MODULATION OF β-ADRENERGIC AGONIST BINDING BY GUANYLNUCLEOTIDES IN AVIAN ERYTHROCYTES

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1. Introduction

The first adenylate cyclase-linked receptor showing a decreased affinity due to the presence of guanine nucleotides, was the glucagon receptor of rat liver plasma membranes [1]. Guanine nucleotide-dependent modulation of agonist binding to a β-adrenergic receptor, was initially shown in plasma membranes from cultured rat glioma cells (C6) [2], and has since been observed in membranes from frog erythrocytes [3], and other cells [4,5]. Guanine nucleotide modulation has also been found for receptors [6–8], which on interaction with adenylate cyclase results in inhibition. Although, the dependence on guanine nucleotides for β receptor-mediated activation of adenylate cyclase [9–11] in avian erythrocyte membranes is well documented, the binding of agonists to the β receptor has been reported to be unaffected by guanine nucleotides [12–14]. To explain the absence of guanine nucleotide-mediated receptor modulation, and the lack of agonist-dependent desensitization of adenylate cyclase, it has been proposed that avian erythrocyte membranes may lack an additional GTP binding protein present in other cells [15]. This study aimed to clarify these controversial points.

2. Materials and methods

2.1. *Materials*

ATP, GTP, GDP, GMP, cAMP, Gpp(NH)p, GTPγS, Gpp(CH_2)p creatine kinase, creatine phosphate were purchased from Boehringer, Mannheim. (±)Propranolol was from Sigma, and L-isoproterenol was obtained from EGA Chemie, Steinheim. GDPβS was a gift from Dr F. Eckstein, Göttingen, phentolamine and (±)-HYP were generously supplied by Eli Lilly Co. and Dr F. Hauser, Sandoz AG, respectively. [α-^{32}P]-ATP (8–15 Ci/mmol) and c[^{3}H]AMP were from the Radiochemical Center, Amersham and (±)-[^{125}I]IHYP was obtained from New England Nuclear. All other chemicals were of the highest purity available. Doubly distilled water was used throughout.

Preparation of both pigeon and turkey membranes was essentially as in [9], omitting DTT*. Prior to lysis, the erythrocytes were resuspended (25% haemotocrit) and incubated in a Krebs phosphate buffer (Ca^{2+} free) containing 5 mM glucose, 0.1 mg/ml streptomycin, 0.1 mg/ml penicillin, 150 μg/l aprotinin for 1 h at 37°C. The membranes were stored in liquid N_2 in a buffer containing 20 imOsm. phosphate, 150 mM NaCl, 3 mM $MgSO_4$, 1 mM EDTA (pH 7.4) (buffer A). Protein was measured according to [16] using bovine serum albumin as standard.

2.2. *Adenylate cyclase*

Adenylate cyclase activity was measured as in [17] and c[^{32}P]AMP isolated according to [18].

2.3. *β-adrenergic receptor*

Binding of [^{125}I]IHYP to pigeon or turkey erythrocyte membranes was measured according to [2]. Pigeon or turkey erythrocyte membranes (20–50 μg) were incubated with 70–120 pM [^{125}I]IHYP in

Abbreviations: Gpp(NH)p, guanosine 5′-(β, γ-imino) triphosphate; Gpp(CH_2)p, guanosine 5′-(β, γ-methylene) triphosphonate; GTPγS, guanosine 5′-(3-thiotriphosphate); GDPβS, guanosine 5′ (2-thiodiphosphate); IHYP, iodohydroxypindolol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol

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* DTT was omitted throughout the membrane preparation and also for measurement of receptor binding and adenylate cyclase activity, since it interfered with binding of [^{125}I]-IHYP to membranes

250 μl total vol. buffer A containing 10 μM phentolamine [19] in the presence and absence of appropriate concentrations of L-isoproterenol and nucleotides for 6 min at 37°C. Specific binding (~80–90% total) was defined as the difference in amount of [^{125}I]-IHYP bound in the absence and presence of 20 nM HYP. The incubation was terminated by dilution with 1 mL 20 mM phosphate, 1 mM $MgSO_4$ (pH 7.4) containing 0.1 mM (D,L)-propranolol, followed by vacuum filtration through a Whatman GF/C filter. The reaction vessel was washed with a further 1 ml of the same solution and the filter rapidly washed with 25 ml of the same buffer (propanalol omitted). All solutions were maintained at 37°C. The filters were placed in a Triton X-100–toluene-based scintillant and the bound [^{125}I]IHYP measured in a β-scintillation counter with an efficiency of 50%. Binding experiments were performed in duplicates. Purity and recovery of nucleotides on exposure to membranes were checked by thin-layer chromatography on PEI-cellulose sheets developed in 0.75 M LiCl.

3. Results

Typical results demonstrating guanine nucleotide-dependent modulation of L-isoproterenol binding to both turkey and pigeon erythrocyte membranes are shown in fig.1. It can be seen that the shift in the [^{125}I]IHYP displacement curve to higher isoproterenol concentrations, induced by the presence of 10 μM

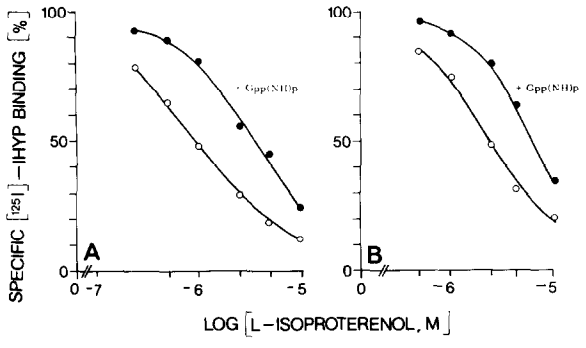

Fig.1. Modulation by Gpp(NH)p of L-isoproterenol binding to pigeon (A) and turkey (B) erythrocyte membranes. Displacement of [^{125}I]IHYP-binding in the presence (●——●) and absence (○——○) of 10 μM Gpp(NH)p was measured with 40 μg membrane protein and 147 pM (±)-[^{125}I]IHYP (pigeon) and 50 μg membrane protein and 250 pM (±)-[^{125}I]-IHYP (turkey) as in section 2.

Table 1
Effect of various nucleotides on displacement by L-isoproterenol of specific [^{125}I]IHYP-binding to pigeon erythrocyte membranes

Nucleotide (μM)		Shift of EC_{50} for L-isoproterenol alone (-fold)
Gpp(NH)p	10	3.6
GTP	0.1	2.5
GTP	10	3.7
GDP[a]	1	3.55
GMP	1000	2.9
Gpp(CH_2)p	80	3.2
GTPγS	0.1	3.65
GTPβS	100	3.6
ATP	1000	2.2

[a] GDP was degraded by <5% (to GMP) under the conditions applied

Binding experiments were done with the same batch of membranes (35 μg/assay) as in section 2.

GppHNp, is about the same for membranes from either species, i.e., for pigeon erythrocyte membranes, the EC_{50} for L-isoproterenol is shifted from 1.05–3.5 μM, and for turkey membranes from 2.5–7.1 μM. The magnitude of this shift varies for different membrane preparations and is dependent on the nature and concentration of the nucleotide. For pigeon erythrocyte membranes, the observed shift corresponds to a change in the app. K_d for L-isoproterenol from 0.6–2.1 μM, based on a K_d for (–)-[^{125}I]IHYP of 120 pM, measured by equilibrium binding. The K_{act} for L-isoproterenol and pigeon erythrocyte adenylate cyclase in the presence of 10 μM GTP, is 2 μM. In contrast to guanosine triphosphates and its analogues GDP and its derivatives, although tightly bound, inhibit adenylate cyclase [11,20,21]. We have therefore studied the ability of various nucleotides to affect L-isoproterenol binding in pigeon erythrocyte membranes. Table 1 indicated that guanine nucleotide modulation of β-receptor affinity exhibits specificity which is different from that for adenylate cyclase activation. The partial efficacy of ATP to cause modulation must be viewed with caution, as, although no regenerating system was included, the possibility of trace contaminants of guanine nucleotides which could be converted to GDP or GTP, by membrane-bound phosphotransferases cannot be excluded.

Using plasma membranes from S49 lymphoma cells, it was demonstrated [5] that, unlike activation

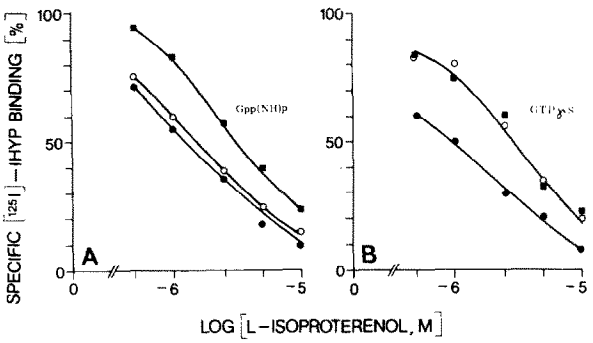

Fig.2. Binding of L-isoproterenol to pigeon erythrocyte membranes: reversibility of nucleotide modulation. Membranes (2 mg/ml) in buffer A were preincubated with 10 μM L-isoproterenol and with (○,■) or without (●) 10 μM Gpp(NH)p or 1 μM GTPγS at 37°C for 10 min. Membranes were washed 3 times with 10 vol. buffer A at 37°C. Binding of [^{125}I]IHYP in the presence of L-isoproterenol was done with 40 μg membrane protein as in section 2. With membranes pretreated with nucleotide, binding was done in the absence (○–○) and presence (■–■) of 10 μM Gpp(NH)p. (A) Membranes preincubated in the presence (○,■) or absence (●) of 10 μM Gpp(NH)p. (B) Membranes preincubated in the presence (○,■) or absence (■) of 1 μM GTPγS.

of adenylate cyclase by GppNHp, modulation of agonist binding induced by the same ligand could be reversed on washing the membranes, thus inferring some uncoupling of the two events. Using pigeon erythrocyte membranes and Gpp(NH)p, we were able to confirm these observations (fig.2A). Following extensive washing, which did not affect adenylate cyclase activation, the EC_{50} for isoproterenol displacement of [^{125}I]IHYP returned to that observed in the absence of nucleotide. Readdition of 10 μM Gpp(NH)p reestablished full modulation of agonist binding. When a similar washout experiment was carried out with 1 μM GTPγS, persistent activation of adenylate cyclase was found again. However, in contrast to Gpp(NH)p the effect of GTPγS on agonist binding persisted likewise (fig.2B), and subsequent readdition of 1 μM GTPγS had no further effect.

This is in keeping with other differences in response of avian erythrocyte membranes to Gpp(NH)p and GTPγS. For example, it is possible to reverse the Gpp(NH)p activation of adenylate cyclase by incubation with L-isoproterenol and GTP or GDP [22], but it is not possible to deactivate the GTPγS-activated enzyme [22]. Similarly the GTPase activity associated with the activation process is essentially irreversibly blocked by GTPγS, but not by Gpp(NH)p [23].

The extent of modulation by GTPγS is concentration dependent (when the nucleotide is present in the binding assay). The concentration of GTPγS which induces a half-maximal change in EC_{50} for isoproterenol, is ~10 nM. When membranes were exposed to 3 nM–1 μM GTPγS in the presence of 5 μM isoproterenol, and subsequently washed, receptor modulation only persisted at saturating concentrations of GTPγS (100 nM) when adenylate cyclase is fully activated. At subsaturating concentrations of GTPγS, or at saturating (1 μM) GTPγS concentrations but in the absence of hormone, on removal of nucleotide the receptor reverts to or remains in its high affinity state. (It should be noted, that pigeon erythrocyte adenylate cyclase may be activated by GTPγS alone to 65–80% of maximal activity obtainable.)

β-Adrenergic stimulation of adenylate cyclase in turkey erythrocyte membranes has been shown to strongly depend on temperature. The temperature dependence was interpreted to indicate the necessity of a 'fluid membrane' for activation [24,25]. In table 2 temperature dependencies of L-isoproterenol binding in the presence and absence of GTPγS and of activation of adenylate cyclase are compared. The shift of EC_{50} of isoproterenol-mediated displacement of IHYP in the presence of the nucleotide is progressively reduced with decreasing temperature and finally disappears at 17°C. The lack of modulation by the nucleotide at 17°C could be due to a retarded binding of GTPγS at the lower temperature. However membranes pretreated at 37°C with saturating concentrations of both nucleotide and L-isoproterenol and then assayed for binding at 17°C, likewise failed to show an EC_{50} value different from that of non-treated membranes.

The most likely explanation therefore is that at 17°C the receptor exists in the low affinity (uncoupled) form, irrespective of the presence of nucleotides. As the temperature is raised to 27°C and 37°C more and more of the high affinity (coupled) state is formed. The failure of the β-adrenergic receptor to form the 'high affinity' (coupled) state at low temperature also agrees with the absence of catecholamine stimulation of adenylate cyclase (table 2).

The increase in affinity of isoproterenol for the β-receptor in the presence of nucleotide on lowering the temperature is in agreement with observations [14] with turkey erythrocytes. Although a nucleotide dependency of agonist binding was not observed [14] the enthalpy (ΔH°) for L-isoproterenol binding in

Table 2
Temperature dependency of L-isoproterenol binding and adenylate cyclase activation

Temp. (°C)	Displacement of specific L-isoproterenol (EC_{50}) (μM)	[^{125}I]IHYP-binding by L-isoproterenol + GTPγS (EC_{50}) (μM)	Adenylate cyclase act. (pmol . mg^{-1} . min^{-1}) in the presence of		
			None	L-isoproterenol + GTP	L-isoproterenol + GTPγS[a]
37	1.15	3.9	2	45	350
27	1.05	2.5	n.d.	n.d.	n.d.
17	1.1	1.15	0.2	0.25	28
17[a]	1.15	1.25	n.d.	n.d.	n.d.

[a] Membranes pretreated with 1 μM GTPγS and 50 μM L-isoproterenol for 10 min at 37°C and washed 3 times with 10 vol. buffer A before determination of binding and adenylate cyclase activity

n.d., not determined

Binding experiments in the presence and absence of GTPγS (0.1 μM) were done as in section 2 except that incubation was 20 min at 27°C and 30 min at 17°C. Maximal binding of [^{125}I]IHYP was the same within ± 2% at each temperature

the presence of GTP was about the same as the ΔH° value we found with pigeon erythrocyte membranes in the presence of GTPγS.

4. Discussion

These results demonstrate that guanine nucleotide modulation of agonist binding also plays a role in β-adrenergic stimulation of adenylate cyclase in avian erythrocytes. The two forms of the β-adrenergic receptor in the presence and absence of guanylnucleotides, may be designated as 'low' and 'high-affinity' states although they are not defined in molecular terms. From observations with cells lacking the GTP-binding protein [5] and with solubilized β-receptors from frog erythrocytes [26] it seems, that the 'high-affinity' form of the receptor is a receptor–GTP-binding protein complex. In any event, the high affinity form is probably an obligatory intermediate in the activation of adenylate cyclase and might be needed for the recharging of the GTP-binding protein with GTP and the displacement of GDP, formed by the GTPase reaction [23,27]. However it must be borne in mind that GDP is likewise able to induce the low affinity form of the receptor (see table 1). This suggests the existence of an 'empty' GTP-binding site which is functional in interacting with the receptor. Whether this site represents a second guanine nucleotide binding site, residing in the same or another protein as suggested by others, is not as yet clear. The observation that activation of adenylate cyclase by Gpp(NH)p is quasi-irreversible whereas receptor modulation is not, may be explained by assuming only one GTP-binding protein represented by the protein with M_r 42 000 [17,28], if one considers further that this GTP-binding protein is in excess over catalytic sites (and receptors). In that case one could speculate that the fraction of GTP-binding protein which has become coupled to the catalytic moiety during activation [29] binds Gpp(NH)p irreversibly in a 'locked' conformation whereas the remainder of the GTP-binding protein binds the nucleotide reversibly making it capable of interaction with the receptor to form a 'high affinity' state. But whatever the molecular basis of modulation of agonist–receptor interaction by nucleotides may be, it seems likely that the ability of the hormone receptor to return to its high affinity state is a prerequisite for productive coupling to adenylate cyclase. Uncoupling of hormonal response by genetic or chemical manipulation or changes of the membrane environment appears to abolish or to prevent formation of a high affinity state of the hormone receptor. An example is the desensitization of hormone receptors as a consequence of prolonged exposure to agonists [30,31]. Another example is the action of a eucaryotic ADP-ribosyltransferase from turkey erythrocyte cytosol [32] which uncouples hormonal stimulation in avian erythrocytes accompanied by a loss in nucleotide modulation of β-adrenergic receptors [33].

Addendum

During preparation of this manuscript a paper

appeared [34] demonstrating modulation of β-adrenergic agonist binding by guanylnucleotides in turkey erythrocyte membranes.

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