Receptor-mediated endocytosis of asialoglycoproteins and diferric transferrin is independent of second messengers

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The intracellular concentrations of cyclic AMP, polyphosphoinositides and free Ca^{2+} were unaffected during receptor-mediated endocytosis of the neoglycoprotein β -D-galactosyl-bovine serum albumin (D-Gal-BSA) by isolated hepatocytes. Elevation of either intracellular cyclic AMP by glucagon or inositol phosphates and Ca^{2+} by vasopressin were without effect on the binding and internalization of D-Gal-BSA. The normal response of this cell to glucagon- and vasopressin-mediated mobilization of these second messengers was not modified in the presence of saturating concentrations of D-Gal-BSA. Receptor-mediated endocytosis of diferric transferrin (Fe^{3+} -TRF) by both hepatocytes and HL60 cells was also shown to be independent of second messengers, although the unequivocal expression of the transferrin receptor by hepatocytes could not be satisfactorily demonstrated. The results of the present study are at variance with a suggested regulatory role for second messengers in receptor-mediated endocytosis of serum-derived ligands such as asialoglycoproteins and Fe^{3+} -TRF. Receptor phosphorylation by protein kinase C in particular has been proposed to regulate the distribution and recycling of these receptors in actively endocytosing cells. We would suggest that if receptor phosphorylation has a regulatory role during endocytosis, it is likely to be mediated by a second-messenger-independent protein kinase analogous to casein kinase II. An alternative interpretation is that phosphorylation has no physiological significance and receptor-mediated endocytosis is a constitutive event coupled to membrane turnover.

INTRODUCTION

Many receptors for extracellular ligands, such as growth factors, peptide hormones and pharmacologically active compounds, elicit their transmembrane signals by manipulation of the intracellular level of either the cyclic nucleotides, polyphosphoinositides and free cytosolic Ca^{2+} or, more unusually, by ligand-induced tyrosine kinase activity in the intracellular domain of the receptor polypeptide (for reviews, see [1–3]). Expression of the biological response is mediated by selective phosphorylation and dephosphorylation, via a complex cascade of specific kinases and phosphatases, of appropriate target proteins [4,5]. These targets would appear to include the intracellular domain of the receptors themselves, and this may be part of the autoregulatory mechanism that modulates receptor activity [6,7].

The recent identification of phosphorylated species of the receptors for low-density lipoprotein [8], TRF [9], asialoglycoproteins [10] and polymeric IgA [11] has stimulated interest in the significance of this modification in the endocytic process. Evidence to date, extrapolated primarily from kinetic studies on TRF receptor expression in cells treated with phorbol esters and Ca^{2+} ionophores, has suggested that phosphorylation may control intracellular receptor traffic by modifying the distribution between cell surface and intracellular receptors in favour of the intracellular pool [12,13]. This would imply that modulation of receptor distribution by transient phosphorylation/dephosphorylation is tightly coupled

to the endocytic pathway. However, it is clear, from studies on both the TRF and asialoglycoprotein receptors, that occupation of the ligand-binding site does not provide the signal for phosphorylation and therefore, by inference, for internalization [10,14]. This has invoked a role for intracellular second messengers in the endocytic process. If receptor phosphorylation has a regulatory function which is normally coupled to a second-messenger-dependent kinase, then this should be demonstrable either by mobilization of one of the established second messengers during receptor-mediated endocytosis or by modifying the kinetics of endocytosis by the simultaneous stimulation of cells with physiologically appropriate ligands which are known to mobilize second messengers. Conversely, if receptor-mediated endocytosis is independent of second-messenger mobilization or modulation, then this would suggest that receptor phosphorylation is either coupled to second-messenger-independent kinases or that it is a constitutive modification with no physiological significance.

The hepatocyte would appear to be an ideal cell in which to study this possible interrelationship. Receptor-mediated endocytosis of asialoglycoproteins [15] and Fe^{3+} -TRF [16], and the mobilization of cyclic AMP by glucagon [17] and polyphosphoinositides and Ca^{2+} by vasopressin [18] have all been described in this cell. However, like others, we have been unable to demonstrate the unequivocal expression of the TRF receptor in this cell [19,20] and, at best, our estimate of B_{max} was 14000 receptors/cell. In order to preclude possible

Abbreviations used: D-gal-BSA, D-galactosyl-bovine serum albumin; (Fe^{3+})-TRF, (diferric) transferrin; TBS is defined in the text; K_D , equilibrium binding constant; B_{max} , apparent maximum number of binding sites; k_e , endocytotic rate constant; IBMX, 3-isobutyl-1-methylxanthine.

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ambiguities arising from the uncertain interaction between Fe^{3+} -TRF and hepatocytes, the same studies have also been carried out in HL60 cells [21].

We have concluded that the endocytosis of asialoglycoproteins and Fe^{3+} -TRF is independent of the recognized second messengers. However, this is not to say that receptor phosphorylation, possibly by a casein kinase II type enzyme [22], does not have a role in regulating subcellular distribution or recycling, for example.

MATERIALS AND METHODS

The neoglycoprotein β -D-galactosyl-bovine serum albumin (D-Gal-BSA) was prepared as previously described [23]. Fe^{3+} -TRF was prepared essentially by the method of Bates & Schlabach [24]. Briefly, Fe^{3+} -nitrilotriacetic acid at a nitrilotriacetic acid/iron ratio of 2:1 was rapidly mixed with apotransferrin (10 mg/ml, Behringwerke, Marburg, Germany) in 0.1 M- NaClO_4 /10 mM-Tris/HCl, pH 7.4, to which 0.1 M- NaHCO_3 had been freshly added to make a final concentration of 5 mM. The amount of iron added to the mix corresponded to an approximate Fe/apo-TRF molar ratio of 1.2:1. After 1 h at room temperature chelate-free Fe^{3+} -TRF was separated in a Sephadex G-25 column equilibrated in 0.1 M- NaClO_4 /10 mM-Tris/HCl, pH 7.4, and dialysed extensively against 20 mM-Tris/HCl/0.15 M-NaCl, pH 7.4 (TBS). Saturation of transferrin with iron was confirmed by an A_{465}/A_{280} ratio of 0.046. ^{125}I -labelled ligands were prepared as described previously [23]. myo -[2- ^3H]-Inositol, [^3H]acetic anhydride, [^{32}P] P_i , $^{45}\text{Ca}^{2+}$ as $^{45}\text{CaCl}_2$, Na^{125}I and cyclic AMP-[^{125}I]Amersham-M assay system were from Amersham International.

Analysis of ligand binding and uptake

Isolated hepatocytes were prepared from male Wistar rats as described previously [25]. After a 45 min pre-incubation period to allow for re-expression of cell-surface receptor, the cells were resuspended at a concentration of $(4-6) \times 10^6$ cells/ml in Earle's basal salt solution, gassed, and either placed on ice until required or incubated at 37°C in a shaking water bath before experimentation. Cell viability was assessed at the end of every study by measuring the exclusion of 0.2% Trypan Blue. In all cases viability was estimated to be greater than 90% for the duration of the experiments.

HL60 cells were kindly provided by Dr. M. Clemens, Department of Biochemistry, St. George's Hospital Medical School, and were cultured in RPMI 1640 medium supplemented with 10 mM-glutamine and 10% (v/v) fetal-calf serum. The cells were pelleted by centrifugation at 850 g for 1 min, washed with Earle's basal salt solution and resuspended at a density of approx. 5×10^6 cells/ml before experimentation.

For studies on ligand binding to cell-surface receptors, 2 ml aliquots of hepatocytes or HL60 cells were diluted 2-fold with ice-cold medium. Aliquots (0.5 ml) of the diluted cells were then added to individual wells of a 25-well culture dish containing 10 μl of medium and 0.02 μCi of an appropriate ^{125}I -labelled ligand mixed with homologous unlabelled ligand so that the final ligand concentration was in the range 0.3–30 nM. The dishes were shaken for 1 h at 4°C (we had previously established that this was sufficient for equilibration of ligand binding) before layering 0.1 ml of the cell suspension on top of 0.5 ml of silicone oil (relative density 1.04). The cells were

immediately pelleted in a Beckman Microfuge. For hepatocytes, non-specific binding, which was estimated in the presence of 200- and 1000-fold molar excesses of unlabelled D-Gal-BSA and Fe^{3+} -TRF respectively, was 2% for D-Gal-BSA and 35% for Fe^{3+} -TRF. Non-specific binding of Fe^{3+} -TRF in HL60 cells was less than 4% in the presence of a 1000-fold molar excess of unlabelled ligand. The amount of bound ligand in the pellet and the concentration of free ligand in the supernatant was used to estimate the apparent equilibrium binding constant, K_D , and the apparent maximum number of binding sites, B_{max} , by Scatchard analysis. In all cases Scatchard plots were linear.

The endocytotic rate constant, k_e , for the internalization of D-Gal-BSA by hepatocytes was measured as described in [25]. Surface and internalized ligand were distinguished by stripping surface-bound ligand with NaEDTA, which removed over 90% of the radioactivity associated with the cell surface. The amount of ligand internalized and the total amount bound by the cells was calculated from the radioactivity in cells that had been treated with or without NaEDTA respectively; the difference in these values represented the surface-bound ligand. The values of k_e for the internalization of Fe^{3+} -TRF by hepatocytes and HL60 cells were measured in a similar fashion, except that surface and internalized ligand were distinguished as follows. Four 0.2 ml aliquots were taken at various time intervals from suspensions of hepatocytes or HL60 cells containing 0.05 μCi of ^{125}I - Fe^{3+} -TRF/ml. Two aliquots were added to 1.4 ml of an ice-cold iron-chelating buffer (137 mM-NaCl/3.5 mM-KCl/20 mM-sodium citrate, pH 5, containing 0.1% ovalbumin and 5 mM-nitrilotriacetic acid) [26] and two were added to 1.4 ml of ice-cold TBS in Eppendorf tubes. After 10 min the pH of the chelating buffer was adjusted to pH 7.4 by the addition of 25 μl of 1 M-Tris and, after a further 10 min, all samples were centrifuged for 1 min in a Beckman Microfuge. The pellets were gently washed with 1 ml of TBS and counted for radioactivity in a LKB Compugamma γ -radiation counter. The amount of surface-bound ligand was calculated from the difference in radioactivity between the pellets that had been treated with the iron chelator and those that had not, whereas the amount internalized was indicated by the cells which had been stripped by the chelator.

Measurement of second messengers

(i) **Polyphosphoinositides and inositol phosphates.** The possible breakdown of the polyphosphoinositides coincident with receptor-mediated endocytosis of D-Gal-BSA or Fe^{3+} -TRF by hepatocytes was compared with the well-described effects of vasopressin on mobilization of this second messenger [18,27]. Aliquots (15 ml) of freshly isolated hepatocytes were preincubated with 0.5 mCi of [^{32}P] P_i for 1 h at 37°C. Experiments were started by the addition of 0.5 ml aliquots of cells to separate vials containing 10 μl of stock solutions of the appropriate ligands, so that the final concentration of vasopressin was in the range 1–100 nM and those of D-Gal-BSA and Fe^{3+} -TRF were 0.1–10 μM .

Incubations were stopped at 0.5, 1, 3 and 5 min by the addition of 1.5 ml of chloroform/methanol/conc. HCl (200:100:1, by vol.), left for 10 min and then vortex-mixed vigorously for 15 s. A portion (0.5 ml) of water was added to each sample, vigorously mixed and the two phases were separated by centrifugation in an MSE

Chilspin-2 centrifuge. The lower organic phase was washed once and the ^{32}P -labelled phospholipids were separated on silica-gel 60 plates (Merck). Before use the plates were sprayed with methanol/water (2:3, v/v) containing 1% potassium oxalate, dried at room temperature and then activated at 115 °C for 15 min. Two sets of plates were prepared. One set was developed in ethyl acetate/acetic acid/2,2,4-trimethylpentane (9:2:5, by vol.) for separation of phosphatidic acid as a single spot; the other phospholipids remained at the origin [28]. The second set of plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) for the separation of ^{32}P -labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-bisphosphate [29]. ^{32}P -labelled phospholipids were detected by autoradiography on Kodak X-Omat film, and the spots corresponding to the phosphoinositides were scraped from the plate and counted for radioactivity.

In another series of experiments the generation of inositol phosphates by the same three ligands was investigated by incubating hepatocytes with 2 μCi of *myo*-[2- ^3H]inositol/ml for 40 min at 37 °C and for a further 10 min in the presence or absence of 10 mM-LiCl before transferring 1 ml aliquots to separate vials containing 20 μl of the stock solutions of ligands. The vials were incubated, with shaking, for a further 15 min before the addition of 1 ml of ice-cold 1 M-HClO₄. The supernatants were neutralized with 1 M-KOH before separation of the inositol phosphates on 1 ml columns of Dowex AG-1 (X8; formate form), exactly as described by Berridge *et al.* [30].

The accumulation of diacylglycerol in hepatocytes after stimulation with 100 nM-vasopressin or 10 μM -D-Gal-BSA or -Fe³⁺-TRF was investigated by incubating 2 ml of cells with the appropriate ligand for 10 min before the addition of 6 ml of chloroform/methanol (2:1, v/v). The lipid extract was washed and the total content of diacylglycerol was measured by acetylation with [^3H]acetic anhydride [31].

For kinetic studies 30 nM-vasopressin was added to cells (both in the presence and in the absence of 10 mM-LiCl) at 10 min, 5 min and 0 min before the addition of 2 nM- ^{125}I -D-Gal-BSA or ^{125}I -Fe³⁺-TRF. The estimation of k_e was as described above.

For HL60 cells the breakdown of ^{32}P -labelled polyphosphoinositides and generation of ^3H -labelled inositol phosphates during receptor-mediated endocytosis of Fe³⁺-TRF was investigated exactly as described for hepatocytes.

(ii) Calcium. (a) Cytoplasmic free Ca²⁺. Cytoplasmic free Ca²⁺ concentrations (nM) were measured in individual aequorin-loaded hepatocytes exactly as described by Woods *et al.* [32]. Individual cells were superfused with William's medium E containing 1.8 mM-Ca²⁺ and either 2 μM -phenylephrine, 2 μM -ADP, 10 μM -D-Gal-BSA or 1 μM -Fe³⁺-TRF alone or in combination with one of the other ligands. Photon counts were detected by a low-noise photomultiplier and sampled every 50 ms by a Sirius microcomputer. Data was plotted using exponential smoothing as described previously [32]. HL60 cells were loaded with quin 2, and fluorescence measurements in the presence and absence of 1 μM -Fe³⁺-TRF were made exactly as described by Iacopetta *et al.* [33].

(b) Calcium flux. A portion (1 μCi) of $^{45}\text{Ca}^{2+}$ (10 μl) was added to 2 ml aliquots of hepatocytes or HL60 cells

which were gently stirred in a small thermostatically controlled cup at 37 °C for 15 min before the addition of 10 μl aliquots of 2 mM-D-Gal-BSA, 2 mM-Fe³⁺-TRF or 0.2 mM-A23187, either singly or in combination, to give final concentrations of ligand or ionophore of 100 μM , 10 μM and 1 μM respectively. Duplicate 0.1 ml samples were withdrawn at timed intervals over a 15 min period and spun through 0.5 ml of silicone oil (relative density 1.04) into 0.2 ml of 1.6 M-HClO₄ to lyse the cells. A 0.1 ml portion of the acid-soluble material was then counted.

(iii) Cyclic AMP. The effect of receptor-mediated endocytosis of D-Gal-BSA and Fe³⁺-TRF by hepatocytes on the intracellular concentration of cyclic AMP was compared with the well-described stimulation of cyclic AMP levels by glucagon [17]. Aliquots (8 ml) of cells were preincubated with shaking at 37 °C for 10 min with 1 mM-3-isobutyl-1-methylxanthine (IBMX) before the addition of 50 μl aliquots of appropriate stock ligand so that the final concentration of glucagon was 10 nM and both D-Gal-BSA and Fe³⁺-TRF were in the range 0.1–10 μM . The possible antagonism of glucagon action by these ligands was assessed in the absence of IBMX. Duplicate 0.5 ml samples were withdrawn at timed intervals over 15 min and rapidly mixed with 0.5 ml of ice-cold 1.6 M-HClO₄. The supernatant was neutralized with 1.6 M-KOH before cyclic AMP determination using the Amerlex-M assay kit. For kinetic studies, 10 nM-glucagon was added to cells (in the presence of 1 mM-IBMX) at –10 min, –5 min and 0 min before the addition of 2 nM- ^{125}I -D-Gal-BSA or ^{125}I -Fe³⁺-TRF. The value of k_e was then estimated for these ligands as described above. Adenylate cyclase activity was also demonstrated in both hepatocytes and HL60 cells by stimulation with forskolin [34]. A 40 μl portion of 2 mM-forskolin in dimethyl sulphoxide was added to 8 ml aliquots of cells at 37 °C, and duplicate 0.5 ml samples were processed for the measurement of cyclic AMP exactly as described above. The concentration of intracellular cyclic AMP in HL60 cells was also measured over a 15 min period after the addition of up to 10 μM -Fe³⁺-TRF to the extracellular medium. The k_e for Fe³⁺-TRF uptake by HL60 cells was compared between unstimulated cells and cells that had been preincubated with 10 μM -forskolin for 10 min before the addition of ligand.

RESULTS

Analysis of ligand binding and uptake

The accurate measurement of K_D , B_{max} and k_e for the binding and internalization of D-Gal-BSA by hepatocytes was facilitated both by the exceptionally low non-specific binding of ligand to the cell surface and by the sensitivity of ligand binding to NaEDTA [25] (Table 1). By contrast, the amount of ^{125}I -Fe³⁺-TRF taken up per cell over a 20 min period was at least 100-fold less than for ^{125}I -D-Gal-BSA, despite only an apparent 10-fold difference in reported B_{max} values for the two receptors in this cell [25,35] (Fig. 1). Stripping hepatocytes with an acidic, iron-chelating buffer only removed 60% of cell-surface-associated ^{125}I -Fe³⁺-TRF. Furthermore, the distribution of ^{125}I -Fe³⁺-TRF between the cell surface and an acid-resistant, presumed internalized, pool was maintained as a 1:1 ratio, even in the presence of a 3000-molar excess of unlabelled Fe³⁺-TRF. The unequivocal

Table 1. Parameters for ligand binding and uptake in hepatocytes and HL60 cells

Isolated hepatocytes or HL60 cells were suspended in Earle's basal salt solution. K_D and B_{max} were estimated by Scatchard analysis of ^{125}I -D-Gal-BSA or ^{125}I -Fe $^{3+}$ -TRF binding to the cells at 4 °C for 60 min over the range of 0.3–30 nM. k_e was determined at 37 °C as described in the Materials and methods section. The results are expressed as means $\pm$ s.e.m., with the number of separate determinations given in parentheses.

Cell type	Ligand used	K_D (nM)	B_{max} (no./cell)	k_e (min $^{-1}$)
Hepatocytes	^{125}I -D-Gal-BSA	1.72 ± 0.11 (26)	$175\,600 \pm 8\,900$ (26)	0.21 ± 0.04 (13)
	^{125}I -Fe $^{3+}$ -TRF	4.4 ± 0.6 (6)	$14\,000 \pm 1\,200$ (6)	—
HL60	^{125}I -Fe $^{3+}$ -TRF	5.6 ± 2.4 (6)	$154\,000 \pm 23\,000$ (6)	0.10 ± 0.03 (4)

Fig. 1. Surface binding and internalization of D-Gal-BSA and Fe $^{3+}$ -TRF in hepatocytes and HL60 cells

Hepatocytes were incubated at 37 °C in the presence of 20 nM- ^{125}I -D-Gal-BSA (a) or 20 nM- ^{125}I -Fe $^{3+}$ -TRF (b), whereas HL60 cells were incubated with 20 nM- ^{125}I -Fe $^{3+}$ -TRF only (c). At each time point the amount of ligand bound to the surface (■) and internalized (●) was determined as described in the Materials and methods section. The results are means $\pm$ s.e.m. of three separate determinations.

presence of TRF receptors in the terminally differentiated non-proliferating hepatocyte could not, therefore, be satisfactorily demonstrated. For these reasons the measurement of K_D , B_{max} and k_e for the binding and internalization of Fe $^{3+}$ -TRF by hepatocytes was difficult

Fig. 2. Effect of vasopressin and D-Gal-BSA on ^{32}P phosphatidyl-myoinositol mobilization in hepatocytes

Phosphatidylinositol (PI) mobilization was monitored in hepatocytes prelabelled with ^{32}P orthophosphate under control conditions (a) or after stimulation with either 10 μM -D-Gal-BSA (b) or 30 nM-vasopressin (c). Samples were removed at given time intervals and ^{32}P phosphatidylinositol phosphates were separated by t.l.c. from the hepatocyte lipid extract as described in the Materials and methods section. Further abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE/PS, phosphatidylethanolamine/phosphatidylserine; PIP, phosphatidylinositol monophosphate; PIP $_2$, phosphatidylinositol bisphosphate.

to perform consistently. Despite only an apparent 5-fold difference in reported B_{max} values for the TRF receptor in hepatocytes and HL60 cells [21,35] the amount of ^{125}I -Fe $^{3+}$ -TRF bound and internalized per cell was up to 60-fold greater for HL60 compared with hepatocytes (Fig. 1). Non-specific binding of ^{125}I -Fe $^{3+}$ -TRF to HL60 cells was less than 4%, and acid-stripping removed 98% of cell-surface associated ligand. Under these conditions a k_e value of 0.1 min $^{-1}$ was estimated for the endocytosis of Fe $^{3+}$ -TRF by HL60 cells (Table 1).

Measurement of second messengers

(i) **Polyphosphoinositides.** The unequivocal presence of phospholipase C activity could only be satisfactorily demonstrated in hepatocytes. In the absence of LiCl the breakdown and resynthesis of ^{32}P phosphatidylinositol 4,5-bisphosphate could be demonstrated within 5 min of the addition of 30 nM-vasopressin to the cells (Fig. 2). By 5 min the cellular content of ^{32}P phosphatidylinositol 4,5-bisphosphate had returned to more than 80% of the basal level. Both 10 μM -D-Gal-BSA and Fe $^{3+}$ -TRF failed to trigger this transient turnover of phosphatidylinositol 4,5-bisphosphate. In the presence of 10 mM-LiCl, a dose-

Fig. 3. Effect of vasopressin, D-Gal-BSA and Fe^{3+} -TRF on the incorporation of $[^{32}\text{P}]\text{P}_i$ into phosphatidic acid and the release of $[^3\text{H}]\text{inositol}$ phosphates from rat hepatocytes

Dose-response curves are shown for the incorporation of $[^{32}\text{P}]\text{P}_i$ into phosphatidic acid (PA) (a) and the release of $[^3\text{H}]\text{inositol}$ phosphates (IPs) (b) from hepatocytes by 1–100 nM-D-Gal-BSA or Fe^{3+} -TRF ($\bullet$, $\circ$) or by 1–100 nM-vasopressin alone or in combination with 10 μM -D-Gal-BSA or 10 μM - Fe^{3+} -TRF ($\blacksquare$, $\square$). The results are means $\pm$ S.E.M. for three experiments.

dependent increase, up to 30% above control value, in the amount of $[^{32}\text{P}]\text{phosphatidic acid}$ released from prelabelled cells by vasopressin could also be demonstrated (Fig. 3a). Under the same conditions there was a dose-dependent increase in the production of $[^3\text{H}]\text{inositol}$ phosphates within 5 min of stimulating prelabelled hepatocytes with 1–100 nM-vasopressin (Fig. 3b). Maximal stimulation with 30 nM-vasopressin doubled the total inositol phosphates and diacylglycerol (Table 2), which was in close agreement with previous estimates [27]. Neither 10 μM -D-Gal-BSA nor Fe^{3+} -TRF elicited any change in the basal level of inositol phosphates or phosphatidic acid up to 15 min after addition to hepatocytes (Figs. 3a and 3b). The simultaneous administration of these ligands together with vasopressin failed to attenuate or enhance the production of inositol phosphates or phosphatidic acid measured with vasopressin alone.

Mobilization of the polyphosphoinositides by stimulation of hepatocytes with 30 nM-vasopressin had no effect on the k_e value for the internalization of D-Gal-BSA by hepatocytes. The value of k_e remained unaffected, irrespective of the presence or absence of 10 mM-LiCl or whether the ligand was administered simultaneously with, or up to 10 min after, vasopressin. Similarly K_D and B_{max} values for ligand binding were unaltered from normal,

Table 2. Effect of vasopressin and other ligands on diacylglycerol content of hepatocytes

Isolated hepatocytes were incubated in 2 ml aliquots at 37 $^{\circ}\text{C}$ for 10 min with vasopressin, D-Gal-BSA or Fe^{3+} -TRF either on their own or in combination as indicated. The incubation was quenched by the addition of 6 ml of chloroform/methanol (2:1, v/v) and the diacylglycerol content was measured in the lipid extract as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M. for three separate experiments.

Ligand added	Diacylglycerol content of hepatocytes (nmol/mg of protein)
Control	2.5 ± 0.7
D-Gal-BSA (10 μM)	2.0 ± 0.8
Fe^{3+} -TRF (10 μM)	2.8 ± 0.4
Vasopressin (100 nM)	8.4 ± 1.2
+ D-Gal-BSA (10 μM)	8.1 ± 1.0
+ Fe^{3+} -TRF (10 μM)	9.0 ± 1.4

irrespective of the mode of vasopressin administration.

It was not possible to demonstrate the presence of phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate in HL60 cells. No breakdown of $[^{32}\text{P}]\text{phosphatidylinositol}$ 4,5-bisphosphate, generation of $[^3\text{H}]\text{inositol}$ phosphates or accumulation of diacylglycerol could be demonstrated in prelabelled cells by the addition of vasopressin (100 nM), f-Met-Leu-Phe (2 nM), phenylephrine (10 μM) or epidermal growth factor (100 nM) either singly or in combination, to the extracellular medium. Similarly, the addition of 20% fetal-calf serum, with or without some of these ligands, also failed to elicit any breakdown of phosphatidylinositol 4,5-bisphosphate in cells that had been grown in serum-free medium. Consequently the addition of 10 μM - Fe^{3+} -TRF to the extracellular medium also failed to elicit any demonstrable change in the basal level of inositol phospholipids in these cells.

(ii) **Calcium.** (a) **Cytoplasmic free Ca^{2+} .** The mean resting signal in single isolated hepatocytes injected with aequorin was 0.6 photon counts/s above background, and the mean total radioactivity per cell was 8×10^4 c.p.m., which gave an upper limit for mean resting free Ca^{2+} concentration of 180 nM. Up to 10 μM extracellular concentrations of D-Gal-BSA or Fe^{3+} -TRF failed to raise the resting signal. By contrast, 2 μM -phenylephrine or 2 μM -ADP induced transients in the same cells at approx. 20 s and 30 s intervals, reaching free Ca^{2+} concentrations of about 600 nM and 1000 nM respectively. The simultaneous administration of 10 μM -D-Gal-BSA or Fe^{3+} -TRF with these agonists did not alter the frequency or amplitude of the transients recorded in the presence of agonist alone (Fig. 4a). Fe^{3+} -TRF at 1 μM had no effect on quin 2 fluorescence in HL60 cells.

(b) **Ca^{2+} flux.** The equilibration of $^{45}\text{Ca}^{2+}$ across the plasma membrane of isolated hepatocytes was not perturbed by the addition of up to 10 μM -D-Gal-BSA or Fe^{3+} -TRF to the extracellular medium. Influx of $^{45}\text{Ca}^{2+}$ into this cell was demonstrated by the addition of the Ca^{2+} ionophore A23187 (1 μM) to the medium (Fig. 4b). A23187-mediated $^{45}\text{Ca}^{2+}$ influx was not modified by the

Fig. 4. Effect of D-Gal-BSA and Fe^{3+} -TRF on calcium mobilization

Cytoplasmic free calcium in hepatocytes (a) was monitored by using the aequorin signal from individual prelabelled cells perfused sequentially with: A, 0.5 μM Fe^{3+} -TRF; B, normal medium; C, 1 μM D-Gal-BSA; D, normal medium; and E, 2 μM ADP. The trace is representative of those given in a number of similar studies. Influx of $^{45}Ca^{2+}$ into hepatocytes (b) was measured in the presence of 10 μM D-Gal-BSA or 10 μM Fe^{3+} -TRF (●) or 1 μM A23187 with or without 10 μM D-Gal-BSA or 10 μM Fe^{3+} -TRF (■). Results are means $\pm$ S.E.M. for three experiments.

simultaneous addition of both the ionophore and either 10 μM D-Gal-BSA or 10 μM Fe^{3+} -TRF. Fe^{3+} -TRF also failed to perturb the Ca^{2+} flux across the plasma membrane of HL60 cells, whereas A23187 had a similar effect to that seen with hepatocytes.

(iii) **Cyclic AMP.** In the presence of the phosphodiesterase inhibitor IBMX, extracellular concentrations of up to 10 μM of D-Gal-BSA and Fe^{3+} -TRF failed to elicit any change in the basal concentration of cyclic AMP in hepatocytes (Fig. 5a). Similar concentrations of Fe^{3+} -TRF failed to raise cyclic AMP levels above control in HL60 cells. The intracellular concentrations of cyclic AMP in hepatocytes can be greatly increased by stimulation with glucagon [17]. In the present study, stimulation of hepatocytes with 10 nM-glucagon in the presence or

Fig. 5. Effect of D-Gal-BSA and Fe^{3+} -TRF on accumulation of cyclic AMP in hepatocytes and HL60 cells

A time course of cyclic AMP accumulation in hepatocytes (a) was measured in the presence (●) or absence (○) of 1 mM-IBMX after stimulation with 10 nM-glucagon alone or in combination with 10 μM D-Gal-BSA or 10 μM Fe^{3+} -TRF (●, ■). (b) Cyclic AMP accumulation was determined after stimulation by 10 μM -forskolin in hepatocytes (○) and HL60 cells (□). The presence of 10 μM D-Gal-BSA or 10 μM Fe^{3+} -TRF did not alter the control values in hepatocytes (a, ▲) or HL60 cells (b, △). The results are means $\pm$ S.E.M. of three to five determinations.

absence of IBMX, elicited transient 40-fold and 8-fold increases respectively in the intracellular cyclic AMP concentration (Fig. 5a). The prior or simultaneous addition of 10 nM-glucagon with up to 10 μM D-Gal-BSA or Fe^{3+} -TRF did not antagonize or amplify the response to glucagon in either the presence or absence of IBMX.

Adenylate cyclase activity could be maximally stimulated in both hepatocytes and HL60 cells by incubation with 10 μM -forskolin (Fig. 5b). On a per-cell basis, hepatocytes generated approximately twice the amount of cyclic AMP compared with HL60 cells, but if the same results were expressed in terms of cellular protein, the cyclic AMP content of HL60 cells was approx. 7-fold greater than that of hepatocytes. In the presence of IBMX, stimulation of hepatocytes and HL60 cells with 10 μM -forskolin for 10 min before measurement of K_D , B_{max} , and k_e for D-Gal-BSA and Fe^{3+} -TRF respectively

failed to cause any significant change in these parameters compared with unstimulated cells (Table 1).

DISCUSSION

We have shown that receptor-mediated endocytosis of asialoglycoproteins (and presumably transferrin) by isolated hepatocytes was not coupled to either the simultaneous breakdown of the polyphosphoinositides, the generation of inositol phosphates or the mobilization of cytoplasmic free Ca^{2+} . Furthermore, the generation of diacylglycerol or phosphatidic acid independently of phosphatidylinositol 4,5-bisphosphate breakdown could not be demonstrated, which discounted a role for these compounds as unique second messengers linked to the endocytotic process [36]. This would suggest that the endocytotic activity of the hepatocyte *per se* was not associated with the mobilization of these second messengers, and therefore, by implication, with protein kinase C involvement. Under normal circumstances a prerequisite for stimulation of protein kinase C activity would be the obligatory turnover of the polyphosphoinositides to release diacylglycerol and the inositol phosphates [1]. Studies on transformed cell lines treated with phorbol esters and/or Ca^{2+} ionophores have suggested that down-regulation of the asialoglycoprotein and TRF receptors may be modulated by protein kinase C-catalysed receptor hyperphosphorylation [12,13,36]. But deletion of the putative protein kinase C phosphorylation site (Ser-24) in the cytoplasmic domain of the TRF receptor by site-directed mutagenesis has no effect on the kinetics of internalization and recycling of the mutagenized receptors in cells transfected with the appropriate mutant cDNA [37–39]. Furthermore, recycling of these phosphorylation-defective receptors is regulated by phorbol esters in a similar manner to the wild-type receptor [37,39], indicating that regulation of TRF receptor recycling is independent of phosphorylation at Ser-24. Destabilization of the cytoskeleton inhibits phorbol-ester-induced down-regulation, but not hyperphosphorylation, of the TRF receptor [40]. Thus the effects of phorbol esters and Ca^{2+} ionophores on the activity of these endocytosing receptors would appear to be unrepresentative of the physiological response and secondary to more direct effects of protein kinase C on other cellular functions.

Another possibility was that the asialoglycoprotein and TRF receptors were coupled to protein kinase C as a consequence of phosphatidylinositol 4,5-bisphosphate breakdown in response to other ligands. However, in the present study, vasopressin, at concentrations shown by others to elicit maximum turnover of the polyphosphoinositides [18,27], was without effect in changing any of the parameters selected to measure endocytotic activity. Furthermore, the absence of a modified response to vasopressin in actively endocytosing cells would suggest that signal transduction by phospholipase C/inositol-phospholipid-coupled events was completely independent of cell-surface endocytotic activity. Similar conclusions on the role of the inositol phospholipids in receptor-mediated endocytosis of Fe^{3+} -TRF by HL60 cells could not be made with certainty, owing to our inability to demonstrate constitutive phospholipase C activity, despite the presentation of a variety of ligands to the cell. (This was consistent with ligand-induced inositol phospholipid metabolism only occurring in terminally

differentiated HL60 cells [41].) Nonetheless, the failure of supersaturating concentrations of Fe^{3+} -TRF to perturb the polyphosphoinositides and the refractoriness of the endocytotic process to a variety of heterologous ligands was consistent with the independence of Fe^{3+} -TRF endocytosis from protein kinase C involvement.

This conclusion was reinforced by our measurements of intracellular calcium, $[\text{Ca}^{2+}]_i$, coincident with receptor-mediated endocytosis of D-Gal-BSA and Fe^{3+} -TRF. In the hepatocyte, both ligands failed to raise $[\text{Ca}^{2+}]_i$ by either generating a net influx of Ca^{2+} or by releasing cytoplasmic free Ca^{2+} . The absence of an effect of receptor-mediated endocytosis on cytoplasmic free Ca^{2+} was particularly interesting. D-Gal-BSA and Fe^{3+} -TRF failed to modify the frequency or amplitude of calcium transients generated by ligands such as vasopressin, phenylephrine and ADP, which was at variance with the inhibition of hormone-induced transients by low concentrations of phorbol esters [42], but consistent with the independence of endocytosis from protein kinase C-mediated regulation. The complete absence of transients, indicative of the release of free Ca^{2+} from intracellular stores in aequorin-loaded hepatocytes, in response to the prolonged presentation of supersaturating concentrations of D-Gal-BSA and Fe^{3+} -TRF was unlikely to have been due to constraints on signal detection. We have estimated that even if the change in cytoplasmic free Ca^{2+} had been restricted to a $1\ \mu\text{m}$ zone underlying the plasma membrane, the minimal signal expected from a rise in cytoplasmic free Ca^{2+} comparable with that elicited by physiological ligands such as phenylephrine and vasopressin ($\sim 1000\ \text{nM}$) would have been approx. 11 photon counts/s and easily detected above the level of 1–2 counts/s. If lateral zonation of this region had occurred, as uncoated pits for example, the signal would have been proportionately lower. Zones $1\ \mu\text{m}$ deep under 10% of the plasmalemmal layer would have generated signals of approx. 0.8 photon counts/s. This would have been detected after integration over 1 min or so. The absence of a regulatory role for $[\text{Ca}^{2+}]_i$ was consistent with an earlier study on changes in $[\text{Ca}^{2+}]_i$ during Fe^{3+} -TRF endocytosis by HL60 cells [33]. The authors demonstrated that raising or lowering $[\text{Ca}^{2+}]_i$ 10-fold from the resting level had no significant effect on Fe^{3+} -TRF endocytosis. Our studies on $[\text{Ca}^{2+}]_i$ during endocytosis of Fe^{3+} -TRF by HL60 cells were compatible with these observations and were exactly analogous to those obtained in hepatocytes, i.e. there was no change in the flux of $^{45}\text{Ca}^{2+}$ across the plasma membrane, nor was there any evidence of raised $[\text{Ca}^{2+}]_i$ in quin 2-loaded cells, although it should be noted that the sensitivity of this technique is not comparable with the measurement of calcium transients in single cells.

The results of our studies on a possible regulatory role for cyclic AMP in receptor-mediated endocytosis were analogous to those obtained with the inositol phospholipids and Ca^{2+} . The failure of elevated levels of intracellular cyclic AMP to affect either B_{max} , K_D or k_e for the binding or internalization of D-Gal-BSA and Fe^{3+} -TRF by hepatocytes and HL60 cells respectively was compatible with the absence of consensus sequences for cyclic AMP-dependent protein kinase (two or more adjacent basic amino acids *N*-terminal to the phosphorylation site) in the cytoplasmic domains of the asialoglycoprotein and TRF receptors [43,44]. Furthermore, the failure of the cyclic AMP-mobilizing hormone, glucagon, to

modify the endocytotic process in hepatocytes, together with the absence of a modified response to glucagon in actively endocytosing cells, would suggest that adenylate cyclase activity is completely independent of other cell-surface events such as ligand internalization.

The absence of a regulatory function for the recognized second messengers in receptor-mediated endocytosis of extracellular ligands by hepatocytes, and by implication by HL60 cells, was apparently incompatible with a role for receptor phosphorylation in this process. However, these results would support receptor phosphorylation by a second-messenger-independent protein kinase analogous to casein kinase II [8,22]. Phosphorylation of Ser-833 in the cytoplasmic domain of the low-density-lipoprotein receptor is carried out by a second-messenger-independent kinase which shares several properties with casein kinase II, including the requirement for the close juxtaposition of a cluster of acidic amino acids C-terminal to the phosphorylated serine residue [8]. The short cytoplasmic domains of the two asialoglycoprotein receptor polypeptides in both the rat and man also contain, with one exception, clusters of acidic amino acids C-terminal to serine residues [43]. The TRF receptor contains four other serine residues, in addition to Ser-24, in its cytoplasmic domain, one of which, namely Ser-34, is nine residues away from a C-terminal cluster of four acidic amino acids [44]. Although less compelling than the sequence analogies between the asialoglycoprotein and low-density-lipoprotein receptors, secondary-structure modifications may favour phosphorylation of this serine residue by a second-messenger-independent kinase. A kinase with properties identical with those of casein kinase II, the activity of which cannot be modified by cyclic AMP, phospholipids or calmodulin, has been shown to co-purify with clathrin-coated vesicles [45]. The function of this kinase remains speculative, but its association with the initial stage of the endocytotic pathway could be suggestive of a regulatory role coupled to receptor phosphorylation.

If receptor phosphorylation has no significance in the endocytotic activity of the cell, then the internalization of serum-derived ligands such as asialoglycoproteins and TRF should be a constitutive event coupled to membrane turnover. The escalator concept for receptor-mediated endocytosis does not require occupation of receptors as a prerequisite for their internalization, since constitutive membrane turnover maintains a continuous throughput of receptors at the cell surface, and only some of these may fortuitously bind ligand [26]. We have estimated that k_e for the uptake of asialoglycoproteins by hepatocytes is 0.21 min^{-1} . This indicates that there is a 1 in 5 chance of an occupied receptor being internalized every minute. This implies that the entire plasma membrane, or specialized regions involved in the endocytic process, could be completely replaced every 5 min. This is in close agreement with an estimate for the rate of membrane internalization coincident with the endocytosis of the fluid-phase marker inulin by hepatocytes [46]. (This is not to say, however, that fluid-phase endocytosis and receptor-mediated endocytosis follow the same intracellular pathway [47].) Therefore the continuous and rapid turnover of the plasma membrane, or specialized regions contained therein, would preclude the requirement for specific signals to trigger receptor-mediated endocytosis. The occupation of a receptor would inevitably be followed by its internalization within a short

time period and could occur quite independently of the recognized signal transduction mechanisms. A possible regulatory role for receptor phosphorylation in controlling the subcellular distribution of receptors in actively endocytosing cells remains its most likely function.

Since this paper was originally submitted for publication we have become aware of the fact that the means of signal transduction has yet to be identified for the growth-hormone and prolactin receptors [48]. The homologous ligands are apparently without effect on cyclic AMP, cyclic GMP, inositol phospholipids, phosphorylation, Ca^{2+} or ion channels [48]. A recent report has also shown that the haemopoietic growth factor interleukin 3 stimulates the translocation of protein kinase C without invoking inositol-lipid turnover [49]. Our inability to demonstrate TRF-mediated mobilization of the recognized intracellular second messengers is therefore consistent with this apparent association between novel transduction mechanisms and certain types of growth factors. It is intriguing to also include the asialoglycoprotein receptor in this group of receptors and to speculate on its function in the terminally differentiated hepatocyte.

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