

# Lipid requirements for reconstitution of the delipidated $\beta$ -adrenergic receptor and the regulatory protein

Jorge Kirilovsky, Sonia Steiner-Mordoch, Zvi Selinger and Michael Schramm

*Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

Received 16 January 1985

The role of lipids in the interaction of the  $\beta$ -adrenergic receptor (R) with the regulatory protein ( $G_s$ ) was investigated. Solubilized preparations of R and of  $G_s$  from turkey erythrocytes were delipidated by gel filtration. They were subsequently combined and reconstituted by the addition of various lipids. When reconstitution was carried out in the presence of soybean lipids,  $G_s$  could be fully activated via R by addition of hormone plus  $GTP\gamma S$ . In contrast, purified phospholipids or a phospholipid fraction from soybean failed to produce an active system. Fractionation of soybean lipids revealed that acetone-soluble neutral lipids are essential for the reconstitution of a hormone responsive system. The acetone fraction could be replaced by specific neutral lipids such as  $\alpha$ -tocopherol or cholesteryl arachidonate while a mixture of phosphatidylethanolamine, -choline and -serine satisfied the phospholipid requirement of the system.

<i>Delipidation</i>	<i>Reconstitution</i>	<i><math>\beta</math>-Adrenergic receptor</i>	<i>Guanyl nucleotide binding protein</i>	<i>Regulatory protein</i>
			<i>Lipid requirement</i>	

## 1. INTRODUCTION

The adenylate cyclase system is composed of 3 separate proteins: the neurotransmitter or hormone receptor, the guanyl nucleotide binding protein ( $G_s$ ), and the catalytic unit (C) [1,2]. All 3 components are membranous proteins and thus it seems likely that lipids may have a regulatory role in this system. Earlier studies [3] suggested that acidic phospholipids might be required for glucagon activation of adenylate cyclase in phospholipase-treated liver membranes. Recently, it was shown that uptake of phosphatidylinositol by turkey erythrocytes uncouples the  $\beta$ -adrenergic receptor (R) from the adenylate cyclase [4]. Segregation of cholesterol in erythrocyte mem-

branes by Filipin uncoupled R [5] while its removal from liver membranes drastically reduced all adenylate cyclase activities [6]. Several studies also indicate that membrane fluidity regulates the activation of adenylate cyclase by hormones [7–9].

To study the role of lipids in receptor function, we developed a procedure for delipidation of a deoxycholate-solubilized preparation of R [10]. We showed that phosphatidylethanolamine was the only phospholipid among all those tested that was capable of fully reconstituting the agonist and antagonist binding to the receptor. Cholesteryl hemisuccinate, when mixed with an appropriate phospholipid, was also effective. Once the lipid requirements for the reconstitution of hormone binding to the receptor were established we tried to look at the second stage of the process, namely, receptor activation of  $G_s$ .

A procedure for reconstitution of a hormone responsive system by combining non-purified preparations of R and  $G_s$  has been described by our laboratory [11] and others [12,13]. Recently,

*Abbreviations:* R,  $\beta$ -adrenergic receptor;  $G_s$ , stimulatory guanyl nucleotide binding protein;  $GTP\gamma S$ , guanosine 5'-O-(3-thiotriphosphate);  $G_sGTP\gamma S$ ,  $G_s$  protein to which  $GTP\gamma S$  is bound; cyc<sup>-</sup>, an S49 lymphoma variant clone deficient in functional  $G_s$ .

active purified preparations of R and  $G_s$  were successfully reconstituted in phospholipid vesicles [12,13]. There were several reasons, however, which led us to attempt the delipidation of R and  $G_s$  with non-purified preparations. The chances of inactivation of the purified R after delipidation, by self aggregation, seemed considerable; relatively large amounts of R and  $G_s$  are required to develop the procedures, and the lipid requirements which will be established for the crude system would most likely hold also for the purified preparations.

Here we show that delipidated preparations of R and  $G_s$  can be reconstituted together into lipid vesicles, yielding a hormone-responsive system. The lipid requirements for the R- $G_s$  interaction are much more extensive than those of ligand binding to R [10]. While in the latter system phosphatidylethanolamine could efficiently replace the endogenous lipids of the native membrane, the R- $G_s$  interaction requires more than one phospholipid and a neutral lipid as well.

## 2. EXPERIMENTAL

For most of the materials, see [10]. Sodium cholate and neutral lipids were purchased from Sigma. Unless otherwise stated, all operations were performed at 4°C.

### 2.1. Biological preparations

The preparation from turkey erythrocyte membranes of a deoxycholate extract containing R [14] and the procedure for its delipidation were as described [10]. The pooled delipidated fractions contained 0.2 mg protein/ml and 1.5 pmol R/mg protein.  $G_s$  was solubilized in buffered cholate [15]. For  $G_s$  delipidation, the cholate extract was applied to an Ultrogel AcA-34 column as used for R delipidation; the column was previously equilibrated with cholate and elution was performed with the cholate buffer. Between 50 and 75% of  $G_s$  was recovered in the delipidated fraction (0.17 mg protein/ml). Its activity was  $940 \pm 230$  pmol cyclic AMP/min per mg protein, measured in  $S_{49}$   $cyc^-$  membranes in the presence of fluoride. Fluoride was used in this assay since it is known to activate  $G_s$  directly and does not require a receptor or a specific guanyl nucleotide [1]. Delipidation was estimated by the addition of traces of labeled phospholipids or cholesterol [10]

to the sample applied to the column and measurement of radioactivity in the delipidated fractions. The validity of these determinations was confirmed by extracting total lipids from the delipidated  $G_s$  preparation in a chloroform/methanol mixture [10] and colorimetric determination of phospholipids in this fraction [16]. From these analyses, it was calculated that the delipidated  $G_s$  contains about 0.5 and 0.3% of the original phospholipid and cholesterol, respectively (0.02 mg phospholipid, or less, per mg protein).

Phospholipids were dispersed by sonication as described [10], at a concentration of 30 mg/ml in buffer containing cholate. Neutral lipids were dispersed together with phospholipids at the desired concentrations.

### 2.2. Reconstitution of the R- $G_s$ system

Delipidated  $G_s$  (0.6 ml), delipidated R (0.3 ml) and lipid suspension (0.1 ml, 3 mg lipids) were combined. Detergents were removed by treatment with XAD-2 beads [17].  $MgCl_2$  was added to a final concentration of 15 mM. Activation of  $G_s$  was carried out at 30°C in the presence of 10  $\mu$ M  $GTP\gamma S$ , (a) without further additions, or with 10  $\mu$ M cyanopindolol, which gives the basal activation of  $G_s$ , (b) 0.1 mM isoproterenol, and (c) 100 mM  $MgCl_2 \pm 0.1\%$  Lubrol, which determines the total  $G_s$  present in the system [18]. After the desired incubation time, activation was stopped by transferring the systems to 4°C and addition of 10  $\mu$ M cyanopindolol to the hormone-activated systems.

### 2.3. Assay of the amount of activated $G_s$

The procedure was essentially as in [19] but with some modifications which allowed the use of  $cyc^-$  cell ghosts instead of membranes as the source of the catalytic unit of adenylate cyclase. Detergent addition to the assay was not required. Adenylate cyclase activity was determined by incubation at 30°C for 30 min.

## 3. RESULTS AND DISCUSSION

Delipidated preparations of R and  $G_s$  were combined and reconstituted in the presence of a soybean lipid mixture. As shown in fig.1,  $G_s$  was readily activated in the presence of isoproterenol and  $GTP\gamma S$ . The activation was critically depen-

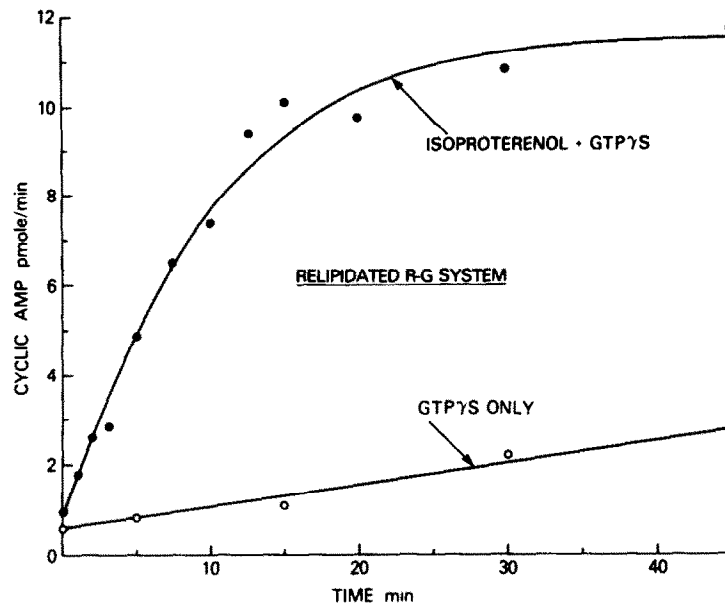


Fig.1. Kinetics of activation of  $G_s$  by isoproterenol and  $GTP\gamma S$ . Reconstitution of delipidated R and  $G_s$  in the presence of crude soybean lipid was performed as described in section 2. The reconstituted system was incubated at  $30^\circ C$  in the presence of  $10 \mu M$   $GTP\gamma S$  and  $0.1 mM$  isoproterenol. Control system (basal activity) was without addition of agonist or antagonist. At the times indicated, the reaction was stopped by the addition of cyanopindolol to a concentration of  $10 \mu M$  and transfer to  $4^\circ C$ . The amount of  $G_s GTP\gamma S$  produced was determined as described in section 2.

dent on the addition of the agonist. In the absence of agonist, or when the specific R antagonist cyanopindolol was added, the rate of  $G_s$  activation was only 5–10% of that attained in the presence of isoproterenol. Activation by hormone plus  $GTP\gamma S$  followed first-order kinetics with a rate constant of  $0.1 \text{ min}^{-1}$ . Maximal activation was achieved after 20 min when all the available  $G_s$  was activated. This was concluded from the finding that addition of  $100 mM$   $MgCl_2$  [18] produced a comparable level of  $G_s GTP\gamma S$  as the addition of hormone (see table 1). The lower activity displayed by  $Mg^{2+}$ -activated  $G_s$  reflects some inhibition of the adenylate cyclase in  $cyc^-$  membranes by the rather high  $Mg^{2+}$  carried over into the final assay. When systems which had been fully activated by hormone were assayed in the presence of such a high  $Mg^{2+}$  concentration, they exhibited about the same activity as the  $Mg^{2+}$ -activated systems. Addition of 0.1% Lubrol in the  $100 mM$   $MgCl_2$  activation did not uncover additional 'cryptic'  $G_s$ . It is shown in table 1 that when delipidated R and  $G_s$  were reconstituted in the absence of added lipids, isoproterenol failed to produce significant activa-

Table 1  
Lipid requirement for  $G_s$  activation

Stage of lipid addition	Activation system	
	Iso-proterenol plus $GTP\gamma S$	$100 mM$ $Mg^{2+}$ plus $GTP\gamma S$
Before detergent removal	9.8	6.2
After detergent removal	6.7	6.5
After addition of $15 mM$ $Mg^{2+}$	4.5	6.4
After activation	0.6	5.8

Delipidated preparations of R and  $G_s$  were combined. Crude soybean phospholipid was added before XAD-2 treatment as described in section 2, or at a later stage as shown in the table. In all systems the final lipid concentration was  $3 \text{ mg/ml}$ . Each system was activated at  $30^\circ C$  in the presence of  $10 \mu M$   $GTP\gamma S$  plus  $0.1 mM$  isoproterenol for 20 min, or  $10 \mu M$   $GTP\gamma S$  plus  $100 mM$   $MgCl_2$  for 30 min. Hormone activation was stopped by the addition of cyanopindolol to a final concentration of  $10 \mu M$ .  $G_s$  was assayed in  $cyc^-$  cells as described in section 2. Values expressed as  $pmol$  cAMP/min

tion of  $G_s$ . However, full activation of  $G_s$  could still be obtained by addition of 100 mM  $MgCl_2$  indicating that such activation of  $G_s$ , which is not dependent on a receptor [18], does not require lipids. Thus, it is only the activation of  $G_s$  by R which is lipid-dependent. Efficient coupling to R required that the soybean lipids be added prior to detergent removal. Delaying lipid addition until after the XAD-2 treatment already caused a 30% reduction in the R-induced activation of  $G_s$  (see also [10]).

Various defined phospholipids were tested individually or in mixtures, for their ability to replace the soybean lipid in the reconstitution of delipidated R with  $G_s$ . Surprisingly, none of these experiments demonstrated efficient reconstitution of the R- $G_s$  interaction (as shown below). Since specific agonist binding to delipidated R could be fully restored by phosphatidylethanolamine [10], it became evident that the reconstitution of the R- $G_s$  interaction with delipidated components is much more demanding in lipid requirements.

It was clear that some constituent of the crude soybean lipid preparation was necessary, in addition to phospholipids, for the hormone-dependent activation of  $G_s$ . Therefore, an acetone fraction containing neutral lipids and an ether fraction containing the polar lipids were prepared from the soybean lipid mixture as previously described for reconstitution of the nicotinic acetylcholine receptor [20]. Each of these lipid fractions, tested separately, failed to confer significant isoproterenol-induced activity on the R- $G_s$  system (fig.2). However, when the neutral and polar lipid fractions were added in combination to the delipidated preparations of R and  $G_s$ , full activation of  $G_s$  was regained. The ether fraction, containing mainly phospholipids could also be effectively combined with defined neutral lipids. Fig.2 shows that  $\alpha$ -tocopherol, as well as cholesteryl arachidonate, can substitute efficiently for the acetone fraction. Vitamin  $K_1$ , coenzyme  $Q_{10}$ , *trans*-retinol and cholesteryl oleate were also effective in reconstitution when added together with the ether fraction. These findings are similar to those obtained by Paul Mueller, Efraim Racker and co-workers in the reconstitution of the nicotinic acetylcholine receptor [20]. However, in the latter system, cholesteryl hemisuccinate [21] or cholesterol [22] could also satisfy the neutral lipid requirement for

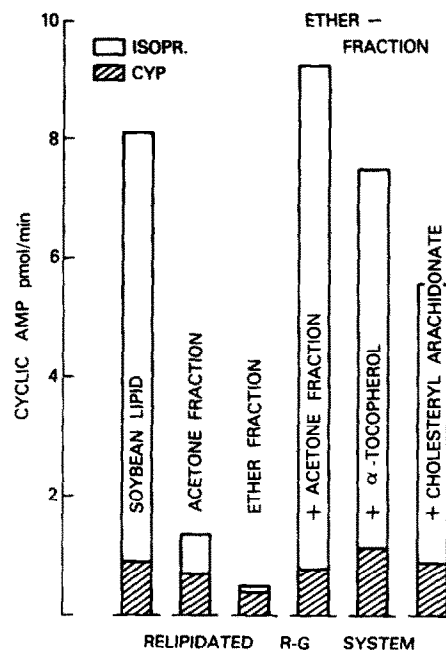


Fig.2. Requirement for neutral lipid in the reconstitution of the R- $G_s$  system. Delipidated preparations of R and  $G_s$  were reconstituted in the presence of crude soybean lipid (3 mg/ml), acetone extract from soybean lipid (1 mg/ml), or the ether soluble fraction from soybean lipid (3 mg/ml) with or without the addition of  $\alpha$ -tocopherol (0.2 mg/ml) or cholesteryl arachidonate (0.075 mg/ml), as shown in the figure. Activation of  $G_s$  was performed at 30°C for 20 min in the presence of 10  $\mu$ M cyanopindolol (hatched bars) or 0.1 mM isoproterenol.

correct incorporation of the receptor into phospholipid vesicles. So far we have been unable to obtain a satisfactory R- $G_s$  interaction using these sterols as the neutral lipid. Our most recent experiments also indicate that the phospholipid specificity for reconstitution of the R- $G_s$  system is different from that of the nicotinic acetylcholine receptor. The R- $G_s$  interaction can be reconstituted to full activity in a completely defined lipid medium shown in table 2. Phosphatidylethanolamine, -choline, and -serine are required in addition to  $\alpha$ -tocopherol. Any other combination of these phospholipids, with or without tocopherol, did not produce an active R- $G_s$  system.

The role of neutral lipids in the receptor-mediated activation of  $G_s$  is not yet clear. The possibility that a neutral lipid increases permeability

Table 2  
Defined phospholipids required for reconstitution of the R-G<sub>s</sub> system

Phospholipids added to delipidated components	Addition of $\alpha$ -tocopherol	Activity of relipidated system		Net activation due to hormone (b - a)
		Cyanopindolol plus GTP $\gamma$ S (a)	Isoproterenol plus GTP $\gamma$ S (b)	
PE	-	1.4	1.5	0.1
	+	1.2	1.7	0.5
PC	-	1.1	1.2	0.1
	+	1.1	1.3	0.2
PE:PC (2:1)	-	1.4	1.4	0.0
	+	1.8	2.0	0.2
PE:PS (3:1)	-	0.2	0.6	0.4
	+	0.4	1.0	0.6
PE:PC:PS (3:2.2:1)	-	1.1	1.4	0.3
	+	1.3	8.3	7.0

Delipidated preparations of R and G<sub>s</sub> were reconstituted with or without  $\alpha$ -tocopherol (0.25 mg/ml) and different phospholipids. In all systems the final phospholipid concentration was 3 mg/ml. The numbers in parentheses indicate phospholipid weight ratios. Activation was performed as described in the legend to fig.2. Net activation due to hormone was obtained by subtracting the basal activity determined in the presence of cyanopindolol from that obtained in the presence of isoproterenol. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine. Results expressed as pmol cAMP/min

ty of the reconstituted vesicles to Mg<sup>2+</sup> of GTP $\gamma$ S seems unlikely since  $\alpha$ -tocopherol has been shown to decrease permeability of liposomes [23]. Furthermore, negative staining with phosphotungstic acid indicates that vesicles obtained by our reconstitution procedures are rather open structures, even in the absence of neutral lipid. It is nevertheless possible that the neutral lipids are necessary for proper positioning of R and G<sub>s</sub> in the reconstituted system. We speculate, however, that the receptor specifically requires neutral lipid so as to recognize, and perhaps to activate, the regulatory protein. With this idea in mind, special attention will be given to the requirement for specific lipids in the partial reactions involved in the R-G<sub>s</sub> interaction [2,24].

#### ACKNOWLEDGEMENTS

We are grateful to Aviva Petcho, Tirza Fibich and Nomi Goldstein for participation in some of the experiments. The work was supported by grant

AM-10451 to M.S. from the National Institutes of Health, USA.

#### REFERENCES

- [1] Ross, E.M. and Gilman, A.G. (1980) *Annu. Rev. Biochem.* 49, 533-564.
- [2] Schramm, M. and Selinger, Z. (1984) *Science* 255, 1350-1356.
- [3] Rubalcava, B. and Rodbell, M. (1973) *J. Biol. Chem.* 248, 3831-3837.
- [4] McOsker, C.C., Weiland, G.A. and Zilversmit, D.B. (1983) *J. Biol. Chem.* 258, 13017-13026.
- [5] Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M. (1974) *J. Biol. Chem.* 249, 3232-3240.
- [6] Whetton, A.D., Gordon, L.M. and Houslay, M.D. (1983) *Biochem. J.* 212, 331-338.
- [7] Orly, J. and Schramm, M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3433-3437.
- [8] Rimón, G., Hanski, E., Braun, S. and Levitzki, A. (1978) *Nature* 276, 394-396.
- [9] Bakardjieva, A., Galla, H.J. and Helmreich, E.J.M. (1979) *Biochemistry* 18, 3016-3023.

- [10] Kirilovsky, J. and Schramm, M. (1983) *J. Biol. Chem.* 258, 6841–6849.
- [11] Citri, Y. and Schramm, M. (1980) *Nature* 287, 297–300.
- [12] Brandt, D.R., Asano, T., Pedersen, S.E. and Ross, E.M. (1983) *Biochemistry* 22, 4357–4362.
- [13] Cerione, R.A., Codina, J., Benovic, J.L., Lefkowitz, R.J., Birnbaumer, L. and Caron, M.G. (1984) *Biochemistry* 23, 4519–4525.
- [14] Nedivi, E. and Schramm, M. (1984) *J. Biol. Chem.* 259, 5803–5808.
- [15] Citri, Y. and Schramm, M. (1982) *J. Biol. Chem.* 257, 13257–13262.
- [16] Stewart, J.C.M. (1980) *Anal. Biochem.* 104, 10–14.
- [17] Eimerl, S., Neufeld, G., Korner, M. and Schramm, M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 760–764.
- [18] Northup, J.K., Smigel, M.D. and Gilman, A.G. (1982) *J. Biol. Chem.* 257, 11416–11423.
- [19] Hanski, E., Sternweis, P.C., Northup, J.K., Dromerick, A.W. and Gilman, A.G. (1981) *J. Biol. Chem.* 256, 12911–12919.
- [20] Kilian, P.L., Dunlap, C.R., Mueller, P., Schell, M.A., Haganir, R.L. and Racker, E. (1980) *Biochem. Biophys. Res. Commun.* 93, 409–414.
- [21] Criado, M., Eibl, H. and Barrantes, F.J. (1982) *Biochemistry* 21, 3622–3629.
- [22] Ochoa, E.L.M., Dalziel, A.W. and McNamee, M.G. (1983) *Biochim. Biophys. Acta* 727, 151–162.
- [23] Diplock, A.T., Lucy, J.A., Verrinder, M. and Zieleniewski, A. (1977) *FEBS Lett.* 82, 341–344.
- [24] Schramm, M., Korner, G. and Nedivi, E. (1983) *Cold Spring Harbor-Symp.* 48, 187–191.