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# Functional implantation of a solubilized β-adrenergic receptor in the membrane of a cell

(receptor-coupled adenylate cyclase/cyclic AMP/membrane fusion/hybridization of membrane components/hormone and neurotransmitter action)

SARA EIMERL, GERA NEUFELD, MIRA KORNER, AND MICHAEL SCHRAMM

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

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**ABSTRACT** When the β-adrenergic receptor of turkey erythrocytes was solubilized by deoxycholate, it retained its potential to activate an adenylate cyclase system. Electron microscopy showed that true solubilization had apparently been achieved; no residual membrane or vesicle structure was found. After removal of deoxycholate and addition of phospholipid, the reprecipitated β-adrenergic receptor was implanted in the cell membrane of Friend erythroleukemia cells by using a chemical fusion method recently developed. Membranes prepared from the cells demonstrated 30-fold stimulation of the Friend cell adenylate cyclase by the implanted β-adrenergic receptor. The function of the indigenous prostaglandin $E_1$ receptor of the Friend cells was not much affected by the implantation of large amounts of the foreign receptor. Activity mediated by the β-adrenergic receptor reached 60% of the activity obtained with fluoride. The implanted receptor is therefore considered to be efficiently coupled to the adenylate cyclase system. The major difficulties hitherto preventing solubilization of hormone receptors and subsequent reconstitution of their function have been overcome by the approach developed in the present work. Conditions of solubilization need preserve only the receptor because all other components, even those unidentified as yet, can be supplied in excess by the adenylate cyclase system of the cell in which the receptor will be implanted. Subsequent recoupling of the receptor to the adenylate cyclase is performed in the native insoluble state of these molecules. Thus, the components need not be subjected to the hazards of solubilization in a common detergent as is usually required in reconstitution procedures. The importance of using implantation as an assay for a functional receptor in the course of purification and the likelihood that the procedure can be adapted to other receptors for hormones and neurotransmitters are discussed.

Solubilization of membrane receptors for hormones and neurotransmitters with subsequent reconstitution of function has remained a rather elusive goal (1–7). Specific binding of bungarotoxin (8), peptide hormones (4, 9), and β-adrenergic receptor blocking agents (2, 3) to solubilized membrane components containing the respective receptors has been demonstrated. Some success in reconstitution experiments has also been reported (8–10), but, even in these instances, evidence of actual solubilization (namely, complete disintegration of the original membrane structure) is often lacking. Expression of receptor function apparently requires the fragile interaction of several components and therefore damage by the solubilizing agent or its insufficient removal from only one of the components could prevent reconstruction of function. Even if some function could be regained, it would be difficult to assess its quantitative significance because there is little information as to which of the components is rate limiting.

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A different approach was therefore developed on the basis of earlier work (11, 12) on the hormone receptors that activate adenylate cyclase. It had been demonstrated that these receptors exist in the cell membrane as independent units. Furthermore, the receptors for different hormones from different cells were interchangeable; the receptor from one cell membrane could be coupled to the adenylate cyclase of another cell membrane by membrane fusion (11). On the basis of this information, for the present work the β-adrenergic receptor was solubilized without regard for the other components of the adenylate cyclase system. After removal of the solubilizing agent the receptor was adsorbed to a phospholipid vehicle and implanted in an intact cell by a modification of a recently developed procedure (11). The receiving cell membrane had high adenylate cyclase activity and thus contained all the components of the adenylate cyclase system but no β-adrenergic receptor (12). With the aid of this design, reestablishment of function of a solubilized β-adrenergic receptor was readily obtained.*

## EXPERIMENTAL

**Cell Preparations.** Friend erythroleukemia cells (Fc) have been described (12). Turkey erythrocyte membranes were prepared by the following modification of the procedure for frog erythrocytes (14). To the washed pellet of cells, DNase was added (1600 units/ml of packed cells) and the slurry was quickly poured into 20 vol of lysis medium (10 mM Tris, pH 8.0/2 mM mercaptoethanol/0.2 mM $MgCl_2$) at 23°C. After 20 min, the cell membranes were washed thrice at 4°C in 10 mM Tris, pH 8.0/0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)/2 mM mercaptoethanol.

**Solubilization of β-Adrenergic Receptor by Deoxycholate (Doc).** Turkey erythrocyte membranes were washed and suspended in (3 mg/ml) 10 mM 3-(*N*-morpholino)propanesulfonate (Mops), pH 7.5/1 mM mercaptoethanol. The membranes were treated with *N*-ethylmaleimide (MalNEt) in the above medium to inactivate the adenylate cyclase (11). After they were washed, the membranes (2 mg/ml) were incubated in the same medium with 6 mM $MgCl_2$ and 20 μM isoproterenol for 15 min at 30°C. After transfer of the reaction mixture to the cold, 1 vol of solubilization buffer (20 mM Mops, pH 7.5/0.5 M sucrose/0.2 mM EDTA/1 mM mercaptoethanol/10 μM phenylmethylsulfonyl fluoride) was added. The membranes were sedimented and resuspended in solubilization

Abbreviations: Fc cells, Friend erythroleukemia cells; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MalNEt, *N*-ethylmaleimide; Mops, 3-(*N*-morpholino)propanesulfonate; Doc, deoxycholate; $PGE_1$, prostaglandin $E_1$.

* A preliminary report of this work has been given at a symposium (13).

buffer. Sodium Doc (Koch Light), in the same buffer mixture, was added to a final concentration of 6 mg/ml; membrane protein was 2 mg/ml. After 10 min at 0°C the suspension was centrifuged at 200,000 × g for 30 min. Only the upper four-fifths of the supernatant was collected (0.9 mg of protein per ml) in order to prevent even the slightest contamination by sedimentable particles. Doc was adsorbed (15) on SM-2 resin (Bio-Rad), 1.2 g wet weight per ml of supernatant. After the suspension was shaken at 14 strokes per min for 1 hr, the SM-2 beads were removed by brief centrifugation. About 150 μg of Doc per ml remained in the supernatant.

**Adsorption of the β-Adrenergic Receptor to a Phospholipid Carrier.** After removal of the resin, to aliquots of the supernatant (0.75 mg of protein per ml) was added a phospholipid mixture without lysolecithin (11). Unless otherwise noted, 0.5 mg of phospholipid was added per ml of receptor preparation. After 5 min at 0°C, 1 M $MgCl_2$ was added to give a final concentration of 10 mM; 15 min later, the mixture was diluted with 2 vol of 10 mM Tris, pH 7.4/1 mM mercaptoethanol. The suspension was centrifuged at 20,000 × g for 15 min and the sediment containing the β-adrenergic receptor was retained. Protein yield was 20% of original membranes. Even without prior MalNEt treatment, the fluoride-stimulated adenylate cyclase activity in the final preparation was only 1–2% of that of the original membranes, and no isoproterenol activity could be detected.

**Implantation of β-Adrenergic Receptor in Fc Cells.** A suspension of $5 \times 10^6$ Fc cells was sedimented on top of the phospholipid-treated β-adrenergic receptor preparation. The implantation procedure was as described (11) for fusion of liver membranes with Fc cells, but $Ca^{2+}$ was omitted and $Mg^{2+}$ was 4 mM. Incubation was at 37°C and dilution was terminated after the cumulative addition of 10.5 ml of medium. The term "implantation" will be used whenever the solubilized resedimented receptor interacted with a cell; the term "fusion" will be used for cell–cell and native membrane–cell interactions.

**Determination of Adenylate Cyclase and β-Adrenergic Receptor Binding Sites.** All operations were at 4°C. After receptor implantation, the cells (which were in the process of lysis) were centrifuged for 10 min at 20,000 × g. The pellet was suspended in 3 ml of 10 mM Tris, pH 7.5/2 mM $MgCl_2$/0.1 mM EGTA/1 mM mercaptoethanol. After 5 min, the suspension was homogenized by six strokes in a loose Dounce homogenizer. The suspension was centrifuged as above and suspended in 0.5 ml of the same medium. Adenylate cyclase was assayed (16) at 37°C. Enzyme activites were measured in the presence of the following additions: 2 μM propranolol for basal activity; 50 μM isoproterenol for the β-adrenergic receptor; 10 μM prostaglandinE$_1$ (PGE$_1$) for the PGE$_1$ receptor; and 10 mM fluoride for enzyme activation bypassing the receptor. The amount of β-adrenergic receptor was determined with [$^{125}$I]iodohydroxybenzylpindolol (17). Most experiments and adenylate cyclase assays within each experiment were run in duplicate. Results varied < ±10%. In some of the figures the extent of variation of duplicates is also indicated by bars. Each experiment was repeated at least once.

**Electron Microscopy.** Samples of the supernatant and of the pellet formed after solubilization of the β-adrenergic receptor by Doc were treated with one-third volume of 8% (wt/vol) glutaraldehyde at 4°C for 2 hr. The glutaraldehyde solution was prepared in the same medium as used for solubilization. The samples were dialyzed against water for 48 hr at 4°C to remove glutaraldehyde, sucrose, Doc, and salts. After staining with 4% (wt/vol) phosphotungstate at pH 7 on Formvar/carbon-coated grids, the samples were examined with a Philips 300 microscope.

## RESULTS

Solubilization of membrane components, including receptors, is usually claimed on the basis of failure to sediment by centrifugation. However, a decrease in specific gravity of a membrane by loss of protein or binding of lipid or detergent and a decrease in size of vesicles might prevent sedimentation even though the structure was not solubilized. It therefore seemed essential to examine the ultrastructure of the material extracted from the turkey erythrocyte membranes by Doc. In the supernatant which contains the receptor, no membrane or vesicle structure could be found (Fig. 1). Because the soluble material becomes aggregated due to cross-linking by the glutaraldehyde fixative there is no information about the true sizes of the solubilized membrane components.

Preliminary titrations showed that the insoluble residue after extraction with Doc contained only 15% of the β-adrenergic receptor binding sites that were present in the native turkey erythrocyte membranes. It therefore seemed probable that the receptor had been solubilized. To check whether the soluble fraction contained functional receptor, Doc was removed, phospholipids were added, and the sediment after centrifugation was implanted in Fc cells. The β-adrenergic receptor solubilized by Doc was functional upon implantation, mediating a 28-fold increase in the activity of the Fc adenylate cyclase in presence of isoproterenol (Fig. 2). Solubilization by Doc without prior incubation of the membranes with isoproterenol gave a product that was nearly inactive upon implantation in Fc cells (not shown). Apparently, isoproterenol protects the β-adrenergic receptor during solubilization. On the basis of protein content, the solubilized receptor preparation was almost twice as active as the original membranes in activation of the Fc enzyme. The stimulation of adenylate cyclase by the β-adrenergic receptor in the original turkey erythrocyte membranes was about 25-fold. The stimulation, in the native Fc cells, by the indigenous PGE$_1$ receptor was about 15-fold. Thus, the technique of solubilization and implantation can produce a receptor-mediated activation of adenylate cyclase that is greater than that found naturally in these cells.

Fc cells subjected to the fusion–implantation procedure without addition of receptor did not demonstrate a response when the β-adrenergic agent isoproterenol was added (Fig. 2).

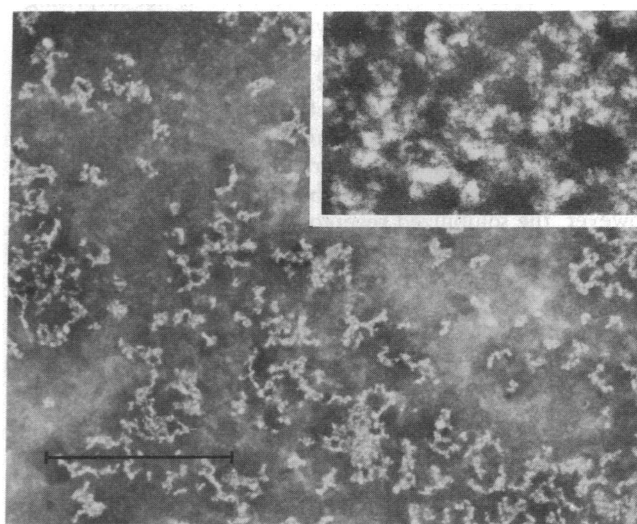

FIG. 1. Electron micrograph of the solubilized β-adrenergic receptor preparation, showing small aggregates formed by glutaraldehyde fixation. (Bar = 1 μm; ×25,000.) (*Inset*) Same preparation but at ×123,000; the material is amorphous. No membrane or vesicle structure was seen at any magnification.

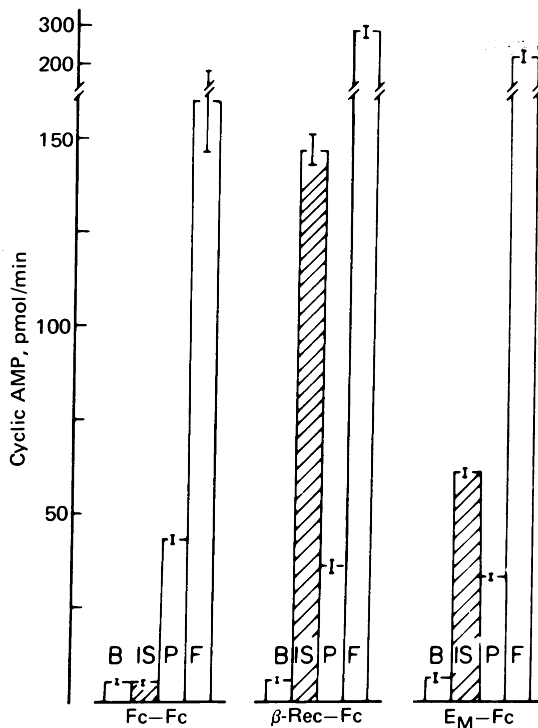


FIG. 2. β-Adrenergic receptor function after solubilization by Doc and coupling with Fc cell adenylate cyclase. An aliquot of MalNEt-treated turkey erythrocyte membrane was used for solubilization of the β-adrenergic receptor. After sedimentation with phospholipid, the β-adrenergic receptor (370 μg of protein) was implanted in Fc cells (β-Rec–Fc). Another aliquot of the erythrocyte membrane (250 μg of protein) was not solubilized but was treated with phospholipid as above and fused with Fc cells ($E_M$–Fc). Activities of membranes from native, untreated Fc cells were (pmol of cyclic AMP per min): basal activity (B), 3; isoproterenol-treated (IS), 3.5; $PGE_1$ receptor (P), 40; fluoride activation (F), 80. Data are shown as mean ± variation of duplicates.

The adenylate cyclase activities of the Fc cells varied somewhat with the culture batch. Therefore, a system of FC cells fused with each other was usually included in the experiments. The phospholipid-treated receptor preparation subjected to the implantation procedure without Fc cells never demonstrated measurable adenylate cyclase activity. This is not surprising because the extract preparation was derived from membranes treated with MalNEt which inactivates the adenylate cyclase irreversibly even in intact cells (12, 18). The MalNEt treatment is not essential, however, because the Doc extract precipitated with phospholipids and $Mg^{2+}$ contained negligible adenylate cyclase activity and yet potent transferable β-adrenergic receptor. Extensive binding studies have not been performed yet. However, the solubilized receptor reprecipitated with phospholipid showed a binding capacity of 140 fmol/mg of protein, compared to 150 fmol/mg for the same batch of original membranes. It is possible, of course, that some of the receptors in the reprecipitated preparation failed to reveal their binding sites because of aggregation.

The implanted β-adrenergic receptor showed a half-maximal response to epinephrine, $K_d$ (activity) = 1.3 μM (Fig. 3), compared to 2 μM in the original erythrocyte membrane (18). The $K_i$ (activity) for DL-propranolol also remained unchanged at 0.01 μM (not shown). It is thus obvious that solubilization of the receptor had no irreversible effect on its affinity for the hormone and the blocker. Dependence of adenylate cyclase activation on the relative amount of receptor added in the implantation system was also examined. A 35-fold stimulation over basal activity by hormone was achieved by the largest amount

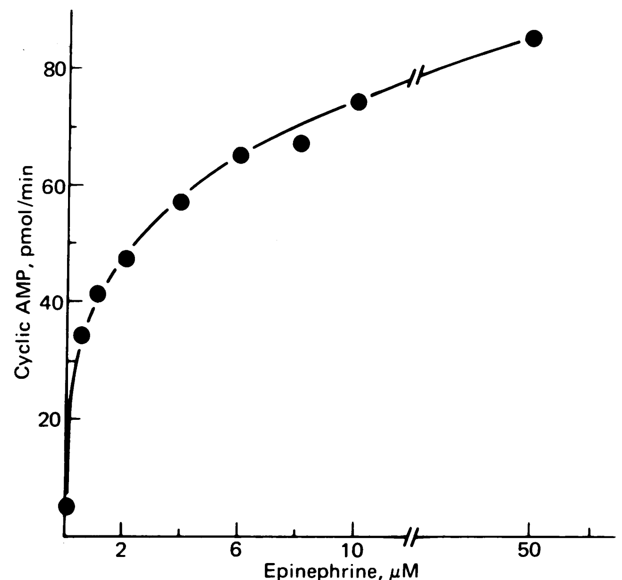


FIG. 3. Activation of Fc cell adenylate cyclase by the implanted β-adrenergic receptor as a function of epinephrine concentration. Phospholipid-adsorbed receptor preparation (185 μg of protein) was used for implantation in $5 \times 10^6$ Fc cells. Two such systems were pooled for adenylate cyclase assays. $K_d$ (activity) was $1.3 \times 10^{-6}$ M, as determined by plotting $1/V$ against 1/hormone concentration.

of receptor preparation added (Fig. 4). Even that amount caused only small increases in the basal and $PGE_1$-stimulated activities of the adenylate cyclase. Thus, implantation of a foreign receptor seems not to interfere much with the function of the indigenous receptor.

Additional experiments were performed to determine the amount of receptor preparation required for maximal adenylate cyclase stimulation. For this purpose the number of Fc cells in the implantation system was decreased and the amount of receptor preparation was kept within the upper range of Fig. 4. This was simpler to achieve than a further increase in the amount of receptor preparation. Apparent saturation of the Fc

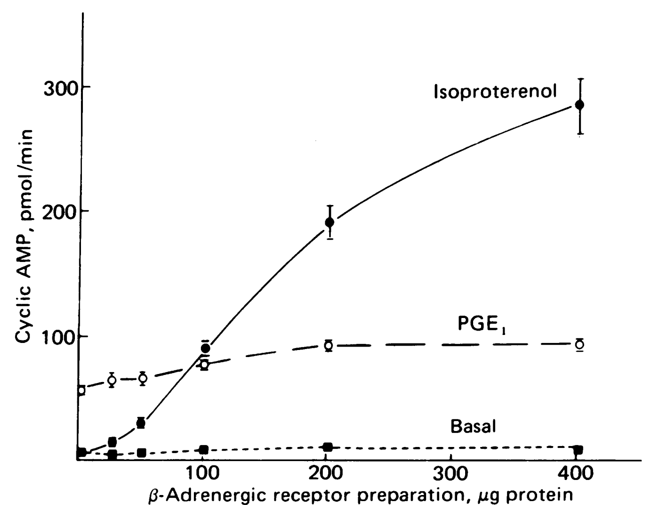


FIG. 4. Fc cell adenylate cyclase activity as a function of the amount of β-adrenergic receptor in the implantation system. Solubilized β-adrenergic receptor preparation was treated with phospholipids. Different amounts were placed in test tubes and sedimented. The pellets (protein content as shown on the abscissa) were implanted in Fc cells. Zero addition of receptor preparation shows the values obtained for Fc cells fused with each other, without phospholipid addition.

Table 1. Amount of solubilized β-adrenergic receptor preparation required to achieve maximal response of Fc cell adenylate cyclase

| β-Adrenergic receptor preparation, μg protein | Adenylate cyclase activity after receptor implantation in Fc cells, pmol cyclic AMP/min | | | | IS/F activity ratio |
|---|---|---|---|---|---|
| | B | IS | $PGE_1$ | F | |
| 0 | 1.5* | 1.4* | 8.4* | 35* | — |
| 150 | 1.7 | 37.0 | 13.0 | 66 | 0.55 |
| 300 | 2.6 | 50.0 | 14.0 | 85 | 0.59 |
| 450 | 2.2 | 43.0 | 11.0 | 77 | 0.56 |

Phospholipid treated β-adrenergic receptor in amounts shown in the table were mixed with Fc cells. The number of Fc cells in each implantation system was only $1.7 \times 10^6$ instead of the usual $5 \times 10^6$.

* Fc cells fused with each other.

system by implanted receptor could be achieved (Table 1). The isoproterenol/fluoride activity ratio shows that the implanted receptor can activate the system with an efficiency approaching 60% of that of fluoride. The activation relative to basal activity was less in the experiment of Table 1 than in the experiment of Fig. 4. The reason is not evident but might be associated with the lower enzyme activity of Fc cells in the experiment of Table 1.

The effect of the amount of added phospholipid on implantation and function of the receptor was also tested (Table 2). Maximal activation by isoproterenol was achieved when 240 μg of phospholipid was added. That amount also caused a marked increase in fluoride activation and a modest decrease in $PGE_1$ activation which decreased further with addition of larger amounts of phospholipid. It is possible that the receptor protein is precipitated preferentially by the phospholipid because most of the isoproterenol activation had already been achieved when 50 μg of protein was precipitated. In the experiment of Fig. 4, 144 μg of phospholipid was added per 200 μg of receptor-containing extract. This relative amount of phospholipid, according to Table 2, may be slightly below the optimum for β-adrenergic receptor implantation but, as discussed above, causes only mild changes in $PGE_1$ and fluoride activation.

## DISCUSSION

The present study shows that a solubilized component from turkey erythrocyte membranes confers β-adrenergic receptor activity on the adenylate cyclase of Fc cells. Several lines of evidence lead to the conclusion that the solubilized transferred component is indeed the β-adrenergic receptor. The Fc clone used was previously checked in various rigorous control experiments for the presence or appearance of β-adrenergic receptors, and no significant amount was detected (12, 19). Fusion of Fc cells with each other in the present study also failed to produce β-adrenergic receptor activity. In addition, the assumption that the turkey erythrocyte might have contributed some essential component but not the β-adrenergic receptor is unlikely because the Fc cells possess a perfectly active adenylate cyclase system coupled to the $PGE_1$ receptor (12, 18, 19) and therefore are not deficient in any relevant component.

In contrast to the absence of β-adrenergic receptor in the Fc cells there is strong evidence for the presence of active β-adrenergic receptor in the solubilized material from the turkey erythrocytes. Solubilization removed most of the β-adrenergic receptor binding activity from the membranes. After reprecipitation the solubilized material contained the β-adrenergic receptor as measured by specific binding. Material obtained from membranes that had not been incubated with isoproterenol prior to solubilization was essentially inactive in the implantation experiment. It would seem unlikely that a component other than the β-adrenergic receptor requires interaction with isoproterenol to withstand solubilization. Taken together, the above considerations leave little doubt that the β-adrenergic receptor from turkey erythrocytes was solubilized and functionally implanted in the Fc cell membrane.

The approach developed in the present work makes no demands on the solubilization procedure except that it should preserve a potentially functional receptor. All other components, including those not known to date, can probably be supplied in excess, in their native state, by the membrane of the intact Fc cell which possesses a normal adenylate cyclase system, as noted above. This approach became feasible because of the special features of the membrane hybridization analysis developed recently (12, 20). After membrane-to-membrane fusion was achieved (11), we further reasoned that perhaps the process could be extended to a situation in which a hydrophobic insoluble molecule would be implanted in a cell membrane. In that case there should be no more need for all the components to interact in an artificially solubilized state. The desirable component might be solubilized for the purpose of purification or other manipulations and subsequently insolubilized for interaction, by the implantation technique, with the other components. Thus, interaction of the components would take place in their native insoluble state.

Additional advantageous features of the implantation approach become evident when the characteristics of hormone receptors are considered. Loss of hormonal response has been reported in almost every paper dealing with solubilization of adenylate cyclase systems. It was therefore tacitly assumed that

Table 2. Effect of increasing amounts of phospholipid on implantation of the β-adrenergic receptor

| Phospholipid added, μg | Protein in phospholipid precipitate, μg | Adenylate cyclase activity after receptor implantation in Fc cells, pmol cyclic AMP/min | | | |
|---|---|---|---|---|---|
| | | B | IS | $PGE_1$ | F |
| 120 | 50 | 10 | 130 | 90 | 370 |
| 240 | 110 | 10 | 180 | 80 | 530 |
| 480 | 140 | 10 | 170 | 60 | 500 |
| 960 | 160 | 6 | 120 | 30 | 420 |
| —* | — | 10 | 10 | 90 | 290 |

After the removal of Doc, 0.3-ml aliquots of receptor-containing extract (220 μg of protein) were treated with increasing amounts of phospholipid in a final volume of 0.5 ml. The pellet after centrifugation was used for fusion with Fc cells; a duplicate tube was used for determination of protein. Column headings as in Fig. 2.

* No receptor-containing extract added. Data are activities of membranes from Fc cells fused with each other.

the receptors are extremely labile molecules. Most surprisingly, the present work shows that the receptor survives solubilization in the presence of Doc at 6 mg/ml or at 3 mg of Doc per mg of membrane protein. Several membrane enzymes that are purified by Doc treatment are quite labile at these detergent concentrations (21, 22). Because adenylate cyclase activity is also lost under such conditions, Doc would have been ruled out as a solubilizing agent for a functional receptor were it not for the implantation approach developed in the present work.

A few additional features are noteworthy. The relative stimulation over basal adenylate cyclase activity obtained through the implanted receptor can reach 30-fold, a potency rarely found for an indigenous receptor. The procedures are readily reproducible in that three of the authors independently solubilized and implanted the receptor without any difficulty. The way now seems open to start purification of a functional receptor by using its action after implantation as an assay. Such a procedure would give assurance that the component that is being purified is indeed a true functional receptor. Our previous studies on hybridization of hormone receptors with adenylate cyclase from different cells revealed that the receptors are a family of interchangeable molecules, suggesting perhaps a similar molecular structure (12, 18, 20). One would expect that the approach of solubilization and implantation described here will also be applicable, after necessary modifications, to other hormone receptors and additional molecules of biological importance in cellular membranes.

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