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 \(\beta\)-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 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 \(B_{\text{max}}\) was 14000 receptors/cell. In order to preclude possible

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

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

We have concluded that the endocytosis of asialo-glycoproteins and Fe\textsuperscript{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-galactosyl-bovine serum albumin (β-Gal-BSA) was prepared as previously described [23]. Fe\textsuperscript{3+}-TRF was prepared essentially by the method of Bates & Schlabach [24]. Briefly, Fe\textsuperscript{3+}-nitrito-tricatic acid at a nitrito-tricatic acid/iron ratio of 2:1 was rapidly mixed with apotransferrin (10 mg/ml, Behringwerke, Marburg, Germany) in 0.1 M-NaClO\textsubscript{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 $A_{280}/A_{365}$ ratio of 0.046.\textsuperscript{125}I-labelled ligands were prepared as described previously [23]. myo-[2\textsuperscript{3}H]-Inositol, [\textsuperscript{3}H]acetic anhydride, [\textsuperscript{3}H]P, \textsuperscript{45}Ca\textsuperscript{2+} as \textsuperscript{45}CaCl\textsubscript{2}, Na\textsuperscript{125}I and cyclic AMP-[\textsuperscript{125}I]Amerlex-M assay system were from Amershon 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) x 10\textsuperscript{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% 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 x 10\textsuperscript{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 \textsuperscript{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\textsuperscript{3+}-TRF respectively, was 2% for d-Gal-BSA and 35% for Fe\textsuperscript{3+}-TRF. Non-specific binding of Fe\textsuperscript{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, $k_t$, 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 $k_t$ for the internalization of Fe\textsuperscript{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 \textsuperscript{125}I-Fe\textsuperscript{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-nitrito-tricatic 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) Polysphosphoinositides and inositol phosphates. The possible breakdown of the polysphosphoinositides coincident with receptor-mediated endocytosis of d-Gal-BSA or Fe\textsuperscript{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 µCi of \textsuperscript{32}PJPP, 1 h at 37°C. Experiments were started by the addition of 0.5 µl 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\textsuperscript{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/acetone/2,2,4-trimethylpentane (9:2:5, \(v/v\)) 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/acetate/water (40:15:13:12:8, \(v/v\)) 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 \(\mu\)Ci of myo-[\(2-3^H\)]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 \(\mu\)l 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-HClO\(_4\). 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 \(\mu\)M-D-Gal-BSA or Fe\(^{3+}\)-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 \([\text{H}]\)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-[\(12^5\)]I-D-Gal-BSA or [\(12^5\)]Fe\(^{3+}\)-TRF. The estimation of \(k_e\) was as described above.

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

(ii) Calcium. (a) Cytoplasmic free Ca\(^{2+}\). Cytoplasmic free Ca\(^{2+}\) 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-Ca\(^{2+}\) and either 2 \(\mu\)M-phenylephrine, 2 \(\mu\)M-ADP, 10 \(\mu\)M-D-Gal-BSA or 1 \(\mu\)M-Fe\(^{3+}\)-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 Sirus 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 \(\mu\)M-Fe\(^{3+}\)-TRF were made exactly as described by Iacopetta et al. [33].

(b) Calcium flux. A portion (1 \(\mu\)Ci) of \(^{42}Ca\(^{2+}\) (10 \(\mu\)l) 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 \(\mu\)l aliquots of 2 mM-D-Gal-BSA, 2 mM-Fe\(^{3+}\)-TRF or 0.2 mM-A23187, either singly or in combination, to give final concentrations of ligand or ionophore of 100 \(\mu\)M, 10 \(\mu\)M and 1 \(\mu\)M 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-HClO\(_4\) 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 Fe\(^{3+}\)-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 \(\mu\)l aliquots of appropriate stock ligand so that the final concentration of glucagon was 10 nM and both D-Gal-BSA and Fe\(^{3+}\)-TRF were in the range 0.1–10 \(\mu\)M. 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-HClO\(_4\). 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-[\(12^5\)]I-D-Gal-BSA or [\(12^5\)]Fe\(^{3+}\)-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 \(\mu\)l 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 \(\mu\)M-Fe\(^{3+}\)-TRF to the extracellular medium. The \(k_e\) for Fe\(^{3+}\)-TRF uptake by HL60 cells was compared between unstimulated cells and cells that had been preincubated with 10 \(\mu\)M-forskolin for 10 min before the addition of ligand.

RESULTS

Analysis of ligand binding and uptake

The accurate measurement of \(K_d\), \(B_{\text{max}}\) 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 [\(12^5\)]I-Fe\(^{3+}\)-TRF taken up per cell over a 20 min period was at least 100-fold less than for [\(12^5\)]I-D-Gal-BSA, despite only an apparent 10-fold difference in reported \(B_{\text{max}}\) 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 [\(12^5\)]I-Fe\(^{3+}\)-TRF. Furthermore, the distribution of [\(12^5\)]I-Fe\(^{3+}\)-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 Fe\(^{3+}\)-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. $K_d$ was determined at 37°C as described in the Materials and methods section. The results are expressed as means ± S.E.M., with the number of separate determinations given in parentheses.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ligand used</th>
<th>$K_d$ (nm)</th>
<th>$B_{max}$ (no./cell)</th>
<th>$k_e$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes</td>
<td>$^{125}$I-D-Gal-BSA</td>
<td>1.72 ± 0.11 (26)</td>
<td>175,000 ± 8,900 (26)</td>
<td>0.21 ± 0.04 (13)</td>
</tr>
<tr>
<td>HL60</td>
<td>$^{125}$I-Fe$^{3+}$-TRF</td>
<td>4.4 ± 0.6 (6)</td>
<td>14,000 ± 1,200 (6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$^{125}$I-Fe$^{3+}$-TRF</td>
<td>5.6 ± 2.4 (6)</td>
<td>154,000 ± 23,000 (6)</td>
<td>0.10 ± 0.03 (4)</td>
</tr>
</tbody>
</table>

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 ± S.E.M. of three separate determinations.

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

Phosphatidylinositol (PI) mobilization was monitored in hepatocytes prelabelled with $^{[32P]}$orthophosphate under control conditions (a) or after stimulation with either 10 μM-D-Gal-BSA (b) or 30 μM-vasopressin (c). Samples were removed at given time intervals and $^{[32P]}$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 $^{[32P]}$phosphatidylinositol 4,5-bisphosphate could be demonstrated within 5 min of the addition of 30 μM-vasopressin to the cells (Fig. 2). By 5 min the cellular content of $^{[32P]}$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-
dependent increase, up to 30% above control value, in the amount of \[^{32}P\]phosphatidic acid released from pre-labelled cells by vasopressin could also be demonstrated (Fig. 3a). Under the same conditions there was a dose-dependent increase in the production of \[^{3}H\]inositol phosphates within 5 min of stimulating prelabelled hepatocytes with 1–100 nm-D-Gal-BSA or Fe\(^{3+}\)-TRF ( ●, ○) or by 1–100 nm-vasopressin alone or in combination with 10 \(\mu\)M-D-Gal-BSA or 10 \(\mu\)M-Fe\(^{3+}\)-TRF ( ●, □). The results are means ± S.E.M. for three experiments.

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

<table>
<thead>
<tr>
<th>Ligand added</th>
<th>Diacylglycerol content of hepatocytes (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>D-Gal-BSA (10 (\mu)M)</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>Fe(^{3+})-TRF (10 (\mu)M)</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Vasopressin (100 nm)</td>
<td>8.4 ± 1.2</td>
</tr>
<tr>
<td>+ D-Gal-BSA (10 (\mu)M)</td>
<td>8.1 ± 1.0</td>
</tr>
<tr>
<td>+ Fe(^{3+})-TRF (10 (\mu)M)</td>
<td>9.0 ± 1.4</td>
</tr>
</tbody>
</table>

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 \[^{32}P\]phosphatidylinositol 4,5-bisphosphate, generation of \[^{3}H\]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 \(\mu\)M) 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 \(\mu\)M 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 \(\mu\)M extracellular concentrations of D-Gal-BSA or Fe\(^{3+}\)-TRF failed to raise the resting signal. By contrast, 2 \(\mu\)M phenylephrine or 2 \(\mu\)M 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 \(\mu\)M 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 \(\mu\)M had no effect on quin 2 fluorescence in HL60 cells.

(b) Ca\(^{2+}\) flux. The equilibration of \(^{45}\)Ca\(^{2+}\) across the plasma membrane of isolated hepatocytes was not perturbed by the addition of up to 10 \(\mu\)M-D-Gal-BSA or Fe\(^{3+}\)-TRF to the extracellular medium. Influx of \(^{45}\)Ca\(^{2+}\) into this cell was demonstrated by the addition of the Ca\(^{2+}\) ionophore A23187 (1 \(\mu\)M) to the medium (Fig. 4b). A23187-mediated \(^{45}\)Ca\(^{2+}\) influx was not modified by the
Cytoplasmic free calcium in hepatocytes (a) was monitored by using the aequorin signal from individual prelabelled cells perfused sequentially with: A, 0.5 μM-Fe³⁺-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 ⁴⁰Ca²⁺ into hepatocytes (b) was measured in the presence of 10 μM-D-Gal-BSA or 10 μM-Fe³⁺-TRF (●) or 1 μM-A23187 with or without 10 μM-D-Gal-BSA or 10 μM-Fe³⁺-TRF (■). Results are means ± S.E.M. for three experiments.

(iii) Cyclic AMP. In the presence of the phosphodiesterase inhibitor IBMX, extracellular concentrations of up to 10 μM of D-Gal-BSA and Fe³⁺-TRF failed to elicit any change in the basal concentration of cyclic AMP in hepatocytes (Fig. 5a). Similar concentrations of Fe³⁺-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 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 Fe³⁺-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 $K_p$, $B_{\text{max}}$, and $k_c$ for D-Gal-BSA and Fe³⁺-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, [Ca$^{2+}$], coincident with receptor-mediated endocytosis of d-Gal-BSA and Fe$^{3+}$-TRF. In the hepatocyte, both ligands failed to raise [Ca$^{2+}$], 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 μm 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 (~1000 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 μm 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 [Ca$^{2+}$], was consistent with an earlier study on changes in [Ca$^{2+}$], during Fe$^{3+}$-TRF endocytosis by HL60 cells [33]. The authors demonstrated that raising or lowering [Ca$^{2+}$], 10-fold from the resting level had no significant effect on Fe$^{3+}$-TRF endocytosis. Our studies on [Ca$^{2+}$], 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 45Ca$^{2+}$ across the plasma membrane, nor was there any evidence of raised [Ca$^{2+}$], 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 $k_r$ 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
with 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 require-
ment 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 recep-
tors, 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 endocytic pathway could be suggestive of a regu-
latory 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 \( k_s \) for the uptake of asialoglycoproteins by hepat-
cytes 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 intra-
cellular pathway [47].) Therefore the continuous and
rapid turnover of the plasma membrane, or specialized
regions contained therein, would preclude the require-
ment for specific signals to trigger receptor-mediated
endocytosis. The occupation of a receptor would inevi-
tably 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 con-
trolling the subcellular distribution of receptors in
actively endocytosing cells remains its most likely
function.

Since this paper was originally submitted for publica-
tion 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 stimu-
lates the translocation of protein kinase C without
invoking inositol-lipid turnover [49]. Our inability to
demonstrate TRF-mediated mobilization of the recog-
nized intracellular second messengers is therefore con-
sistent with this apparent association between novel
transduction mechanisms and certain types of growth
factors. It is intriguing to also include the asialo-
glycoprotein receptor in this group of receptors and to
speculate on its function in the terminally differentiated
hepatocyte.

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