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Unimpaired coupling of phosphorylated, desensitized β-adrenoceptor to G_s in a reconstitution system

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Heterologous desensitization of turkey erythrocyte β-adrenoceptors correlates with receptor phosphorylation and impaired receptor-G_s coupling, as assessed by fusion of purified desensitized receptors with *X. laevis* erythrocytes [(1984) Science 225, 837–840]. We have purified β-receptors from desensitized and untreated turkey erythrocytes and have compared the abilities of these two receptors to couple with pure turkey erythrocyte G_s in a reconstituted system. Functional receptor-G_s coupling was assessed by measuring hormone-dependent (i) G_s activation by GTPγS and (ii) GTPase activity. While in membranes prepared from desensitized cells, receptor-G_s coupling was clearly reduced, this effect was absent when coupling of purified desensitized receptor was measured. We conclude that covalent modification by phosphorylation does not fully explain the functional uncoupling at the membrane level.

β-Adrenoceptor; Desensitization; Phosphorylation; G_s-protein; Reconstitution

1. INTRODUCTION

Heterologous desensitization of β-adrenoceptor-linked adenylate cyclase is associated with reduced ability of the receptor to couple with the adenylate cyclase complex and with diminished responsiveness not only to catecholamines but also to additional hormones such as PGE_1 and to activators of the guanyl nucleotide-binding protein G_s such as fluoride and guanyl nucleotide analogues. Homologous desensitization, however, is agonist-specific and does not result in reduction of receptor-independent G_s-stimulated cyclase activity. In turkey erythrocytes, heterologous desensitization of adenylate cyclase has been correlated with incorporation of ^{32}P-labelled phosphate into serine residues of the receptor and with changes in receptor mobility on SDS-polyacrylamide gels which were ascribed to desensitization-induced changes in receptor conformation [1].

It has been proposed that the desensitized turkey β-receptor which has been modified by phosphorylation, can no longer couple effectively with G_s, since reconstitution of this receptor into phospholipid vesicles, followed by fusion with the β-receptor-deficient cyclase system of *Xenopus laevis* erythrocytes, resulted in a 40–50% reduction of hormone-stimulated cyclase activity, when compared to results obtained with untreated receptor [2]. On the other hand, reconstituted desensitized β-receptors from rat lung interacted with G_s of *X. laevis* erythrocytes to the same extent as control receptors [3]. Additional reconstitution experiments have been used [4] to assay the func-

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Abbreviations: AppNHp, adenylyl imidodiphosphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DTT, dithiothreitol; CYP, cyanopindolol; CGP 12177, Ciba Geigy Product 12177; G_s, guanine nucleotide-binding protein mediating stimulation of adenylate cyclase

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tionality of frog erythrocyte β-receptors after desensitization. These authors incorporated purified receptor with purified bovine brain G_s, into phospholipid vesicles and demonstrated reduced receptor-stimulated GTPase activity.

In the present study we have incorporated purified β-receptors isolated from desensitized and untreated turkey erythrocytes and have compared the abilities of these two receptors to couple with pure turkey erythrocyte G_s in a reconstituted system which is widely used in this laboratory [5-7]. We show that the purified receptor, which has undergone enhanced phosphorylation during desensitization, does not exhibit impaired coupling to G_s in our system. Our findings therefore do not support the generally held view that receptor phosphorylation during heterologous desensitization in turkey erythrocytes is responsible for the uncoupling observed and we discuss alternative mechanisms to explain this process.

2. MATERIALS AND METHODS

[^{35}S]GTPγS (800-1500 Ci/mmol), [γ-^{32}P]GTP (10-50 Ci/mmol) and [α-^{32}P]ATP (600 Ci/mmol) were purchased from New England Nuclear. [^{125}I]-ICYP (2000 Ci/mmol), [^{3}H]CGP 12177 (42 Ci/mmol) and [^{32}P]orthophosphate were from Amersham Buchler. All other chemicals and biochemicals were of the highest grade commercially available and were from the same sources as described before [5,6].

2.1. *Desensitization*

Washed erythrocytes (30% hematocrit) were incubated with 10 μM isoproterenol for 3 h at 37°C in incubation buffer (157.5 mM NaCl, 30 mM Hepes, 2.5 mM KCl, 11.1 mM glucose, 0.2 mM sodium metabisulfite, pH 7.9). Cells were centrifuged at $900 \times g$ for 10 min, the supernatant removed and cells again washed 4 times with 10 vols of incubation buffer.

2.2. *In vivo ^{32}P labelling*

Packed erythrocytes (10 ml) were incubated in buffer containing penicillin (100 E/ml), streptomycin (100 μg/ml) and [^{32}P]orthophosphate (0.5 mCi/ml) for 20 h at 37°C. Following incubation, desensitization was carried out as above.

2.3. *Purification of components*

Tyrkey erythrocyte membranes were prepared according to [8]. β-Adrenoceptor from turkey erythrocytes was purified by affinity chromatography as described in [5]. The purification of turkey erythrocyte G_s was carried out as in [9].

2.4. *Reconstitution*

Reconstitution was performed essentially as described in [5,6]. Briefly, purified β-receptor (3-6 pmol) and G_s (5-10 pmol) from turkey erythrocyte membranes were combined with 24 μl of a lipid mixture (2.5 mg/ml) containing phosphatidylethanolamine, phosphatidylserine and cholesterol hemisuccinate at a ratio of 12:8:5 in the presence of 0.24% lauroyl sucrose in a final volume of 150 μl. Excess detergents were removed on a Sephadex G-50 column which was preequilibrated with 20 mM Hepes (pH 7.8), 20 mM

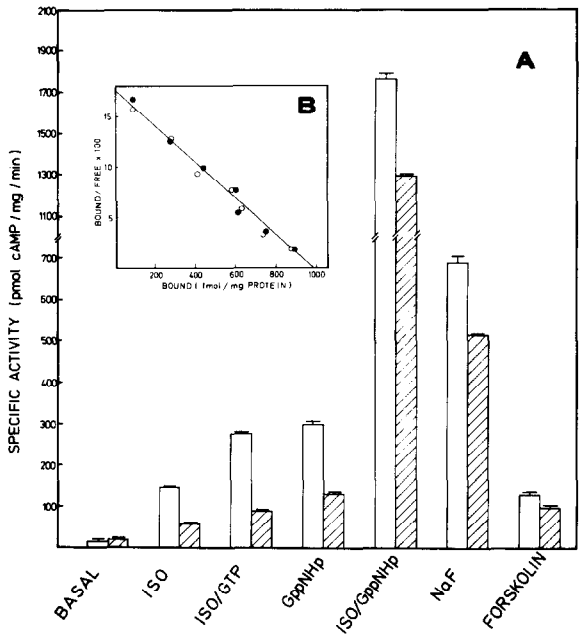

Fig.1. (A) Adenylate cyclase activity in membranes prepared from control (open bars) and desensitized (hatched bars) turkey erythrocytes using the activators indicated at a final concentration of 10 μM with the exception of NaF (10 mM) and forskolin (100 μM). The values shown are means ± SE of three experiments performed in duplicate. (B) Scatchard analysis of [^{3}H]CGP 12177 binding to membranes prepared from control and desensitized cells.

NaCl, 0.1 mM EDTA, 1 mM DTT and 4% glycerin. After vesiculation the NaCl concentration was raised to 150 mM. The amount of β-receptor and G_s incorporated into the lipid vesicles was assayed using 240 pM [^{125}I]ICYP and 1 μM [^{35}S]GTPγS [10], respectively. The incorporation yield was 40–60% for β-receptor and 70–80% for G_s. Hormone-induced activation of G_s was carried out with 10 μM l-isoproterenol, 60 nM GTPγS and 0.5 mM $MgCl_2$ at 30°C. GTPase activity was measured essentially as described in [11] using 10 μM l-isoproterenol, 0.25 μM [γ-^{32}P]GTP, 100 μM AppNHp, 100 μM ascorbic acid and 1 mM $MgCl_2$ at 30°C.

2.5. *Additional assays*

Specific binding to membrane β-receptors was determined using [^{3}H]CGP 12177 (0.05–11.0 nM) and was defined as that binding which could be displaced by 1 μM l-propranolol. Adenylate cyclase activity was measured either directly in membranes, or in β-receptor-G_s vesicles which were previously activated with l-isoproterenol and GTPγS by addition of a crude rabbit myocardial cyclase extract as described in [5]. [^{32}P]cAMP formed was isolated by the method of Salomon et al. [12]. Protein concentrations were determined according to [13] and polyacrylamide gel electrophoresis in the presence of SDS was performed according to [14]. Proteins were visualized by silver staining [15].

3. RESULTS AND DISCUSSION

The presence of heterologous desensitization in our system was verified by examination of cyclase stimulations in membranes prepared from isoproterenol-treated cells. As has been reported [16], all stimulations via receptor or G_s were markedly reduced after desensitization (fig.1A). However the much smaller forskolin stimulation characteristic of the turkey system was almost unaffected

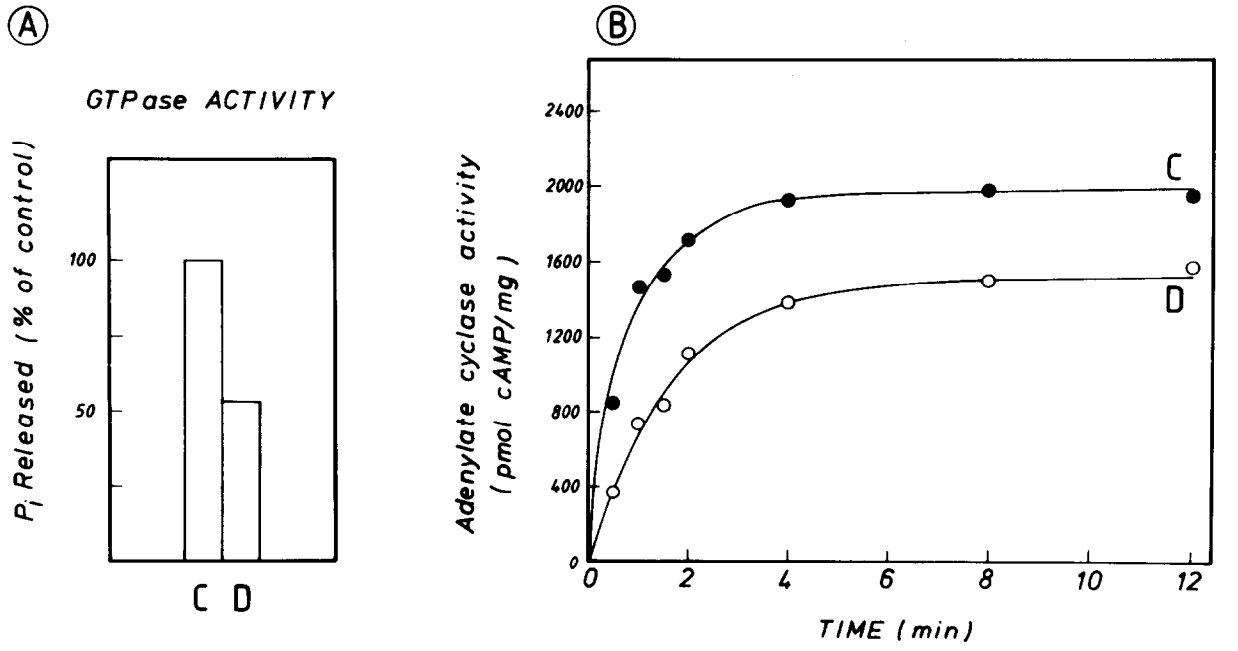

Fig.2. Comparison of β-receptor-G_s coupling efficiency in membranes prepared from control (C) and desensitized (D) cells. (A) GTPase activity in control (C) and desensitized (D) membranes (200 μg/ml) was measured in the presence of 0.25 μM [γ-^{32}P]GTP, 500 μM AppNHp, 2 mM mercaptoethanol, 6 mM $MgCl_2$ and 10 μM l-isoproterenol at 30°C for 20 min as described in [21]. Only hormone-dependent activities are shown. (B) Turkey erythrocyte membranes (1 mg/ml) were activated time-dependently with 60 nM GTPγS and 10 μM l-isoproterenol. At indicated times, aliquots were withdrawn, quenched with 200 μM dl-propranolol and cyclase activity was assayed as described in section 2. A representative example of three experiments is shown.

(fig.1A). Using the hydrophilic ligand [^{3}H]CGP 12177, Scatchard analysis of binding showed no change in membrane receptor number (fig.1B). K_d and B_{max} values were respectively for controls 0.558 nM and 992 fmol/mg, for desensitized receptors 0.551 nM and 979 fmol/mg. Further clarification of the altered receptor-cyclase coupling in membranes was obtained by measurement of hormone-stimulated GTPase activity which was reduced by 50% after desensitization (fig.2A). We also examined the kinetics of hormone-stimulated G_s activation in membranes and found that both the rate and extent of activation induced by isoproterenol/GTPγS were reduced after desensitization (fig.2B). The k_{on} values for activation in control and desensitized membranes were 1.1 and 0.6/min, respectively.

In agreement with previously published reports of receptor mobility changes as assessed by photoaffinity labelling [2,7], the apparent molecular mass shift of desensitized receptor after purification is shown by silver staining (fig.3). While in the intact cell the 50 kDa form of the receptor predominates [7], after cell disruption extensive conversion to the 40 kDa form occurs. After desensitization the latter form comprised polypeptides of molecular masses of 40 and 42 kDa which may reflect different extents of receptor phosphorylation. We also measured a 2.3-fold increase of [^{32}P]phosphate incorporation into receptors purified from desensitized cells in accordance with the results of Stadel et al. [17].

With a view to unambiguous assessment of the contribution made by phosphorylated receptor to uncoupling, we reconstituted this receptor with pure turkey G_s into phospholipid vesicles and measured receptor-G_s coupling efficiency. Time-dependent G_s activation by hormone/GTPγS was not altered following desensitization (fig.4A) nor was there any reduction of hormone-stimulated GTPase activity (fig.4B). We did not find that the altered mobility of receptor on SDS-PAGE, as assessed by photoaffinity labelling, was reversed after reconstitution (not shown), therefore it is unlikely that the equivalent coupling efficiency of control and desensitized receptors was due to dephosphorylation.

Thus we conclude on the basis of these results that covalent modification of receptor by phosphorylation is not sufficient to explain the uncoupling characteristic of heterologous desensitization in turkey erythrocytes. Others have speculated [18] that the desensitization-induced refractoriness may result from events at a site distal to the receptor,

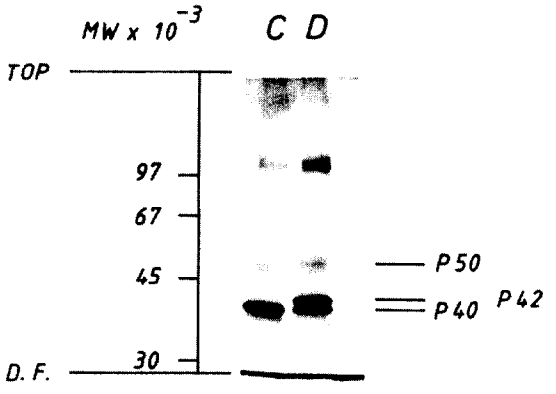

Fig.3. Polyacrylamide gel electrophoresis of β-receptor purified from control (C) and desensitized (D) turkey erythrocyte membranes. Proteins were visualized by silver staining [15].

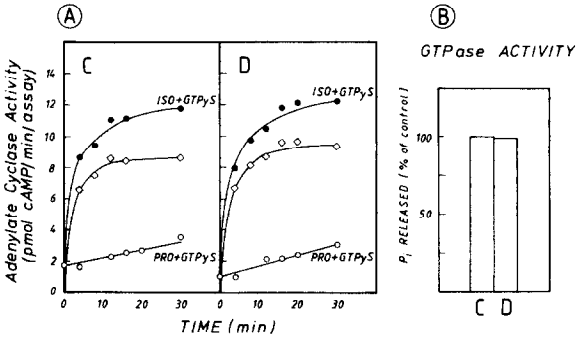

Fig.4. Comparison of β-receptor-G_s coupling efficiency in vesicles containing pure G_s and β-receptor purified from control (C) or desensitized (D) membranes. The phospholipid vesicles were prepared as described in section 2. The molar ratio of G_s to β-receptor after incorporation into the vesicles were 2.92 and 3.14 for control (C) and desensitized (D) β-receptor, respectively. Hormone-dependent G_s activation (A) was carried out at 30°C with 60 nM GTPγS and 10 μM l-isoproterenol (●) or 60 nM GTPγS and 10 μM dl-propranolol (○). The net specific activation is also shown (◇). Hormone-induced GTPase activity (B) was measured using the same β-receptor-G_s vesicles as in (A) at 30°C for 30 min as described in section 2. This experiment was repeated three times with similar results.

since they proposed a delay in the onset of GDP exchange by GTP in desensitized pigeon erythrocyte membranes even in the absence of hormone. Furthermore, Briggs et al. [19] reconstituted crude G_s solubilized from desensitized turkey erythrocytes into S 49 cyc^- membranes and demonstrated a reduction of isoproterenol-stimulated adenylate cyclase activity, which would implicate G_s as a site for the lesion in desensitization.

While previously reported data on the phosphorylated turkey erythrocyte β-receptor point to its decreased functionality [2], it has been more difficult to quantify the importance of receptor phosphorylation in homologous desensitization. In such systems, the contribution of phosphorylation to uncoupling is further complicated by the additional process of sequestration which may itself be regulated by phosphorylation.

A possible alternative site for the lesion in heterologous desensitization is suggested by investigations carried out with the rhodopsin-coupled light transduction system, which shares many structural and functional similarities with β-receptor-coupled adenylate cyclase. Wilden et al. [20] have proposed that a 48 kDa protein (arrestin) binds preferentially to phosphorylated rhodopsin and thereby attenuates its capacity to bind transducin and consequently to activate cGMP phosphodiesterase. In view of the reported similarities between the two systems, it may be that binding of phosphorylated β-receptor to G_s is blocked by interaction of an arrestin-like protein with the phosphorylated receptor. The presence of such a protein in *Xenopus* erythrocytes could also explain the apparent differences between results obtained from our reconstitution experiments and those of the Lefkowitz group [2]. We are at present investigating the possibility that arrestin can alter β-receptor-G_s coupling in our reconstitution sysem.

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