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Endocytosis of β-adrenergic ligands by rat liver

Comparison of β-adrenergic receptor and adenylate cyclase distribution in endosome and plasma-membrane fractions

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The internalization of β-adrenergic receptors was investigated in rat livers perfused with an agonist ([^{3}H]isoprenaline) or an antagonist ([^{125}I]iodocyanopindolol). Analytical centrifugation of liver homogenates indicated that the ligands were transferred rapidly to endosomal and lysosomal positions in sucrose gradients. Endosome fractions contained β-adrenergic binding sites, but adenylate cyclase activity was low and poorly activated by isoprenaline. The results indicate that the receptor–regulatory-protein–adenylate cyclase complex was disassembled during uptake of β-adrenergic ligands, with the adenylate cyclase being retained at the plasma membrane.

Adrenergic receptors and their subtypes have been identified and characterized pharmacologically in a variety of tissues, including liver (Schmelck & Hanoune, 1980; Dickinson & Nahorski, 1983; Harden, 1983). At the plasma membrane the β-adrenergic receptor, on occupation by an agonist, activates the catalytic component of adenylate cyclase via a guanine nucleotide-binding regulatory component that is believed to play a key role in signal transduction and the desensitization of cells to catecholamines (DeLean *et al.*, 1980; Tolkovsky *et al.*, 1982; Stadel *et al.*, 1983; Strulovici *et al.*, 1983).

In general, receptor–ligand complexes formed at the cell surface are internalized, and a variety of labelled ligands have been identified morphologically in membrane-bound vesicles in the cytoplasm (Pastan & Willingham, 1981; Geuze *et al.*, 1983). These vesicles, where ligand–receptor dissociation occurs in a low-pH environment (Tycko *et al.*, 1983; Geisow & Evans, 1984), emerge as important intracellular organelles termed 'endosomes', from where receptors and ligands are processed further along divergent pathways in the cell (Posner *et al.*, 1982; Ashwell & Harford, 1982; Hopkins, 1983). With regard to β-adrenergic-ligand complexes, it is unclear to what extent they are internalized and, if so, whether they maintain their functional association with the regulatory and catalytic subunit. In the present study we show that ligands which combine with plasma-membrane β-adrenergic receptors are rapidly internalized and are recovered in liver endosome fractions. These fractions contain β-adrenergic binding sites, but are depleted of adenylate cyclase activity.

Abbreviation used: p[NH]ppG, guanylyl imidodiphosphate.

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Materials and methods

Materials

DL-[7-^{3}H]Isoprenaline hydrochloride and (−)-3-[^{125}I]iodocyanopindolol were obtained from Amersham International, Amersham, Bucks., U.K. Other reagents were obtained from Sigma and British Drug Houses.

Analytical density-gradient centrifugation of liver homogenates

Female rats were injected via the portal vein with 0.5 ml of phosphate-buffered saline, pH 7.4 (Evans *et al.*, 1980), containing [^{3}H]isoprenaline (9.3ng, approx. 3×10^7 c.p.m.) or [^{125}I]-iodocyanopindolol (0.6ng, approx. 26×10^7 c.p.m.). At 2 and 10min later, livers were removed from the animals, blotted, weighed and then homogenized in 0.25mM-sucrose (3ml/g of tissue) by using ten strokes of a loose-fitting Dounce homogenizer (clearance 0.119mm). The homogenate was filtered through nylon bolting cloth and rehomogenized (six strokes) with a tight-fitting pestle (clearance 0.072mm). A portion (2ml) of the liver homogenate was layered on continuous 15–60% (w/v) sucrose gradients (15ml) and centrifuged for 4h at $97000g_{av.}$ in a Beckman SW27 rotor. The gradients were unloaded and frequency plots constructed (Beaufay & Amar-Costesec 1976).

Preparation of endosome and plasma-membrane fractions

Liver homogenate, prepared as described above from two rats, was centrifuged for 10min at 1000g and the pellet washed by repeating the centrifugation twice. The combined supernatants (100ml) were centrifuged at $33000g_{av.}$ for 8min in a Beckman type 30 rotor. The supernatant was collected and layered on top of 15ml continuous sucrose gradients (15–40%) underlaid with cushions of 43% (6ml) and 70% (1ml) sucrose. After centrifugation in a Beckman SW27 rotor for 4h at $97000g_{av.}$, fractions of density range 1.140–1.120 and 1.117–1.095g/cm^3, designated endosome fractions E and D respectively, were collected. After concentration of fractions by Amicon filtration, they were applied to a Sepharose 2B column (80cm × 1cm) and the turbid peak of membranes, eluted at the column void volume, was collected. Plasma-membrane fractions were prepared as previously described (Evans *et al.*, 1980).

Radioligand-binding assays

Fractions were diluted into 1ml of buffer (10mM-Tris/HCl, pH7.4, 0.154M-NaCl, 1.1mM-ascorbic acid) and incubated for 30min at 37°C with (−)-[^{125}I]iodocyanopindolol, and specific binding was determined as the amount of [^{125}I]-iodocyanopindolol bound in the presence of 0.25μM-(−)-propranolol (Engel *et al.*, 1981). Data analysis was performed with a non-linear least-squares computer program.

Adenylate cyclase activity and other marker enzymes

The standard incubation mixture contained 40mM-Tris/HCl, pH7.5, 5mM-$MgCl_2$, 1mM-EDTA, 0.5mM-isobutylmethylxanthine, 1mM-dithiothreitol, 0.1% bovine serum albumin, 10mM-ATP, 10mM-phosphocreatine, creatine kinase (13.2units/ml) and 5–10μg of membrane protein, and where indicated isoprenaline (1μM), p[NH]ppG (0.1mM) or NaF (10mM). Incubations (30min at 37°C) were stopped by the addition of $HClO_4$ (final concn. 0.5M) and, after neutralization with K_3PO_4 (final conc. 0.23M), cyclic AMP concentration was determined by radioimmunoassay (Steiner *et al.*, 1972; Harper & Brooker, 1975). 5′-Nucleotidase, alkaline phosphodiesterase, galactosyltransferase and acid phosphatase activities and protein were determined by standard methods (Evans, 1978).

Results

The uptake and subcellular processing of radiolabelled ligands bound to cell surfaces were followed by analytical density-gradient centrifugation of liver homogenates. Since this approach showed that various ligands internalized by liver were transferred rapidly to positions in the sucrose gradients of low or high density (Smith *et al.*, 1981; Debanne & Regoeczi, 1981; Evans *et al.*, 1983), it was applied to study the internalization of ligands that bind to β-adrenergic receptors (Harden, 1983).

Subcellular distribution of bound β-adrenergic ligands

The frequency distribution in liver homogenates separated in continuous sucrose gradients of [^{125}I]-iodocyanopindolol and [^{3}H]isoprenaline at 2 and 10min intervals after injection into the portal vein is shown in Fig. 1. Both ligands equilibrated in the low-density region (1.11–1.15g/cm^3) and the high-density regions (1.20–1.22g/cm^3). These density positions corresponded, respectively, to where endosome components (Debanne *et al.* 1982) and lysosome markers (Fig. 1) equilibrated. The density range 1.06–1.08g/cm^3 corresponded to the position where 'free' ligands that did not enter the sucrose gradient were located. Ligand peaks of various heights at density 1.16–1.18g/cm^3 probably indicated material bound to plasma-membrane fragments (Fig. 1). The ligands located in the low-density region of the sucrose gradient also overlapped with a peak of galactosyltransferase, a Golgi-apparatus marker (Fig. 1), but preparative subcellular fraction of ligand-containing vesicles of this density showed that they differed from conventional Golgi apparatus in several respects (Debanne *et al.*, 1982; Evans *et al.*, 1983). When two endosome fractions, D and E, were prepared, approx. 16% of [^{125}I]iodocyanopindolol and 12% of [^{3}H]isoprenaline in the liver homogenates were recovered in the combined fractions; these values were similar to those recorded for the recovery of asialotransferrin, prolactin and insulin in the endosome fraction isolated essentially by the same procedure (Evans *et al.*, 1983).

Subcellular distribution of β-adrenergic receptors in liver

Fig. 2 shows the distribution in a sucrose gradient of [^{125}I]iodocyanopindolol-binding components in the supernatant remaining after first pelleting nuclei, mitochondria, lysosomes, plasma membranes and Golgi cisternal elements from the homogenate (see the Materials and methods section). The two discrete peaks where cyanopindolol bound corresponded to the positions in the gradients where the endosome fractions D and E were recovered, and prompted an investigation of the receptor and adenylate cyclase content of these fractions.

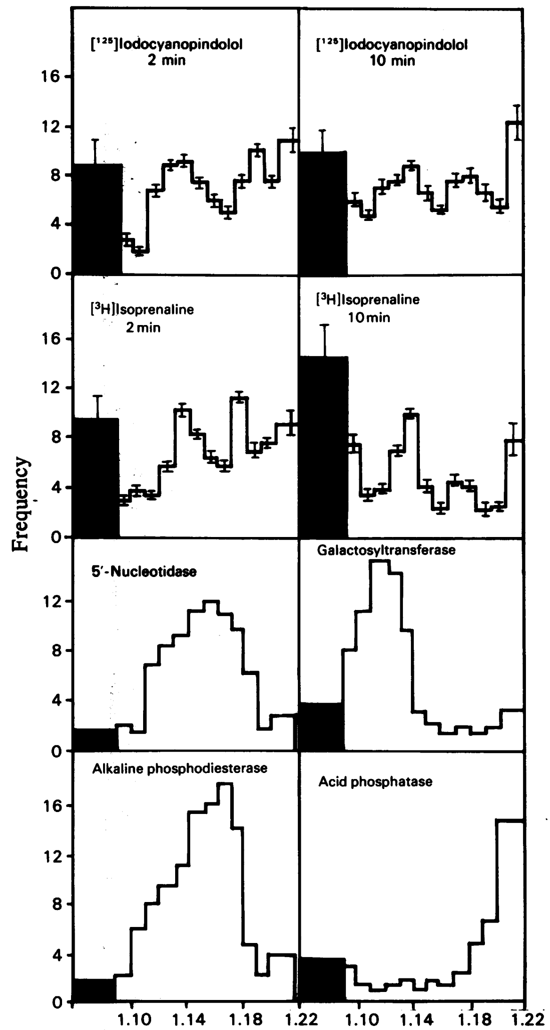

Fig. 1. *Sucrose-density-gradient fractionation of rat liver homogenates*

The frequency–density distributions of [^{125}I]-iodocyanopindolol and [^{3}H]isoprenaline in homogenates prepared 2 and 10 min after injection of radioisotopes and marker enzymes are shown. Results for the radioisotopes are means ± S.D. for three separate experiments. For further details see the Materials and methods section.

The specific binding of [^{125}I]iodocyanopindolol by endosome fractions D and E and by plasma-membrane fractions is shown in Fig. 3. Binding of ligand was saturable, and the maximum number of binding sites (fmol/mg of protein) and equilibrium dissociation constants (K_d) respectively were: plasma membrane, 226.6, 8.2 pM; fraction E, 92.1, 3.2 pM; fraction D, 42.2, 2.7 pM. The binding data could be fitted into a single-site mass-action equation, with the best fit obtained with plasma-membrane and E fractions and suggesting that a single receptor type was present. Although the results obtained with fraction D did not fit so well on the drawn curve, the results did not indicate multiple receptor types on these membranes.

Adenylate cyclase activities

The basal and ligand-stimulated adenylate cyclase activities of plasma membranes and endosome fractions D and E were investigated. Fig. 4 shows that plasma-membrane fractions contained

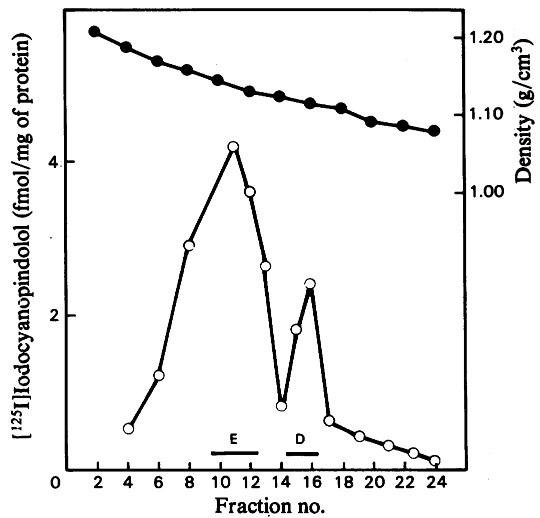

Fig. 2. *Distribution in sucrose gradients of [^{125}I]-iodocyanopindolol-binding sites*

The supernatant remaining after pelleting, from the liver homogenates, nuclei, mitochondria, lysosomes, plasma membranes and Golgi cisternal elements, was centrifuged into a continuous sucrose gradient. Points are means of duplicate determinations from two experiments. The positions in the gradients when endosome fractions D and E were recovered are indicated.

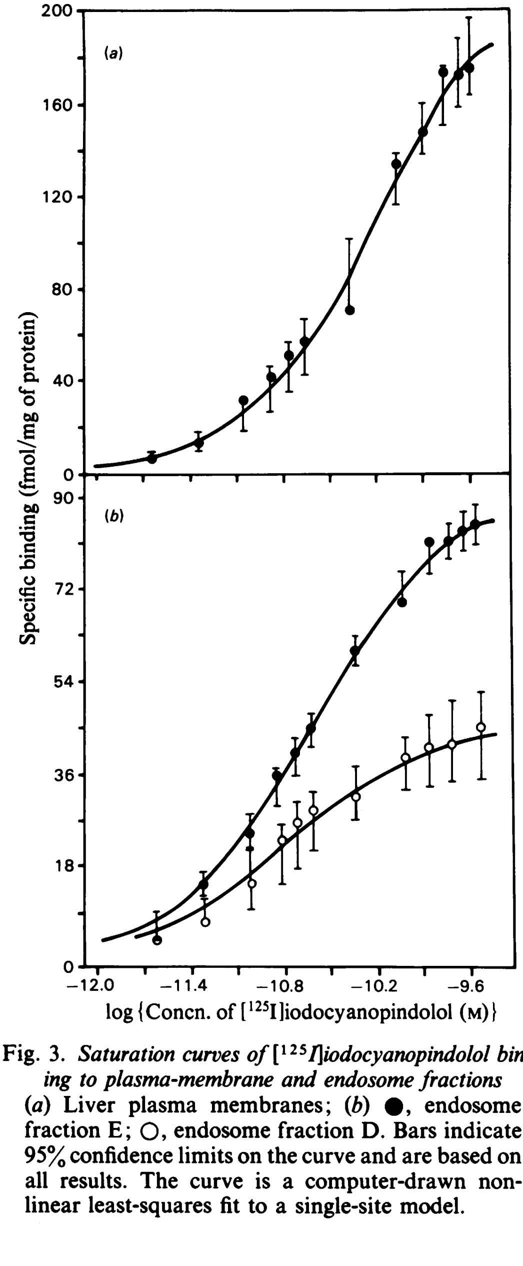

Fig. 3. *Saturation curves of [^{125}I]iodocyanopindolol binding to plasma-membrane and endosome fractions*
(*a*) Liver plasma membranes; (*b*) ●, endosome fraction E; ○, endosome fraction D. Bars indicate 95% confidence limits on the curve and are based on all results. The curve is a computer-drawn non-linear least-squares fit to a single-site model.

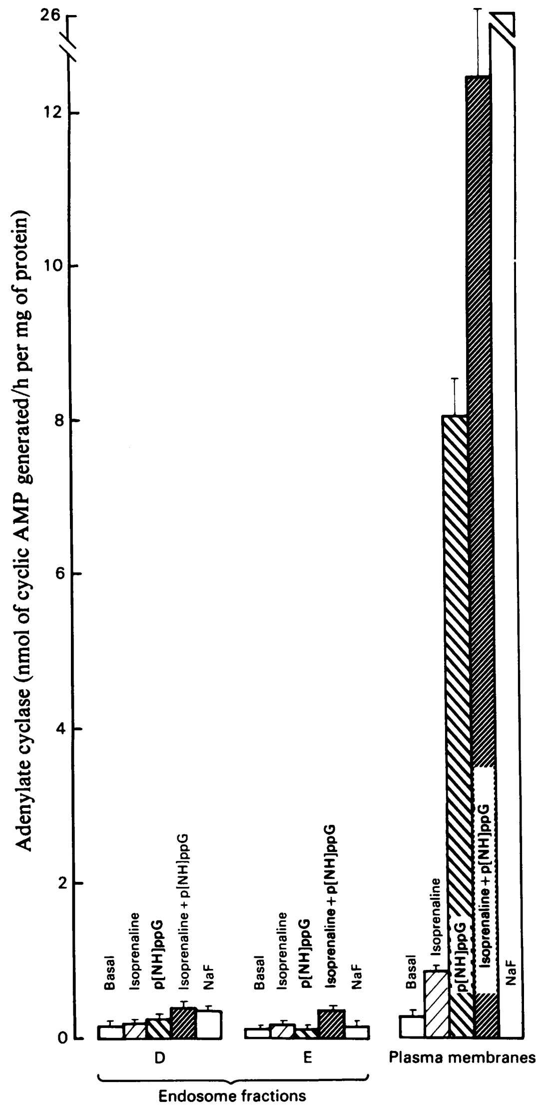

Fig. 4. *Adenylate cyclase activities of plasma-membrane and endosome fractions*
For further details see the Materials and methods section. The results are means ± S.D. of four experiments, each determined in triplicate.

an adenylate cyclase activity that was highly stimulated by isoprenaline, NaF and p[NH]ppG. In contrast, the isoprenaline-stimulated adenylate cyclase activities of the two endosome fractions were 15–18% of that of the plasma-membrane fractions, indicating that only low amounts of β-adrenergic-receptor-coupled adenylate cyclase activity were present. The NaF-stimulated activity in the endosomes was 2% of that in plasma membranes. Addition of p[NH]ppG to membranes in the presence of isoprenaline resulted in a further increase in activity in fractions D and E compared with basal activity, but these activities were low compared with that measured in plasma-membrane fractions.

These results allowed a rough comparison of the ratio of receptor number to adenylate cyclase activity in plasma-membrane and endosome fractions. The ratio of binding sites (calculated from Fig. 3) to adenylate cyclase activity (Fig. 4) was 5.8–14.4-fold higher in the endosomes than in plasma membranes when the enzyme was activated by isoprenaline and p[NH]ppG and 16.8–78-fold higher when the enzyme was activated by NaF. However, these ratios are probably underestimates, for many binding sites in the endosome vesicles would not be accessible to the ligand.

Discussion

The present studies describe the uptake by rat liver of two ligands that bind to cell-surface β-adrenergic receptors, and show by subcellular fractionation that they are transferred into endosomes and lysosomes. The endosome fractions were prepared and shown to contain β-adrenergic receptors, but only low amounts of adenylate cyclase activity. This leads to the conclusion that a dissociation of the receptor from the adenylate cyclase had occurred during endocytosis.

Studies on the binding and internalization of β-adrenergic ligands, with their implication for desensitization and receptor recycling, are confined mainly to avian and frog erythrocytes (Chuang *et al.*, 1980; Stadel *et al.*, 1983) and astrocytoma cells (Harden *et al.*, 1980; Waldo *et al.*, 1983). The present work, with liver, shows, by using analytical and preparative subcellular fractionation, that ligands that bound to β-adrenergic receptors were rapidly transferred into components of low density on sucrose gradients. These components have been shown to originate from the hepatocyte's endocytic networks, for various ligands, internalized after combining with plasma-membrane receptors, were concentrated in these fractions (Debanne *et al.*, 1982). The endosome fractions showed enzymic and morphological properties that distinguished them from plasma membrane, lysosome and, to a lesser extent, Golgi membranes, and a H^+-activated ATPase was shown to be present in these fractions, accounting for their ability to acidify in the presence of Mg^{2+}–ATP (Saermark *et al.*, 1984). Furthermore, the endosome fractions have a high lipid fluidity relative to the plasma membranes (Whetton *et al.*, 1983).

The binding properties of the receptors in the endosome fractions were similar to those of plasma membranes. By comparing the recovery, on a protein basis, of the number of binding sites in the endosome and plasma-membrane fractions, it was estimated that the ratio of cell-surface to intracellular receptors was 5:1. Since internalized receptors are located inside vesicles and are inaccessible to added ligand, the number of receptors in the endosome fraction is probably an underestimate. In frog erythrocytes, it was shown that approx. 50–60% of β-adrenergic receptors were in an intracellular location (Stadel *et al.*, 1983); in astrocytoma cells, approx. 95% of the β-adrenergic receptors were intracellular (Doss *et al.*, 1981). In liver, steady-state conditions apply with respect to cell-surface receptors, but, in frog erythrocytes and astrocytoma cells, receptor distribution was determined in desensitized cells, when presumably a greater proportion of the receptors would be in a cytosolic location. It is likely that the intracellular receptors now identified in liver endosome fractions consist of those internalized with bound ligands, together with a further pre-existing pool. These pools of intracellular receptors probably comprise components of the receptor-recycling mechanisms, operational in many animal cells, that allow fine control of the receptors located at the cell surface.

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