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RECONSTITUTION OF THE TURKEY ERYTHROCYTE ADENYLATE CYCLASE SENSITIVITY TO l-EPINEPHRINE UPON RE-INSERTION OF THE LUBROL SOLUBILIZED COMPONENTS INTO PHOSPHOLIPID VESICLES

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SUMMARY: Turkey erythrocyte adenylate cyclase was activated by GppNHp and l-epinephrine to its stable, highly active form. In this form the enzyme could be solubilized by Lubrol-PX and subsequently re-inserted into phospholipid vesicles concomitantly with the removal of up to 99.3% of the Lubrol. The ability of GTP and l-epinephrine to reverse the GppNHp/epinephrine activated state was taken as a measure for the reappearance of hormone sensitivity in the reconstituted vesicles. An incomplete but significant reappearance of hormone sensitivity in the reconstituted adenylate cyclase was achieved. This hormone sensitivity was found to be stereospecific for (-)epinephrine. The ^{125}I-cyanopindolol binding properties of the reconstituted β-receptor depend on the nature of the detergent and the phospholipids used in the reconstitution.

In recent years there have been several reports of solubilization and resolution of the components making up the hormone-sensitive adenylate cyclase system, from a number of tissues and cell types (1). Rat erythrocyte β-adrenergic receptors, solubilized with digitonin, were reconstituted into DMPC phospholipid vesicles (2). Schramm and his colleagues solubilized turkey erythrocyte β-adrenergic receptors, reincorporated them into phospholipid vesicles, and implanted them into Friend erythroleukemia cells (3). Solubilization and purification of the GTP-binding protein was recently achieved and found to fully reconstitute the hormone sensitive adenylate cyclase in S49 AC$^-$ lymphoma cells which lack a functional GTP-binding protein (4).

Adenylate cyclase from a number of sources has been solubilized with non-ionic detergents, causing some inhibition of cyclase activity but to a

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Abbreviations: PMSF, phenylmethyl-sulfonyl fluoride; TME, 50 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4; GppNHp, guanylyl-imidodiphosphate; CYP, cyanopindolol; HYP, hydroxybenzylpindolol; PE, phosphatidyl-ethanolamine; PS, phosphatidyl-serine; DMPC, dimyristoyl-phosphatidyl-choline.

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much lesser extent than with ionic detergents (1). Solubilization of rat brain cyclase with deoxycholate has produced an inactive preparation, the activity of which can be restored following addition of phospholipids or Triton X-100 (5).

To generalize, reconstitution experiments have involved reincorporation of individual components into a variety of membranes deficient in that particular component of the hormone-sensitive adenylate cyclase system (1, 6-8).

The approach we decided to adopt is to attempt and reconstitute the β-adrenergic receptor sensitive adenylate cyclase from the detergent solubilized components. In this communication, we report our initial success in reconstituting hormone sensitive cyclase activity from the Lubrol-PX solubilized components. The reconstitution of the solubilized catalytic protein and hormone receptors into a hormone sensitive adenylate cyclase has so far been only reported for the dopamine dependent adenylate cyclase, using the cholate dilution method (9, 10).

Rationale: Preliminary experiments indicated to us that the least inhibitory detergent to turkey erythrocyte adenylate cyclase is Lubrol-PX. Furthermore, we found that the GppNHp/l- epinephrine "permanently" activated enzyme from turkey erythrocytes is stable for many days at 4°C when solubilized in Lubrol-PX (11). We therefore decided to solubilize the GppNHp preactivated enzyme in this detergent and attempt the detergent's removal concomitantly with re-insertion of the enzyme system into phospholipid liposomes. Regeneration of β-receptor to cyclase coupling was examined by the ability of GTP and β-agonist to reverse the GppNHp permanently active state. We have previously demonstrated (12, 13) that GTP can reverse the GppNHp activated state in native membranes only in the presence of a β-agonist. This approach allows us to deal with a stable adenylate cyclase and still test for the ability of the β-receptor to recouple with the enzyme system.

EXPERIMENTAL

Materials: Cyanopindolol was obtained from Dr. G. Engel (Sandoz, Basle, Switzerland); egg yolk phosphatidyl-ethanolamine was obtained from Makor

Chemicals (Jerusalem, Israel); asolectin, from Associate Concentrates (Woodside, New York); bovine brain phosphatidyl-serine, synthetic dimyristoyl-phosphatidyl-choline, l-epinephrine-HCl, l-isoproterenol-HCl, creatine phosphate, ATP, creatine kinase, GTP, GppNHp, and Lubrol-PX from Sigma. SM-2 beads were obtained from Bio-Rad and used after methanol activation. All other chemicals were of the highest degree of purity available and all solutions prepared in Corning double distilled water.

GppNHp activated turkey erythrocyte membranes: Turkey red cell membranes, prepared as previously described (14), were thawed, suspended in TME buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and centrifuged twice at 20,000 xg for 10 minutes. The membranes were then activated with 100 μM epinephrine, 100 μM GppNHp in TME containing 10 μM PMSF, at 37°C for 10 minutes (15). Protein concentration in the incubation mixture was 3.0 mg/ml and the final volume 5.0 ml. Excess nucleotide and hormone were removed by washing twice in TME buffer (20,000 xg for 10 minutes).

Solubilization and reconstitution: GppNHp preactivated membranes (4 mg/ml) were solubilized by suspension in TME buffer containing 1 mM DTT, 0.25 M sucrose, 10 μM PMSF, 100 μM isoproterenol, 1 mM catechol, 1.2% (w/v) Lubrol-PX, 150 μM tri-n-butylphosphine. Ten percent of detergent was present as [^{3}H]Lubrol (170 cpm/μg), prepared as previously described (11), and protein (membrane) concentration was 4.0 mg/ml. The mixture was incubated at 32°C for 20 minutes and centrifuged at 200,000 xg for 30 minutes at 4°C. A sonicated dispersion of phospholipid was added to the supernatant (2 ml) to a final concentration of 10.0 mg/ml. The mixture was passed through a column containing methanol pre-activated SM-2 beads and thoroughly washed with TME buffer (0.8 x 15 cm, 4 g net wt.). The turbid fractions were collected (3 ml), diluted 1:1 in TME, and 10 mM $MgCl_2$ were added prior to centrifugation at 200,000 xg for 30 minutes at 4°C. The pellet was resuspended in TME to a final volume of 2 ml, and assayed for cyclase activity, [^{3}H]Lubrol, phospholipids (16) and protein (17).

Reversal assay: Reversal was initiated by adding the resuspended pellet to a preincubated (37°C) reversal mixture in TME, which contained 50 μM GTP, 4 mM $MgCl_2$, 10 mM creatine phosphate (Sigma), 5 mmol/assay creatine phosphokinase (Sigma), and 100 μM l-epinephrine or 10 μM l-propranolol. At successive time intervals, 75 μl samples were aliquoted into test tubes containing 25 μl of 0.1 mM propranolol at 0°C, and then assayed for adenylate cyclase activity.

The phospholipid dispersion was prepared by sonication of a 75 mg/ml suspension in TME 3 times for 5 minutes at 8 to 10°C, immediately before addition to the mixture.

RESULTS

The inhibition of adenylate cyclase by Lubrol-PX: Lubrol-PX was found to strongly inhibit the l-epinephrine dependent adenylate cyclase activity, while the NaF dependent activity and the specific activity of the GppNHp/epinephrine pre-activated state were less sensitive to Lubrol-PX (Fig. 1). It can be seen that 0.01% and 0.025% Lubrol-PX in the assay already inhibit 63% and 93% respectively of the l-isoproterenol dependent activity, but only 3% and 9% of the NaF and of the GppNHp/epinephrine activity. This result in-

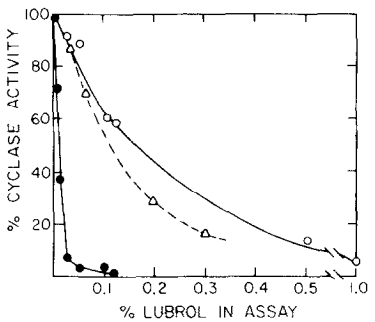

Figure 1: The inhibition of adenylate cyclase activity by Lubrol-PX. Lubrol was incorporated into the assay mixture. (o-o), activity in the presence of 10 mM NaF; (●-●), activity in the presence of 10 μM l-isoproterenol; (Δ-Δ), activity of GppNHp/epinephrine pre-activated membranes (data of Hanski *et al.*, ref. 11).

dicates that if one wishes to obtain a significant recoupling between the receptor and the cyclase system, one must remove the Lubrol-PX quantitatively.

The reconstitution protocol: A representative reconstitution experiment is shown in Figure 2. The final yield of adenylate cyclase units in the reconstituted vesicles, as compared with native membranes, is 15%. The SM-2 treatment removes over 50% of the protein, while phospholipid is recovered quantitatively. [^{3}H]Lubrol is removed down to 1-4% of the initial amount added. We could not achieve a larger extent of Lubrol-PX removal. Namely, the final concentration of the detergent in the reconstituted vesicles is in the range of 0.009% to 0.03%, a range in which the coupling between the receptor and the cyclase system is already significantly, although not completely, inhibited. The activity of the reconstituted cyclase is relatively stable and loses 50% activity after 10 days of storage at 4°C. The presence of GppNHp appears to be necessary for this remarkable stability, since adenylate cyclase, which has not been pre-activated with GppNHp and which undergoes the solubilization-reconstitution cycle, does not exhibit any measurable activity with NaF, Mn^{++}, and GppNHp/epinephrine (data not shown).

Reversal of the GppNHp active state by GTP and l-epinephrine: The ability of GTP plus hormone to reverse the permanently activated GppNHp state was tested. The results are shown in Figure 3. In the native membrane the activity was reduced to a low level steady-state after approximately 20 min.

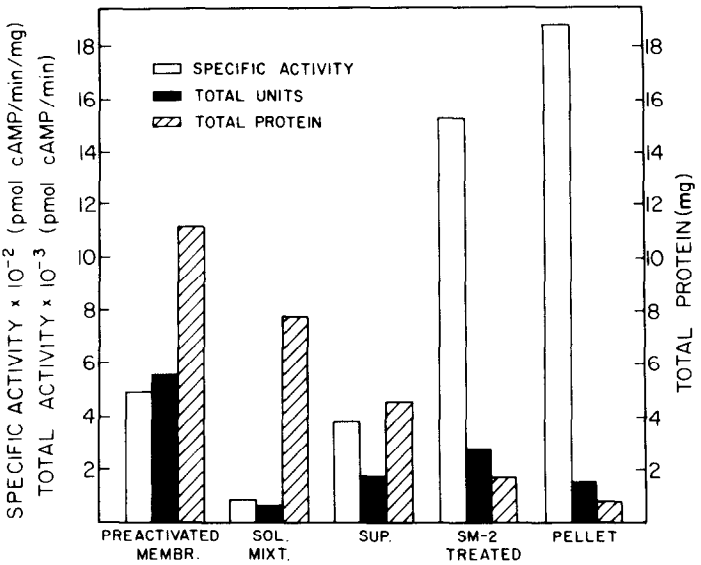

Figure 2: The solubilization-reconstitution cycle. Experimental details are given in the text.

(Fig. 3A). Since this effect is hormone dependent and blocked by propranolol, it is a useful criterion for the presence of a hormone responsive adenylate cyclase (13).

When the same reaction was attempted with the reconstituted material, there was a reduction in specific activity of about 15 to 20% (Fig. 3B), and this was absent in the presence of propranolol (Fig. 2) and (+)epinephrine (Fig. 2C). This stereospecific reduction was small but reproducible whenever 98% to 99.3% of the Lubrol was removed. When Lubrol removal was less than 96%, stable and high specific activity adenylate cyclase was obtained but no hormone dependent reversal could be demonstrated.

The effect of phospholipids: Reconstitution was performed with either asolectin, pure phosphatidyl-choline, a 3:1 mixture of phosphatidyl-ethanolamine and phosphatidyl-serine, 80% asolectin plus 20% cholesterol and pure dimyristoyl-phosphatidyl-choline. All produced similar results.

Binding of ^{125}I-cyanopindolol: Native turkey erythrocyte membranes bind the highly selective ^{125}I-cyanopindolol with marked affinity (Table I). Lubrol-PX reconstituted vesicles revealed only a much smaller binding capa-

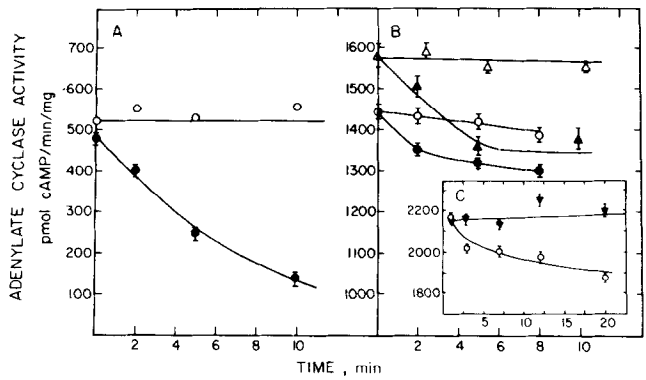

Figure 3: The hormone sensitivity of the reconstituted adenylate cyclase. A. Native membranes: (o-o), 50 μM GTP plus 10 μM l-propranolol; (●-●), 50 μM GTP plus 100 μM l-epinephrine. B. Reconstituted vesicles: (o-o), reconstituted into asolectin with 50 μM GTP and 10 μM l-propranolol; (●-●), reconstituted into asolectin with 50 μM GTP plus 100 μM l-epinephrine; (Δ-Δ), reconstituted into PE/PS (3:1) with 50 μM GTP and 10 μM l-propranolol; (▲-▲), reconstituted into PE/PS (3:1) with 50 μM GTP and 100 μM l-epinephrine. Other experimental details are given in the text. C (inset) Reconstituted vesicles: Reversal was conducted with either 20 μM l-(-)epinephrine (o-o) or d-(+)epinephrine (▼-▼) at 35°C. The rest of the experimental conditions are identical to those in A and B. Data are presented as the average plus the standard error of the mean.

city (Table I). Deoxycholate solubilized and reconstituted vesicles prepared according to Citri and Schramm (18) revealed binding properties similar but not identical to those of native turkey erythrocyte membranes (Table I).

The nature of the vesicles: Electron microscopical examination of the vesicles obtained from Lubrol-PX solubilized cyclase revealed that more than 50% of the vesicles were multilamellar, where the remaining were relatively small monolamellar vesicles, with an average diameter of 30 ± 10 nm.

DISCUSSION

General features of the reconstituted cyclase: A limited but highly reproducible protocol for the solubilization in Lubrol-PX and re-incorporation into phospholipid vesicles of turkey erythrocyte adenylate cyclase has been worked out. Provided that the Lubrol-PX to protein ratio in the final liposomes is below 0.02, one can detect partial hormone responsiveness. So far, we have never obtained vesicles free of Lubrol, and therefore could probably not achieve complete reversal with GTP and l-epinephrine.

Table I

BINDING OF ^{125}I-CYANOPINDOLOL TO NATIVE MEMBRANES AND RECONSTITUTED VESICLES

Nature of preparation	Maximal binding capacity pmol ^{125}I-CYP/mg	K_D pM
Native membranes[a]	1.12 ± 0.042	20.01 ± 2.38
Lubrol-PX vesicles[b]	0.09 ± 0.02	Not determined
Deoxycholate vesicles[c]	1.46 ± 0.25[d]	157 ± 33.2[d]
	0.55 ± 0.14[e]	85.6 ± 22.5[e]

The values were obtained by computer fitting to a Scatchard plot, and they represent an average of 4 independent Scatchard plots plus the standard errors of the mean.

[a]Native turkey erythrocyte membranes were prepared as previously described (14). Maximal binding and the dissociation constant were determined from a Scatchard plot of the binding data. ^{125}I-Cyanopindolol preparation was done according to Engel (19).

[b]Vesicles were those described in the text. The maximal binding was obtained at 0.6 nM ^{125}I-CYP, where the non-specific binding in the presence of 5 μM l-propranolol was 0.40 ± 0.01 pmol/mg and the total binding 0.49 ± 0.01 pmol/mg. The difference between total binding and non-specific binding was much higher at lower saturation of ^{125}I-CYP (for example, at 138 pM, ^{125}I-CYP total binding is 0.17 ± 0.008 pmol/mg and non-specific binding is 0.12 ± 0.005 pmol/mg, yielding specific binding of 0.05 ± 0.013 pmol/mg).

[c]Vesicles prepared by solubilization in deoxycholate and re-insertion into asolectin, as described by Citri and Schramm, Yielded very good binding isotherms for ^{125}I-CYP (18). Unfortunately, so far we could not obtain viable cyclase using deoxycholate instead of Lubrol-PX in the solubilization-reconstitution cycle.

[d]Reconstituted with asolectin.

[e]Reconstituted with phsophatidyl-ethanolamine:phosphatidyl-serine 3:1.

Nevertheless, the partial reversal obtained (Fig. 3) and its kinetics resemble those observed in native membranes. The reconstituted vesicles possess only about 6 to 7% of β-adrenergic receptors available to ^{125}I-CYP (Table I). The absence of more ^{125}I-CYP binding may be due either to the remaining Lubrol which might be strongly attached to the highly hydrophobic receptor or to its selective depletion into the SM-2 beads in the detergent removal step where 50% of the protein is lost (Fig. 2).

The phospholipid reconstituted cyclase activity is extremely stable, which makes the present protocol a reasonable basis for the continuation of these studies.

Phospholipid specificity: So far we were unable to reveal any differences between various phospholipid mixtures in their ability to incorporate and stabilize the reconstituted adenylate cyclase. Also, the different mixtures yielded similar kinetics and extent of hormone plus GTP induced reversal of the GppNHp/epinephrine active state. The possibility still remains that minor phospholipid(s) present in the native turkey erythrocyte membrane may be essential for efficient receptor to cyclase coupling.

The reconstituted β-receptor: From Table I it is apparent that reconstituted β-receptor possesses a significantly lower affinity towards ^{125}I-CYP. The β-receptor reconstituted into asolectin possesses an 8-fold lower affinity, while the receptor reconstituted into PE/PS (3:1) vesicles possesses a 4.2-fold lower affinity towards ^{125}I-CYP than native membranes.

The partial nature of the reversal reaction: The partial nature of the reversal is most probably the result of three phenomena or combination thereof: (a) only a fraction of the vesicles possess a functional receptor which can couple to the adenylate cyclase system; (b) the remaining Lubrol is sufficient to inhibit the coupling of hormone bound receptor with the cyclase system; and (c) the remaining detergent interferes with the binding of the agonist to the hydrophobic receptor.

Future experiments: The choice of a Lubrol type detergent was forced on us because this is so far the best detergent in which the turkey erythrocyte adenylate cyclase can be solubilized in a highly active and stable form. The disadvantage of using Lubrol-PX is the difficulty of its *complete* removal, which is essential for generating total receptor to cyclase coupling.

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