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Coupling of catecholamine receptor from one cell with adenylate cyclase from another cell by cell fusion

(β-adrenergic receptor/hormone receptor/3′:5′-cyclic AMP/guanyl nucleotide site/cell membrane)

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ABSTRACT **The experiments test the hypothesis that β-adrenergic receptor is an independent unit that can be transferred from one adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] system to another. Turkey erythrocytes in which the catalytic activity of adenylate cyclase had been inactivated by *N*-ethylmaleimide or by heat contributed the β-adrenergic receptor. Friend erythroleukemia cells (F cells) that possessed no measurable β-adrenergic receptor contributed the adenylate cyclase. The erythrocytes in which the enzyme had been inactivated were fused with the F cells by Sendai virus. The cell ghosts of the fused preparation demonstrated adenylate cyclase activity which was strikingly enhanced by isoproterenol. Controls of fusion of F cells with each other or with human erythrocytes failed to show a response to isoproterenol. It was therefore concluded that the β-adrenergic receptor of the turkey erythrocytes must have become functionally coupled to the adenylate cyclase of the mouse F cells. Activation by isoproterenol was demonstrable within a few minutes after fusion, and inhibitors of protein synthesis had no effect. Thus, coupling must have occurred between the preexisting components.**

The findings suggest that it may be possible in the future to confer on cells that possess an adenylate cyclase system new hormonal responses by inserting a receptor into their cell membrane. It is proposed that the procedure of massive heterologous cell fusion, as used in the present study, can be used to analyze the function of other cell membrane components.

Adenylate cyclase systems [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in eukaryotic cells are activated by many different hormones (1). In this respect these systems are unique in nature since no other type of enzyme is known that responds to so many different effectors. In certain instances, several hormones all act on the same cell to activate the adenylate cyclase (1). Various theories therefore suggested that the hormone receptors are discrete units, separate from the catalytic unit of the enzyme (2–5). However, to date, no convincing proof for these ideas has been presented. An earlier conclusion that different hormones in the fat cell all activate one adenylate cyclase unit (6) has become less certain in the light of recent findings (7). It became clear that a new experimental approach is needed to establish the relationship between the hormone receptor and the catalytic unit of the adenylate cyclase. We reasoned that it should be possible to combine a hormone receptor from one cell with the catalytic unit of the enzyme from another cell if indeed the receptor is an independent unit. For this purpose two different types of cells would be combined by cell fusion (8–10). One cell would donate the hormone receptor after inactivation of the catalytic enzyme unit. The other cell, not possessing that hormone receptor, would contribute the catalytic unit. It should be emphasized that the experimental approach consists solely of mixing the preexisting components of the two membranes in the absence of function of the genetic apparatus or of protein synthesis. Interesting studies on the genetic control of receptor synthesis of the progeny of heterokaryons formed by cell fusion have been published (11). A recent report also suggests that the β-adrenergic receptor is a product of a gene different from that of the adenylate cyclase (12). The findings communicated in the present paper show that the catecholamine receptor in turkey erythrocytes can be functionally coupled to the catalytic unit of adenylate cyclase in mouse erythroleukemia Friend cells (F cells).

Abbreviations: F cells, Friend T3C12 mouse erythroleukemia cells; MalNEt, *N*-ethylmaleimide; DCC, *N,N′*-dicyclohexylcarbodiimide.

MATERIALS AND METHODS

Reagents. F-12 culture medium was obtained from GIBCO New York; *N,N′*-dicyclohexylcarbodiimide (DCC) was a product of Fluka, Switzerland. Sendai virus was produced in chick embryos (13). Other reagents were as previously described (14).

Turkey Erythrocytes. Cells from fresh blood in heparin were washed twice in K^+ salt medium (mM: KCl 135, NaCl 5, $MgCl_2$ 0.8, Tris buffer 20, pH 7.4). The buffy coat was removed. Final suspension was in K^+ medium at a concentration of 20% vol/vol (8×10^8 cells per ml).

F Cells. The culture was kindly given to us by Dr. P. Leder and was grown in F-12 medium supplemented with 10% calf serum (15). Cells were harvested when their concentration reached 5×10^5 per ml. Cells were washed twice in Na^+ salt medium (mM: NaCl 135, KCl 5, $MgCl_2$ 0.8, Tris buffer 20, pH 7.4) and were finally suspended in the same medium at 1.5×10^7 cells per ml.

***N*-Ethylmaleimide (MalNEt) Treatment.** Previous studies on adenylate cyclase showed that its activity was abolished by reagents that react with SH groups (16, 17). However, MalNEt at 1 mM was not sufficient to fully inactivate the adenylate cyclase of intact erythrocytes (16). One milliliter of the erythrocyte suspension was therefore incubated with 10 mM MalNEt for 10 min at 0° to inactivate the catalytic unit of the enzyme. Subsequently, 10 ml of K^+ medium containing 15 mM mercaptoethanol were added. After 15 min the cells were sedimented by centrifugation for 3 min at $400 \times g$ and resuspended in 1 ml of K^+ medium. No adenylate cyclase activity could be detected after MalNEt treatment.

Fusion System. Two milliliters of incubation mixture in a 20-ml siliconized glass vial contained: 3×10^8 erythrocytes treated with MalNEt, 3×10^6 F cells, 9600 hemagglutinating units of Sendai virus, 2 mM $MnCl_2$ in a mixture of the Na^+:K^+ salt media, 1:4 vol/vol, respectively. Virus was added at 0° as the last component. After 1 min the mixture was transferred to 37° and was incubated with shaking (70 rotations/min). Aliquots were observed by phase microscopy. When most of the F cells fused (20–30 min), the mixture was transferred to ice.

Preparation of Cell Ghosts at 0°. Four milliliters of K^+ salt medium were added to 2 ml of fusion system and the mixture was centrifuged for 3 min at $200 \times g$. The cells were lysed by

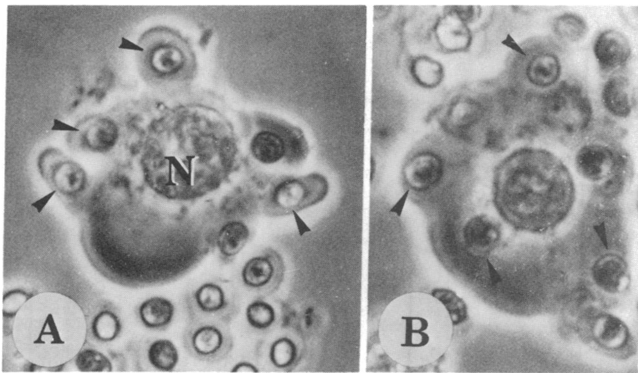

FIG. 1. Heterologous fusion of F cells with turkey erythrocytes. (A) Several erythrocytes (arrows) are in the process of fusion with a single F cell that contains a large nucleus (N). (B) A more advanced stage of fusion. The small nuclei (arrows) of the erythrocytes have become incorporated in the body of the F cell. Magnification ×2000.

suspending the pellet in 5 ml of hypotonic 30 mM Tris medium (pH 8.1) containing 5 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM mercaptoethanol. After centrifugation at 1500 × *g* for 3 min, the pellet was washed once more in 10 mM Tris buffer with additions as above. The pellet was finally suspended in 0.4 ml of 10 mM Tris medium containing the above additions. Mixtures containing only erythrocytes or only F cells were treated exactly by the same procedure. The cell ghosts that still contained the nucleus were used for assay of adenylate cyclase.

Adenylate Cyclase. Activity was measured on a 50-μl aliquot of the cell ghost suspension by the procedure of Salomon *et al.* (18). Basal activity was measured in the presence of 10 μM propranolol to block occasional low endogenous epinephrine present in the erythrocytes. Catecholamine activation was measured in the presence of 50 μM isoproterenol, and fluoride activation in the presence of 10 mM NaF. Incubation was for 10 min at 37°; the reaction was terminated by 3 min of boiling. All results of adenylate cyclase activity were expressed as pmol/min per aliquot of the cell ghost suspension taken for assay (the equivalent of 4×10^5 F cells and/or 4×10^7 erythrocytes). Activity was linear with respect to time and to amount of cell ghosts within the range used for these experiments. The average activities of F cell ghosts were 60, 60, and 400 pmol/mg of protein of cell ghosts per min for basal, isoproterenol, and fluoride activation, respectively. One milligram of protein of F cell ghosts was equivalent to 10^7 cells. Adenylate cyclase activity varied ±50% in different batches of cells. In initial experiments we observed that ghosts of F cells that had been treated with virus showed a higher adenylate cyclase activity than ghosts of cells not treated with virus. The untreated ghosts were perhaps not freely permeable to the ATP substrate required for assay of activity. The activity could be increased by freeze-thawing or by adding a small amount of virus. Therefore, a small amount of virus that does not produce fusion was added to all control systems containing F cells (200 hemagglutinating units per 3×10^6 cells). Erythrocyte ghosts prepared from cells not treated with MalNEt showed rates of 1, 40, and 50 pmol of 3′:5′-cyclic AMP per mg of protein of cell ghosts per min for basal, isoproterenol, and fluoride activation, respectively. One milligram of protein of erythrocyte ghosts was equivalent to 6×10^7 cells.

RESULTS

A phase contrast micrograph of a single F cell in the process of heterologous fusion with several MalNEt-treated erythrocytes is shown in Fig. 1. Cell ghosts prepared from such heterologous fusion products showed strong stimulation of adenylate cyclase by isoproterenol (Fig. 2). This is in spite of the fact that neither of the two cells undergoing fusion showed activation of the

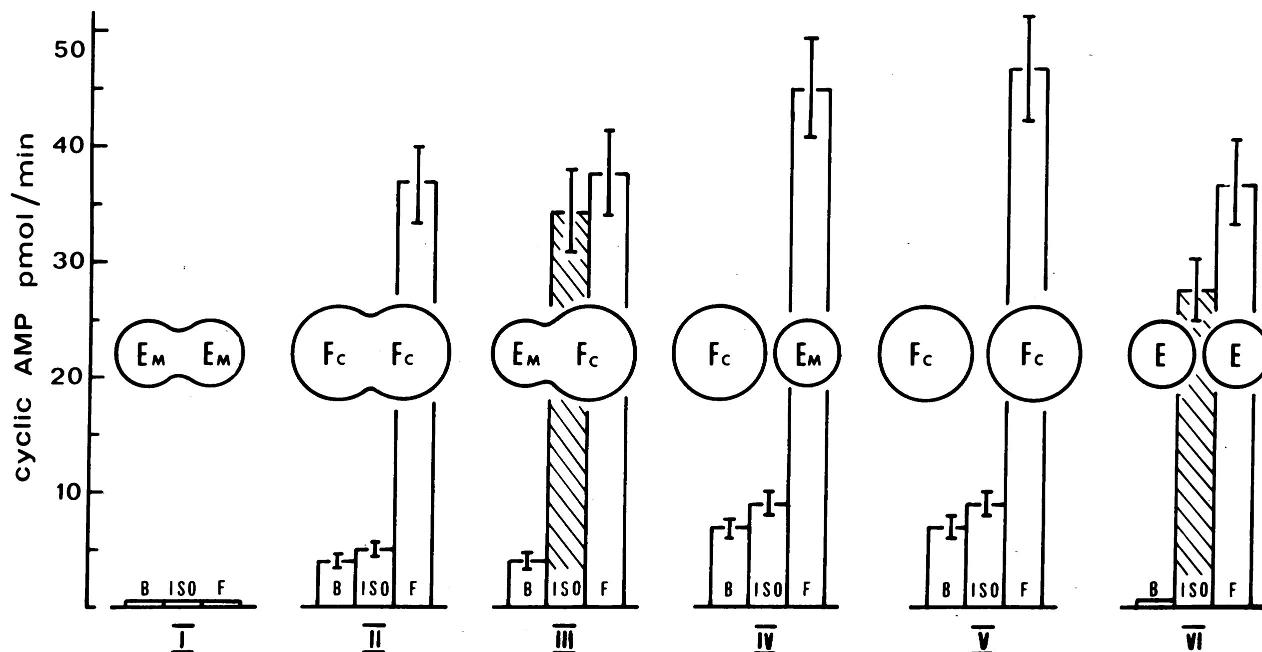

FIG. 2. Coupling of the catecholamine receptor of erythrocytes to adenylate cyclase of F cells by fusion. Incubation systems: (I) MalNEt-treated erythrocytes (E_M) fused among themselves; (II) F cells (Fc) fused among themselves; (III) heterologous fusion of MalNEt-treated erythrocytes with F cells; (IV) cells as in III but without fusion; (V) F cells only; (VI) erythrocytes without MalNEt treatment (E). The conditions in each system are schematically described by the circles or "fused" circles in each set of columns. The figure shows the adenylate cyclase activities of the cell ghosts prepared from the various systems. At the base of each column the activating agent added to the adenylate cyclase assay system is specified: B, basal activity without addition; ISO, isoproterenol; F, fluoride. Bars at the top of the columns show the range of activities of duplicate fusion systems. Significant activation of adenylate cyclase by isoproterenol is emphasized by hatched columns.

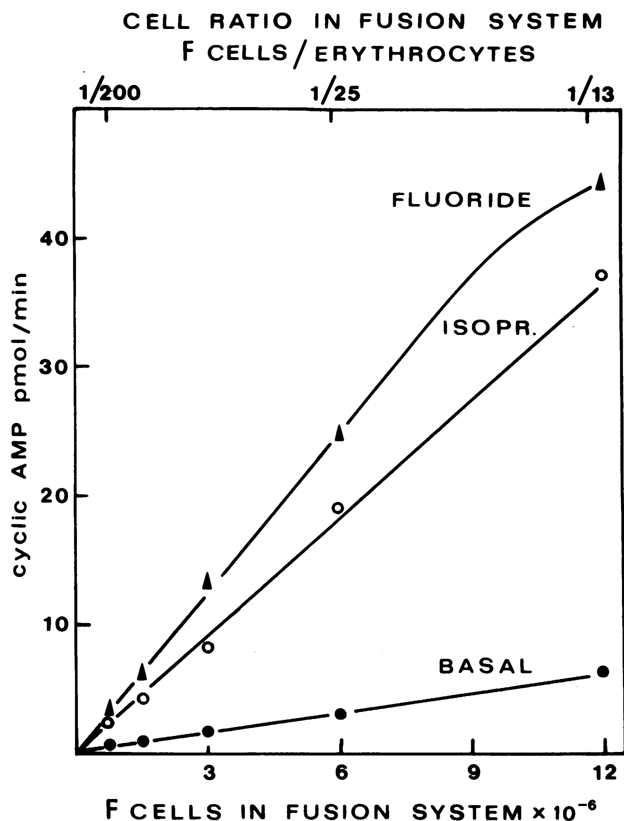

FIG. 3. Coupling of the catecholamine receptor as a function of the number of F cells per erythrocyte in the fusion system. One milliliter of incubation mixture contained 1.5×10^8 turkey erythrocytes treated with MalNEt plus increasing amounts of F cells as shown in the figure. The amount of virus was 4800 hemagglutinating units.

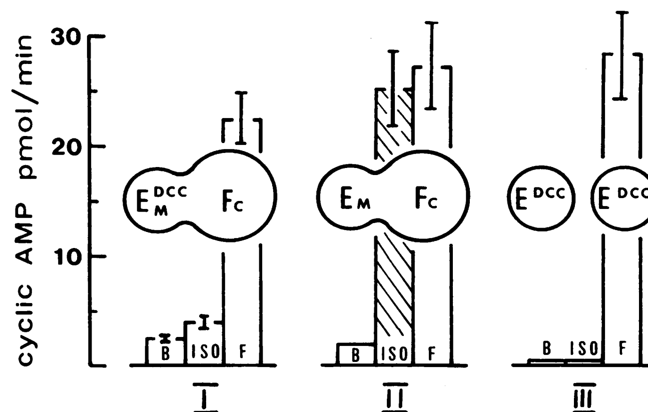

FIG. 4. Failure of DCC-treated erythrocytes to contribute the catecholamine receptor. Erythrocytes (5×10^8 per ml in K^+ medium) received DCC from a fresh solution in ethanol to give a final concentration of 0.2 mM and 1% ethanol. After 30 min at 25° the cell suspension was diluted 10-fold in the K^+ medium, sedimented at $400 \times g$ for 4 min, and resuspended in the K^+ medium at 8×10^8 cells per ml. Aliquots were taken for adenylate cyclase assay as well as for MalNEt treatment followed by cell fusion. No lysis or cell damage was observed at any stage. Incubation systems: (I) erythrocytes treated with DCC followed by MalNEt (E^{DCC}_M) fused with F cells (Fc); (II) MalNEt-treated erythrocytes (E_M) fused with F cells; (III) DCC-treated erythrocytes (E^{DCC}) fused with each other. All other symbols and presentations are as in legend to Fig. 2.

enzyme by catecholamine. The adenylate cyclase activity of the erythrocytes had been abolished by MalNEt, and the enzyme of the F cells did not respond to isoproterenol. Homologous fusion of MalNEt-inactivated erythrocytes among themselves, or fusion of F cells among themselves, or fusion of MalNEt-treated F cells with MalNEt-treated erythrocytes failed to produce isoproterenol activation of adenylate cyclase. In fused or unfused F cells, isoproterenol produced an insignificant 5% increase over the basal rate of adenylate cyclase (T test; $n = 26$, $P < 0.05$). The findings in Fig. 2 therefore justify the conclusion that the catecholamine receptor from the erythrocytes has functionally combined with the catalytic unit of the adenylate cyclase of the F cell. This conclusion is supported by the independent observation that cell ghosts of F cells have no measurable β-receptor. The amount of receptor was determined by measuring the binding of [^{125}I]iodohydroxybenzyl pindolol according to Aurbach and his collaborators (19, 20).

Fig. 3 shows the basal, fluoride, and isoproterenol activities in fusion systems in which a fixed amount of erythrocytes is mixed with increasing amounts of F cells. The ratio of isoproterenol activation to basal and fluoride activation is constant throughout most of the range studied. Activation by isoproterenol is dependent on transfer of the receptor to the adenylate cyclase of F cell, while basal activity and activation by fluoride are not dependent on such a transfer. The finding therefore suggests that within the range investigated sufficient amounts of receptor were transferred to achieve maximal hormonal activation. Further experiments tested whether the intact catecholamine receptor of erythrocytes is essential for hormonal activation of the adenylate cyclase of F cell. Human erythrocytes, which seem to possess no significant quantity of catecholamine-activated adenylate cyclase, failed to cause a catecholamine response upon fusion with F cells. Furthermore, turkey erythrocytes in which the catecholamine response had been inactivated by DCC also failed to produce a hormone response upon fusion with F cells (Fig. 4I). The carbodiimide inactivated the isoproterenol response in the turkey erythrocytes by 98%. In contrast, fluoride activation was inhibited less than 15% by DCC. The extent of heterologous fusion was essentially unaffected by DCC. The interaction of the catecholamine receptor with DCC has been studied in detail and will be published elsewhere.

We have previously shown that hormone activation of adenylate cyclase of turkey erythrocytes drops steeply when the temperature of incubation is reduced below 26° (21). In the present study we found that this temperature sensitivity disappears when the hormone receptor becomes coupled to the adenylate cyclase of the F cells. The ratio of adenylate cyclase activity produced by isoproterenol at 37° to that at 20° was 11 in erythrocytes and dropped to 2.8 after heterologous fusion. These findings suggest that the newly established hormone activation takes place within a more fluid domain contributed by the F cells. Additional experiments showed that transfer of the receptor was not affected by preincubation of erythrocytes and F cells with the inhibitors of protein synthesis, cycloheximide (0.2 mM) or emetine (0.01 mM). Thus, the preexisting components apparently suffice for the coupling of the erythrocyte receptor to the adenylate cyclase of F cells. Finally, it should be noted that experiments can also be conducted with erythrocytes in which the catalytic unit is inactivated by heat treatment instead of by MalNEt. Effective transfer of the receptor after heat treatment is shown in Fig. 5. Washing of the heat-treated erythrocytes or their incubation for 30 min at 37° did not cause reactivation of the adenylate cyclase system in the absence or presence of virus.

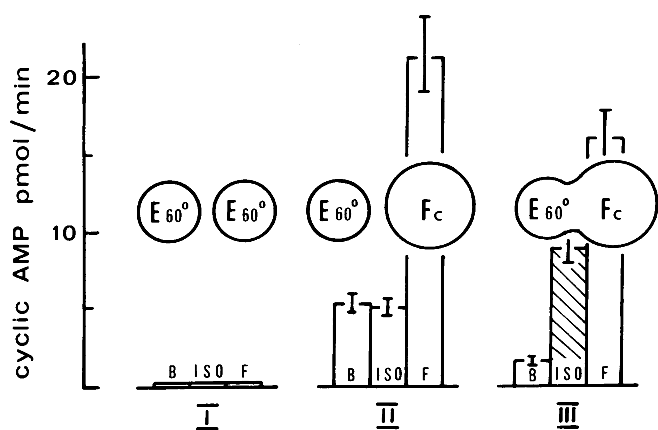

FIG. 5. Ability of heat-treated erythrocytes to contribute the catecholamine receptor. One milliliter of erythrocyte suspension (8 $\times 10^8$ cells per ml) was heated in a water bath at 60° for 5 min followed by transfer to ice. Higher temperatures could not be used for inactivation because of coagulation of cell content. Fusion was performed as described in *Materials and Methods*. Incubation systems: (I) erythrocytes heated at 60° ($E_{60°}$); (II) erythrocytes heated at 60° mixed with F cells; (III) fusion of heated erythrocytes with F cells. All other symbols and presentations are as in legend to Fig. 2.

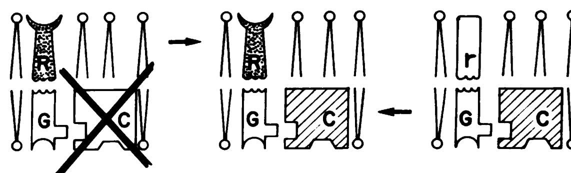

FIG. 6. The adenylate cyclase system is shown embedded in the lipid bilayer of the cell membrane. The lipid bilayer is symbolized by the circles with twin tails. Top faces the outside and bottom faces the inside of the cell. The hormone receptor R, is coupled at its base to the guanyl nucleotide binding component G. The latter in turn interacts with the catalytic enzyme unit C. The adenylate cyclase system on the left represents the turkey erythrocyte system in which the catalytic unit has been inactivated by MalNEt (C is crossed out). The system on the right represents the F cell bearing an unidentified receptor, r. The center of the figure shows a hybrid adenylate cyclase system obtained by heterologous fusion, whereby R is derived from the system on the left and C from the system on the right. The other hybrid system which might form, r coupled to the inactivated catalytic unit, has been omitted for clarity.

DISCUSSION

The present work suggests a novel use of cell fusion: the study of a complex system by combining components from two types of cells brought together by the fusion process. Thus, if each of the two cells lacks a different component, fusion will restore the function of the system. Whether the components were missing because of a mutation or because of intentional specific inactivation is irrelevant to the procedure. This method applied to the cell membrane appears to have great potential for investigating mutants, discovering unknown components of a complex reaction system, exchanging subunits of enzymes, and analyzing compatibility of components from different cell species.

The essence of the present study is that a cell in which the catalytic unit of the adenylate cyclase has been inactivated can contribute its catecholamine receptor to join an active catalytic unit of another cell. Alternative explanations of the results can be ruled out as follows. F cells showed no catecholamine receptor, as determined by binding of iodohydroxybenzyl pindolol (19, 20). Also, F cells fused by virus with each other or with human erythrocytes or with DCC-treated turkey erythrocytes failed to show catecholamine activation of adenylate cyclase. These experiments exclude the possibility that F cells contain a latent catecholamine receptor that can become active after fusion.

The other alternative, that the inactivated adenylate cyclase of the turkey erythrocyte can undergo reactivation to respond to isoproterenol, was also ruled out. Fusion of inactive turkey erythrocytes with each other did not revive the system. Furthermore, heat inactivation of the catalytic unit produced the same results as MalNEt inactivation.

Thus, it should be concluded that indeed the catecholamine receptor of the turkey erythrocytes became coupled to the catalytic unit of the F cells. The results further show that both MalNEt and heat treatment inactivated the enzyme but not the receptor. For clarity of further discussion, a hypothetical scheme of the adenylate cyclase system is presented (Fig. 6). The scheme proposes that the receptor unit in the outer half of the membrane bilayer is coupled to the guanyl nucleotide site located in the inner half of the bilayer. Findings supporting this concept have been published (14, 21-23). The scheme further suggests that the guanyl nucleotide site, when occupied by GTP, interacts with the catalytic unit to activate it. It is not clear which of the two original cells contributes the guanyl nucleotide site to the new hormone coupled system. Further work with various adenylate cyclase systems will be required in order to define the limitations of heterologous coupling between receptors and adenylate cyclase systems of different cells. So far, it has been demonstrated that the catecholamine receptor from a turkey erythrocyte is readily coupled to the adenylate cyclase of a mouse erythroleukemia cell in culture. If receptors to other hormones will also activate the adenylate cyclase of F cells, it is reasonable to assume that they do so through a common mechanism. According to the scheme shown in Fig. 6, all different receptors would have at their base a common attachment site by which they couple to the guanyl nucleotide binding unit. Such receptor molecules could be like the immunoglobulins, which all have a common structure, differing only in the area of the antigen combining site. According to this concept, it may become possible to confer on a cell a new hormone responsiveness by inserting in its membrane a hormone receptor which, though foreign to that cell, will couple to its adenylate cyclase system.

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