Association of turkey erythrocyte β -adrenoceptors with a specific lipid component

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We have recently reported that the highly potent β -adrenergic affinity label [¹²⁵I]bromoacetyl-amino cyanopindolol ([¹²⁵I]BAM-CYP) irreversibly blocks the turkey erythrocyte β -adrenoceptor binding site by combining with a receptor-associated non-protein component. In this communication, we report: (i) lipid labelling is inhibited by β_1 -adrenergic ligands with the potency ratio and stereospecificity characteristic for the turkey erythrocyte β_1 -adrenoceptor; (ii) the tagged component is a glycolipid, probably a ganglioside; (iii) [¹²⁵I]BAM-CYP-blocked receptor, after solubilization in deoxycholate, can be separated from the [¹²⁵I]BAM-CYP–glycolipid with restoration of the binding capacity of the β_1 -adrenoceptor protein; (iv) the tightly associated [¹²⁵I]BAM-CYP-labelled glycolipid can be displaced by a glycolipid mixture extracted from turkey erythrocyte membranes but not by bovine brain gangliosides, when the blocked receptor is solubilized in digitonin. This is the first direct demonstration that a receptor protein is associated with a specific membrane lipid. The possibility that glycolipids play a role in receptor-mediated signal transduction is discussed in view of these findings and in view of data from the literature.

Key words: β_1 -adrenoceptors/glycolipids/ganglioside/cyclase

Introduction

Transmembrane signalling involves the coupling of a receptor with a biochemical effector system within the phospholipid membrane matrix. Membrane proteins, including receptor systems, function best in certain combinations of phospholipids as compared with others. However, the role of specific lipids in the function of membrane enzymes or receptor/effector systems still remains largely elusive. Certain studies suggest that, for example, cholesterol is a specific requirement of the 'fine tuning' of the nicotinic acetylcholine receptor (nAChR) (Criado *et al.*, 1984). Recent reports indicate that gangliosides are associated with a variety of receptor systems and may play a role in the coupling between receptors and their biochemical effector systems.

Gangliosides may play a modulatory role in the stimulation of adenylate cyclase by thyroid stimulating hormone (TSH) (Lacetti *et al.*, 1983, 1984) and by serotonin (Dawson and Berry-Kravis, 1984; Berry-Kravis and Dawson, 1985). In both cases, it is suggested that gangliosides may be essential for the functional coupling between the receptors and the components of adenylate cyclase. Studies indicate ganglioside involvement in the modulation of mitogenic activity of epidermal growth factor

(EGF) and of platelet-derived growth factor (PDGF) on their specific receptors on Swiss 3T3 cells (Bremer *et al.*, 1984), and a role for gangliosides in the internalization of the transferrin receptor (Okada *et al.*, 1985). The specific association of interferon receptors with gangliosides has also been reported (Gupta *et al.*, 1984; Kusharynov *et al.*, 1983).

We have recently reported that a newly synthesized chemical affinity label for the β_1 -adrenoceptor, N-bromoacetyl-amino-methyl-cyanopindolol (BAM-CYP), irreversibly blocks the turkey erythrocyte β_1 -adrenoceptor without affecting the adenylate cyclase components. We have observed that although β_1 -adrenoceptor binding and function were irreversibly blocked, all the specific labelling was associated with a water-soluble lipid fraction and not with a protein. We have therefore hypothesized that the β_1 -adrenoceptor is tightly associated with a glycolipid such that it is in very close proximity to the β_1 -adrenoceptor binding site (Chorev *et al.*, 1985). In this study we clearly demonstrate that the β_1 -adrenoceptor subunit is not labelled by [¹²⁵I]BAM-CYP and we characterize the association of the turkey erythrocyte β_1 -adrenoceptor with the glycolipid.

Results

Affinity labelling with [¹²⁵I]BAM-CYP

Figure 1 contrasts [¹²⁵I]BAM-CYP (Chorev *et al.*, 1985) to the structurally similar β_1 -adrenergic photoaffinity label [¹²⁵I]CYP-diazirine (Burgermeister *et al.*, 1982). Although both affinity labels irreversibly block the β_1 -adrenoceptor (Chorev *et al.*, 1985; Burgermeister *et al.*, 1982) only the [¹²⁵I]CYP-diazirine can be shown to specifically label the turkey erythrocyte 43-kd β_1 -adrenoceptor subunit (Figure 2). The reaction with [¹²⁵I]BAM-CYP results in the irreversible blocking of the β_1 -adrenoceptor by labelling a non-protein component which is found in the aqueous phase of the Folch extraction (Chorev *et al.*, 1985).

Characterization of the [¹²⁵I]BAM-CYP-labelled material

Subsequent to the labelling of turkey erythrocytes with [¹²⁵I]BAM-CYP, the [¹²⁵I]BAM-CYP adduct can be extracted and separated from ¹²⁵I[−] and [¹²⁵I]BAM-CYP on reverse-phase KC-18 t.l.c. plates. Only one labelled glycolipid band can be identified (Figure 3). KC-18 t.l.c. plates were found to give maximal separation of the labelled glycolipid from ¹²⁵I[−] and [¹²⁵I]BAM-CYP. These separations could not be achieved using the silica t.l.c. system routinely used for the separations of glycolipids and gangliosides (Kundu, 1981).

When the labelled material is treated with *Vibrio cholerae* (phospholipase-free) neuraminidase, its migration on reverse phase t.l.c. plates is reproducibly modified, whereas proteinase K or other proteolytic enzymes (pronase, trypsin; data not shown) are without effect (Figure 3). The sensitivity of the [¹²⁵I]BAM-CYP-labelled glycolipid to neuraminidase suggests that it is a ganglioside. Also, upon extensive dialysis of the aqueous phase, one does not lose the labelled material suggesting its association with micelles of glycolipids or gangliosides.

Since the amount of [¹²⁵I]BAM-CYP adduct amenable to analysis is currently in the picomole range, we are as yet unable

AFFINITY LABELS DERIVED FROM CYP

Fig. 1. The structure of BAM-CYP (Chorev *et al.*, 1985) and CYP-diazirine (taken from Burgermeister *et al.*, 1982).

to further identify the chemical nature of the glycolipid. We are currently developing methods to scale up the production of [125 I]BAM-CYP adduct.

The pharmacology of [125 I]BAM-CYP labelling

To explore further the hypothesis that the glycolipid is tightly associated with the β_1 -adrenoceptor, we examined the potency of β_1 -adrenergic ligands in inhibiting the labelling of this glycolipid by [125 I]BAM-CYP. In Figure 4, it is shown that the pharmacologically active R (-) stereoisomers of β_1 -adrenoceptor ligands effectively inhibit the specific labelling of the glycolipid with [125 I]BAM-CYP. The ligand potency ratio and stereospecificity is characteristic for the turkey erythrocyte β_1 -adrenoceptor (Levitzki *et al.*, 1975). Phenylephrine, which is a α_1 -adrenergic agonist and a very weak partial β_1 -agonist in the turkey erythrocyte system (Levitzki *et al.*, 1975; Arad and Levitzki, 1979), is very ineffective. The α_1 -adrenergic blocker, prazosin, and the α_2 -adrenergic blocker, yohimbine, are without effect (data not shown). Under the experimental conditions used, > 80% of the incorporated [125 I]BAM-CYP is found on the specifically labelled glycolipid.

Displacement of the [125 I]BAM-CYP-glycolipid from its complex with the receptor

The specifically labelled [125 I]BAM-CYP can be displaced from the digitonin-solubilized receptor by a water-soluble lipid fraction extracted from the turkey erythrocyte membranes but not by a mixture of bovine brain gangliosides (Figure 5)

Reconstitution of the blocked β_1 -adrenoceptor

In view of these findings which demonstrate a tight complex between the β_1 -adrenoceptor and a glycolipid, we attempted to separate the β_1 -adrenoceptor protein from the [125 I]BAM-CYP-labelled glycolipid and reconstitute the binding activity of the receptor. For these experiments, we first labelled the membranes with a low concentration (0.2 nM) of [125 I]BAM-CYP to obtain minimal non-specific binding, and then treated the membranes with a high concentration (10 nM) of non-radioactive BAM-CYP to completely block receptor binding. Figure 6A shows that when the [125 I]BAM-CYP-labelled turkey erythrocyte membranes are solubilized in deoxycholate and the proteins delipidated by chromatography on an Aca34 Ultrogel permeation column (Kirilovsky and Schramm, 1983), the β_1 -adrenoceptor binding activity can be quantitatively restored upon re-lipidation with soybean lipids. The reconstituted binding activity appears in two peaks: at ~40 kd and 170 kd. These two peaks most probably represent the β_1 -adrenoceptor subunit (Atlas and Levitzki, 1978)

Fig. 2. Labelling of turkey erythrocyte membranes with [125 I]BAM-CYP and [125 I]CYP-diazirine. Membranes were labelled with [125 I]BAM-CYP as described (Chorev *et al.*, 1985) at a membrane concentration of 1 mg/ml and [125 I]BAM-CYP at 1.0 nM. Briefly, the membranes were suspended in 30 mM phosphate buffer, pH 7.8 and 1 mM MgCl_2 (PM buffer) and incubated with [125 I]BAM-CYP for 30 min at 30°C. The membranes were diluted with 10 volumes of PM buffer and centrifuged at 23 000 g for 20 min at 25°C. The pellets were then washed twice more with 10 volumes of PM buffer. Membranes were labelled with [125 I]CYP-diazirine as described by Burgermeister *et al.* (1982). d,l-Propranolol, when used, was 10 μM . The final pellet was suspended in sample buffer and electrophoresed according to Laemmli (1970), on a 7.5–15% polyacrylamide gradient gel.

Fig. 3. Identification of the [125 I]BAM-CYP-labelled glycolipid on reverse-phase RP-18 t.l.c. plates. Autoradiograph of a t.l.c. of the aqueous fraction of a Folch extraction of [125 I]BAM-CYP-labelled turkey erythrocyte membranes. 1 mg of membranes were labelled with 1.0 pmol of [125 I]BAM-CYP as described earlier (Chorev *et al.*, 1985). 315 fmol were specifically bound and 605 ± 10 fmol were recovered in the supernatant. In the presence of 1.0 μ M d,l-propranolol, irreversible labelling was 71 ± 5 fmol/mg where the remaining radioactivity was recovered in the supernatant. The labelled membranes were extracted as described (Chorev *et al.*, 1985) and the aqueous phase was lyophilized under vacuum centrifugation, resuspended in methanol, clarified by centrifugation and dried again under argon. The untreated and neuraminidase-treated Folch aqueous fractions were prepared as follows. The precipitate was resuspended in 50 mM acetate buffer, pH 5.8, 150 mM NaCl, 1.0 mM CaCl_2 and, where indicated, 0.01 unit/ml neuraminidase added. The mixture was incubated for 60 min at 37°C, dried under argon, resuspended in methanol and chromatographed on RP-18 t.l.c. plates in 70% ethanol–30% 1 mM HCl. The proteinase K-treated Folch aqueous fractions were prepared as follows. The precipitate was resuspended in 10 mM Tris, pH 7.4, 1 mM EDTA, and 10 μ g/ml proteinase K. The reaction mixtures were incubated for 3 h at 37°C and chromatographed as above. Unt., untreated; Prase K, proteinase K-treated; Neudase, neuraminidase-treated.

and an oligomeric species of the receptor which we have noted previously (Feder *et al.*, 1984). The [125 I]BAM-CYP-labelled glycolipid migrates on the column as a broad peak, probably because it is associated with phospholipid/detergent mixed micelles. The incorporation of the [125 I]BAM-CYP–glycolipid into the soybean lipid-reconstituted material is negligible.

Figure 6B shows that AcA34 chromatography followed by SM-2 treatment in the presence of soybean lipids (asolectin) restores >80% of the binding capacity of the BAM-CYP-treated β_1 -adrenoceptor. Seventy percent of the [125 I]BAM-CYP-labelled glycolipid remains in solution subsequent to SM-2 reconstitution and ~30% remains absorbed to the beads. The remaining 125 I in the SM-2-reconstituted fractions was <10% of the specific

[125 I]-CYP binding measured in these fractions. Treatment of the [125 I]BAM-CYP-treated deoxycholate (DOC)-solubilized receptor with asolectin and then SM-2 biobeads, without prior chromatography on an AcA34 column, did not restore binding activity (Figure 6B). We attempted to use other detergents such as digitonin and sodium cholate for reconstitution but these were found to be ineffective (data not shown).

Discussion

Lipid requirements for the β -adrenoceptor

Among the receptors which stimulate adenylate cyclase, the β_1 -adrenoceptors have been the most investigated, mainly the turkey erythrocyte β_1 -adrenoceptor (Levitzki, 1982, 1986) and the frog erythrocyte β_2 -adrenoceptor (Lefkowitz *et al.*, 1983). The β_1 -adrenoceptor has recently been purified from a number of sources (Feder *et al.*, 1984; Shorr *et al.*, 1982; Homcy *et al.*, 1983; Cubero and Malbon, 1984) and functionally reconstituted with purified GTP stimulatory protein G_s (N_s) (Brandt *et al.*, 1983; Cerione *et al.*, 1984a, b) as well as with the catalytic unit (Cerione *et al.*, 1984b; May *et al.*, 1985; Feder *et al.*, 1986). Although the phospholipid specificity of the β -adrenoceptor to G_s coupling has not been extensively investigated for the purified receptor, certain phospholipid combinations were found to be preferred in the reconstitution of β_1 -adrenoceptor binding activity from the turkey erythrocyte (Kirilovsky and Schramm, 1983; Keenan *et al.*, 1982) and for the functional coupling between crude turkey erythrocyte β_1 -adrenoceptor with crude G_s from the same source (Kirilovsky *et al.*, 1985). It remains to be established whether glycolipids play a role in the functional coupling between the β -adrenoceptor and the components of adenylate cyclase or have another role such as receptor stabilization and maintenance within the bilayer.

Tagging of the associated lipid and its characteristics

Among the numerous β -adrenoceptor affinity labels which were synthesized in the past decade (Atlas and Levitzki, 1978; Atlas *et al.*, 1976; Pitha *et al.*, 1980; Lavin *et al.*, 1982; Rashidibaigi and Ruoho, 1981; Burgermeister *et al.*, 1982), one other affinity label (Pitha *et al.*, 1980) was also reported not to label a protein although it irreversibly blocked the β_2 -adrenoceptor binding site of pigeon erythrocytes. The authors observed that most of the radioactively-labelled material migrated as low mol. wt. components separated from the protein components in sucrose density gradients. It is possible that the bromoacetyl derivative of the β -blocker alprenolol, used by Pitha *et al.*, (1980), also labels a lipid moiety strongly associated with the receptor.

We do not postulate an affinity between the ligand (BAM-CYP in this case) and a glycolipid, as was hypothesized for the case of the TSH receptor (Lacetti *et al.*, 1984). Alternatively, we suggest that the β -adrenoceptor may have preferential affinity for a glycolipid such that the reactive bromoacetyl moiety of the BAM-CYP may, coincidentally, covalently label the glycolipid when the ligand binds to the receptor. The likely groups to be attacked by the active bromine are hydroxyl and carboxyl groups within the sugar residues or the double bond within the sphingosine moiety. The sensitivity of the [125 I]BAM-CYP–glycolipid to neuraminidase suggests the presence of a sialic acid residue. This finding suggests that the glycolipid is a ganglioside. Neuraminidase treatment converts the [125 I]BAM-CYP-labelled lipid to a compound which migrates more slowly on reverse phase t.l.c. This is expected of a compound from which a hydrophilic residue (sialic acid) has been removed. The neuraminidase used is phospholipase C-free and phospholipase C treatment does not

Fig. 4. Inhibition of [125 I]BAM-CYP–glycolipid formation by β -adrenergic ligands. Turkey erythrocyte membranes (0.2 mg/ml) were incubated with the appropriate adrenergic ligand and 0.2 nM [125 I]BAM-CYP for 30 min at 30°C in 30 mM phosphate buffer, pH 7.8, and 1 mM MgCl_2 (PM buffer). The reaction mixture was diluted with 10 volumes of PM buffer and centrifuged for 15 min at 27 000 g . The pellet was resuspended in 10 volumes of PM buffer, incubated for 30 min at 30°C and re-centrifuged as above. This wash was repeated, and the [125 I] radioactivity counted in a gamma counter. Each point represents the average of triplicate determinations where the standard error of the mean ranges between 5 and 7%. Under the experimental conditions used, >80% of the total label incorporated is found in the specific glycolipid fraction. Control labelling (100%) was 135 fmol/mg protein. At higher concentrations of [125 I]BAM-CYP non-specific labelling of both protein (Figure 2) and non-protein components increases to 35% (see also Chorev *et al.*, 1985).

Fig. 5. Displacement of the [125 I]BAM-CYP–glycolipid from its protein associated state by turkey erythrocyte glycolipids and by brain gangliosides. Crude glycolipids were prepared by Folch extraction (Christie, 1982) of turkey erythrocyte membranes with chloroform:methanol:HCl 2:1:0.01 and partition against 1/4 volume 0.2 M KCl as described (Chorev *et al.*, 1985). The aqueous fraction was separated, dialysed in a spectrophor dialysis membrane with a 12 000 mol. wt cut-off for 36 h at room temperature and lyophilized. The lyophilizate was resuspended in methanol and stored at -20°C under argon. Prior to use, the methanol was dried under argon and the water-soluble lipids resuspended in the appropriate buffer. Membranes were labelled as described in Figure 2, except that the final wash was in 10 mM Tris, pH 7.4 and 90 mM NaCl (90/10 buffer). The final pellet was resuspended to 2.5 mg/ml membrane protein in 1% digitonin in 90/10 buffer, and incubated for 2 h at 4°C with constant agitation. The suspension was then centrifuged for 30 min at 200 000 g and the supernatant (solubilized receptor) removed. Aliquots of the labelled solubilized receptor, containing ~ 6000 c.p.m., were incubated in 90/10 buffer plus 10 μM propranolol containing the indicated amount of the aqueous fraction. The final volume was 1 ml, and the reaction was started by the addition of the crude ganglioside fraction. At the indicated time points, 150 μl of the mixture was removed and filtered through polyethyleneimine (PEI)-treated GF/B glass filters (Bruns *et al.*, 1983). The retained radioactivity was measured in a gamma-counter. The data points represent the average of triplicate determinations where the standard error of the mean was 7–10% of the measured value. The 10 μM propranolol prevents the rebinding of the displaced [125 I]BAM-CYP–glycolipid.

RECONSTITUTION OF β -ADRENERGIC BINDING ACTIVITY SUBSEQUENT TO BAM-CYP LABELLING

Fig. 6. Dissociation of the β_1 -adrenoceptor protein from its [125 I]BAM-CYP-labelled glycolipid and the restoration of its binding activity. (A) turkey erythrocyte membranes (1 mg/ml) were incubated with 0.2 nM [125 I]BAM-CYP for 30 min at 30°C in 30 mM phosphate buffer, pH 7.8 and 1.0 mM MgCl₂ (PM buffer). The reaction mixture was diluted with 10 volumes of PM buffer and centrifuged for 15 min at 27 000 g. The pellet was resuspended in 1 volume of PM buffer and non-radioactive BAM-CYP was added to 10 nM, and incubated for 30 min at 30°C. The reaction was diluted with 10 volumes of PM buffer and centrifuged as above. The pellet was washed twice in PM buffer as described in Figure 2. Control membranes went through all manipulations without [125 I]BAM-CYP and BAM-CYP treatment. The membrane pellet was resuspended to 2 mg/ml, and solubilized in 0.6% sodium deoxycholate. The equivalent of 150 fmol of solubilized receptor was loaded on an Ultragel AcA 34 column (10 ml, 0.5 cm $\times$ 6.5 cm), run at 15 ml/h, and 0.75 ml fractions collected. Soybean lipids were immediately added to each fraction to a final concentration of 0.6 mg/ml and the fractions treated with 1.2 g of freshly prepared (Keenan *et al.*, 1982) SM2 biobeads/1.0 ml solution. All procedures were carried out at 4°C. Binding activity of each fraction was then monitored using 150 pM [125 I]CYP as described (Keenan *et al.*, 1982). Data are shown as the average of triplicate determinations plus the standard error of the mean. Typically, 35–40% of the control DOC-solubilized receptor activity was recovered from the AcA 34 column. (B) AL: affinity-labelled AL + DOC/ASO + SM2: affinity-labelled treated sequentially with DOC soybean lipids ('Asolectin') and SM2 biobeads but without chromatography. AL + DOC/ASO + CHROM + SM2 affinity-labelled membranes solubilized in deoxycholate and chromatographed on an AcA 34 Ultragel column in the presence of DOC and then treated with soybean lipids and SM2 biobeads. The results indicate recovery of binding summed across chromatographed fractions.

have any effect on the chromatographic behaviour of the [125 I]BAM-CYP-labelled material (data not shown). Also, proteolytic enzymes such as proteinase K (Figure 3), pronase or trypsin were found not to have any effect on the migration of the labelled glycolipid (data not shown).

The generality of the finding

The finding that the turkey erythrocyte β_1 -adrenoceptor is tightly associated with a glycolipid, probably a ganglioside, and the evidence that other receptors linked to adenylate cyclase (Lacetti *et al.*, 1983, 1984; Dawson and Berry-Kravis, 1984; Berry-Kravis and Dawson, 1985) as well as receptors for EGF and PDGF (Bremer *et al.*, 1984) transferrin (Okada *et al.*, 1985) and interferon (Gupta *et al.*, 1984; Kusharynov *et al.*, 1983) are also associated with gangliosides raises interesting possibilities. Thus these complex glycolipids may function in transmembrane signalling, or receptor stabilization and maintenance within the membrane bilayer.

The observation that gangliosides from bovine brain are unable to displace [125 I]BAM-CYP–glycolipid from the β_1 -adrenoceptor but turkey erythrocyte glycolipids are effective in that capacity (Figure 5) suggests that different receptors associate with specific glycolipids or gangliosides. Indeed, recently Okada *et al.* (1985) have provided strong evidence for the association of the transferrin receptor with a specific ganglioside.

Our aim at present is to establish the identity of the glycolipid

labelled with [125 I]BAM-CYP and examine the possible role of glycolipids in the reconstitution of the purified β -adrenoceptors with the purified components of adenylate cyclase.

Materials and methods

Materials

Cyanopindolol was obtained from Dr. G.Engel (Sandoz, Basel), d- and l-propranolol were from ICI, UK, d- and l-soterenol were from Mead Johnson. Sodium deoxycholate, sodium cholate and soybean lipids (asolectin) were from Sigma (St. Louis, MO) and Ultragel AcA 34 was obtained from LKB (Sweden). *V. cholerae* neuraminidase (phospholipase C-free) was from Serva. SM-2 biobeads were obtained from BioRad. All other chemicals were of the highest chemical purity available and usually obtained from Sigma. Silica t.l.c. plates were obtained from Merck (Darmstadt) and reverse phase (KC-18) plates from Whatman. Organic chemicals and solvents were obtained from Aldrich, Sigma or Fluka and were of the highest purity available. BAM-CYP and [125 I]BAM-CYP (figure 1) were synthesized as described by Chorev *et al.*, 1985).

Methods

Purified turkey erythrocyte membranes were prepared as described (Steer and Levitzki, 1975). Protein was determined according to Lowry *et al.* (1951) and cAMP according to Salomon *et al.* (1974). Hexose content of the glycolipid was determined by the anthrone reaction, using glucose as a standard (Yamamoto and Rouser, 1970). [125 I]Cyanopindolol was prepared as described (Engel *et al.*, 1981) and receptor binding assays were performed as previously described (Chorev *et al.*, 1985).

Special techniques

The specialized techniques used in this study are described in the legends to the figures.

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