14862–14866
4. Casey, J. L., DiJeso, B., Rao, K., Klausner, R. D. & Harford, J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1787–1791
5. Mattia, E., Rao, K., Shapiro, D. S., Sossman, H. H. & Klausner, R. D. (1984) J. Biol. Chem. **259**, 2689–2692
6. Buys, S. S., Keogh, E. A. & Kaplan, J. (1984) Cell **38**, 569–576
7. Tanner, K. & Lienhard, G. E. (1987) J. Biol. Chem. **262**, 8975–8980
8. Davis, R. J. & Meisner, H. (1987) J. Biol. Chem. **262**, 16041–16047
9. McGraw, T. E., Dunn, K. W. & Maxfield, F. R. (1988) J. Cell Biol. **106**, 1061–1066
10. May, W. S., Jacobs, S. & Cuatrecasas, P. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 2016–2020
11. May, W. S., Sahyoun, N., Wolf, M. & Cuatrecasas, P. (1985) Nature (London) **317**, 549–551

12. Klausner, R. D., Harford, J. & van Renswoude, J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3005–3009
13. Rothenberger, S., Iacopetta, B. & Kuhn, L. C. (1987) Cell **49**, 423–431
14. Zerial, M., Suomalainan, M., Zanetti-Schneider, M., Schneider, C. & Garoff, H. (1987) EMBO J. **6**, 2661–2667
15. Sibley, D. R., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1987) Cell **48**, 913–922
16. Schulman, H. (1988) Second Messenger Phosphoprotein Res. **22**, 39–112
17. Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3035–3039
18. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-Lian, Rich, D. P., Smith, M. K. & Soderling, T. R. (1989) Biochem. J. **258**, 313–325
19. Lorand, L. & Conrad, S. M. (1984) Mol. Cell. Biochem. **58**, 9–35
20. Bar-Zvi, D., Mosley, S. T. & Branton, D. (1988) J. Biol. Chem. **263**, 4408–4415
21. Adams, M. L., Ostapiuk, I. & Grasso, J. A. (1989) Biochim. Biophys. Acta **1012**, 243–253
22. Young, S. P. & Aisen, P. (1980) Biochim. Biophys. Acta **633**, 145–153
23. Eppig, J. J. & Dumont, J. N. (1976) In Vitro **12**, 418–427
24. Okhuna, S. & Poole, B. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3327–3331
25. Morgan, E. H. (1981) Biochim. Biophys. Acta **642**, 119–134
26. Harding, C. & Stahl, P. (1983) Biochem. Biophys. Res. Commun. **113**, 650–658
27. Davies, P. J. A., Davies, D. R., Levitski, A., Maxfield, F. R., Milhaud, P., Willingham, M. C. & Pastan, I. (1980) Nature (London) **283**, 164–167
28. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
29. Kawamoto, S. & Hidaka, H. (1984) Biochem. Biophys. Res. Commun. **125**, 258–264
30. Prozialeck, W. C. & Weiss, B. (1982) J. Pharmacol. Exp. Ther. **222**, 509–516
31. Weiss, B., Prozialeck, W. C. & Wallace, T. L. (1982) Biochem. Pharmacol. **31**, 2217–2226
32. Hidaka, H., Asano, M. & Tanaka, T. (1981) Mol. Pharmacol. **22**, 571–578
33. Tanaka, T., Ohmura, T. & Hidaka, H. (1982) Mol. Pharmacol. **22**, 403–407
34. Iacopetta, B. & Morgan, E. H. (1983) J. Biol. Chem. **258**, 9108–9115
35. Stein, B., Bensch, K. & Sussman, H. H. (1984) J. Biol. Chem. **259**, 14762–14772
36. Brown, B. L., Walker, S. W. & Tomlinson, S. (1985) Clin. Endocrinol. **23**, 201–218
37. Schettini, G., Judd, A. M. & MacLeod, R. M. (1983) Endocrinology (Baltimore) **112**, 64–70
38. Schettini, G., Judd, A. M. & MacLeod, R. M. (1983) Neuroendocrinology **37**, 229–234
39. Aguila, M. C. & McCann, S. M. (1988) Endocrinology (Baltimore) **12**, 305–309
40. Henquin, J.-C. (1981) Biochem. J. **196**, 771–780
41. Steinhardt, R. A. & Alderton, J. M. (1982) Nature (London) **295**, 154–155
42. Nishikawa, M., Tanaka, T. & Hidaka, H. (1980) Nature (London) **287**, 863–865
43. Tanaka, T., Saitoh, M., Ito, M., Shin, T., Naka, M., Endo, K. & Hidaka, H. (1988) Biochem. Pharmacol. **37**, 2537–2542
44. Watanabe, S., Tomono, M., Takeuchi, M,. Kitamura, T., Hirose, M., Miyazaka, A. & Namihisa, T. (1988) Liver **8**, 178–183
45. Nolan, J. C., Gathright, C. E. & Wagner, L. E. (1988) Agents Actions **25**, 71–76
46. Dasarathy, Y. & Fanburg, B. L. (1988) Biochim. Biophys. Acta **1010**, 16–19
47. Dasarathy, Y. & Fanburg, B. L. (1988) J. Cell. Physiol. **137**, 179–184
48. Arruda, J. A., Talor, Z. & Dytko, C. (1988) Arch. Int. Pharmacodyn. Ther. **293**, 273–283
49. Hebbert, D. & Morgan, E. H. (1985) Blood **65**, 758–763
50. King, A. C. & Cuatrecasas, P. (1983) in Receptor-Mediated Endocytosis (Cuatrecasas, P. & Roth, T., eds) New York, pp. 45–81, Chapman and Hall, London
51. Pan, B. T. & Johnstone, R. (1984) J. Biol. Chem. **259**, 9776–9782
52. Tanaka, T., Ohmura, T., Yamakado, T. & Hidaka, H. (1982) Mol. Pharmacol. **22**, 408–412
53. Schatzman, R. C., Wise, B. C. & Kuo, J. F. (1981) Biochem. Biophys. Res. Commun. **98**, 667–676
54. Mazzei, G. J., Schatzman, R. C., Turner, R. S., Vogler, W. R. & Kuo, J. F. (1984) Biochem. Pharmacol. **33**, 125–130
55. Iacopetta, B., Carpentier, J.-L., Pozzan, T., Lew, D. P., Gorden, P. & Orci, L. (1986) J. Cell Biol. **103**, 851–856
56. Iacopetta, B. & Morgan, E. H. (1985) Biochim. Biophys. Acta **805**, 211–216
57. Cox, T. M., O'Donnell, M. W., Aisen, P. & London, I. M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5170–5174
58. Gonzales, E., Yates, A. & Grasso, J. A. (1988) Blood **72**, 27a
59. Volpi, M., Sha'afi, R., Epstein, P. M., Andrenyak, D. M. & Feinstein, M. B. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 795–799
60. Thompson, W. J., Terasaki, W., Epstein, P. M. & Strada, S. J. (1979) Adv. Cyclic Nucleotide Res. **10**, 69–92

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