A PROBE FOR THE ORGANIZATION OF THE β-ADRENERGIC RECEPTOR-REGULATED ADENYLATE CYCLASE SYSTEM IN TURKEY ERYTHROCYTE MEMBRANES BY THE USE OF A COMPLEMENTATION ASSAY

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1. Introduction

From investigations of the adenylate cyclase systems in several membranes it is now clear that the system consists of at least 3 protein components: the hormone receptor (R), the guanine nucleotide regulatory component (N), and the catalytic unit (C) [1–5]. Despite the existence of these 3 types of units in all systems studied so far, the nucleotide component shows several functional differences in different types of plasma membranes [6,7]. For example, the adenylate cyclase system of the turkey erythrocyte plasma membranes, in contrast to the liver and fat cell plasma membranes, displays the unusual property of binding the guanine nucleotide GDP tightly [6], presumably to the N component. Possibly as a result of this tight binding, Gpp(NH)p, which activates many adenylate cyclase systems rapidly and completely, activates turkey erythrocyte membrane adenylate cyclase poorly and then only with a lag of several hours. If the activation of the catalytic unit were limited by the stoichiometry of the N unit relative to C, a lag in activation would be expected. A way of testing this proposition would be to add exogenous nucleotide unit to the turkey erythrocyte membrane and to examine the functional consequences. Three questions need to be answered:

(i) Would the added N unit be capable of functional linkage with the catalytic unit?

(ii) Could the tight binding of GDP, an inhibitor of Gpp(NH)p activation, be reversed by the addition?

(iii) Would the added N unit influence the guanine nucleotide exchange reaction, which is specifically promoted by the β-adrenergic receptor [8,9]?

In order to answer these questions it was necessary to choose a source for donor N and a method for complementation. Possibilities for these were suggested by recent experiments [7,10] with the human erythrocyte membrane which contains N as judged by the following criteria:

(i) It reconstitutes both Gpp(NH)p and fluoride responses with cyc^-.

(ii) Labeling with cholera toxin and [α-^{32}P]NAD leads to the incorporation of label into a single band of M_r 42 000 which is thought to represent the N unit.

(iii) Toxin modification also leads to a diminution of fluoride response and an enhancement of GTP activation when the modified human erythrocyte membrane is complemented with cyc^-.

It can also be shown that the human erythrocyte membrane contains the N units in an amount equivalent to that found in other membranes but by contrast contains very little adenylate cyclase catalytic activity or β-adrenergic receptor [10]. The mode of complementation is also suggested by these experiments in which functional linkage was obtained merely by mixing vesicles in the presence of activating ligands.

In these experiments we show that the human erythrocyte membrane upon addition to the turkey erythrocyte membrane causes an increase in Gpp(NH)p activation. The functional linkage between donor N and the acceptor C occurs without the release of GDP from the recipient membrane N unit and without

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participation of the hormone promoted guanine nucleotide exchange reaction.

2. Experimental

ATP synthesized from adenosine was purchased from Sigma; [α-^{32}P]ATP was purchased from ICN. Human erythrocyte membranes were prepared and the adenylate cyclase assayed as in [6,12]. Treatment of membranes with cholera toxin was by a modification [10] of the method in [13]. Protein measurements were following [14]. The complementation assay was done by the general method in [10], except that here we used turkey erythrocyte membranes as acceptor and human erythrocyte membranes as N component donor. Functional interaction occurred upon mixing, as described below.

3. Results

Fig.1 shows the time course of Gpp(NH)p-activation of adenylate cyclase for the turkey erythrocyte membrane alone and in the presence of a saturating amount of the human erythrocyte membrane. Increased concentrations of the donor human erythrocyte membrane caused increased activation, and the process was saturable. Under the conditions used, saturation of the response occurs at a ratio of 3.3 for human erythrocyte protein to turkey erythrocyte protein (not shown). This value reflects a contribution relating to the relative amounts of cyclase components in these two membranes [10]. The lag observed in approaching steady state is characteristic of the complementation process when it involves only vesicle mixing, and is thought to represent the time required for functional linkage between components [10]. Here, steady state of complementation is reached in ~10 min.

The dose–response for activation by Gpp(NH)p is shown in fig.2. Because of the complex nature of the assay, full time courses are shown. The half-maximal concentration for Gpp(NH)p activation was 2 μM. At these concentrations, no activation by Gpp(NH)p of the acceptor turkey erythrocyte membrane was observed, in the absence of the hormone. This concentration is close to the value obtained for the activation process in other membranes [3].

These results suggested that the nucleotide component in the donor human erythrocyte membrane became functionally coupled to the catalytic unit in the turkey erythrocyte membrane. As a further test, the donor membrane was treated with cholera toxin and NAD, prior to addition to the turkey erythrocyte membrane. When the control membrane is added to the acceptor turkey erythrocyte membrane, some GTP activation is indeed conferred but this is well below the levels obtained with Gpp(NH)p as the activating ligand. As seen in table 1, after modification with cholera toxin, the GTP response of the system was magnified. A variety of other membranes containing complete adenylate cyclase systems show similar functional responses to cholera toxin [16]. Thus, the effects in complementation reported here are indeed due to the cyclase-related guanine nucleotide component in the

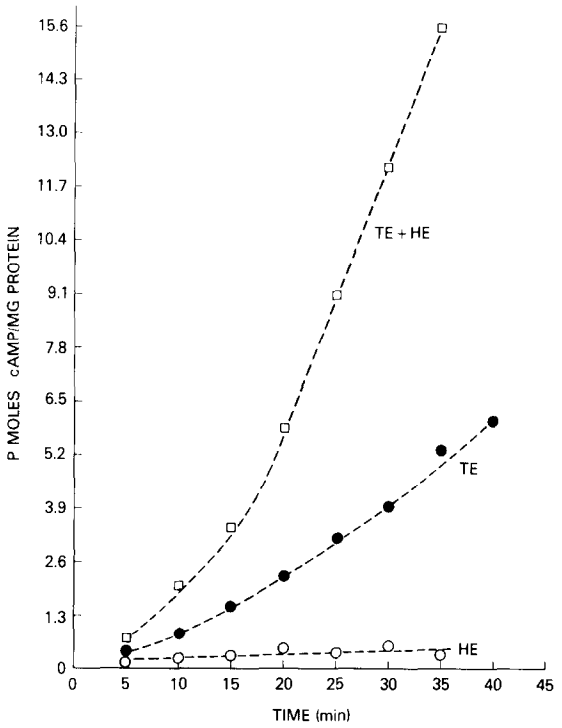

Fig.1. Turkey erthyrocyte membranes (•), human erythrocyte membranes (○), or both turkey and human erythrocyte membranes (□) were diluted at 0°C to form a suspension with the following composition: 0.17 mg/ml of turkey erythrocyte membrane protein; 1.07 mg/ml of human erythrocyte membrane protein; [α-^{32}P]ATP (100–500 cpm/pmol); 0.1 mM ATP; 10 mM $MgCl_2$; 0.1 mM Gpp(NH)p; 1mM DTT; 5 mM creatine phosphate; 33 units/ml of creatine phosphokinase; 10 mM Tris–HCl pH 7.5. The mixture was incubated at 30°C; at the times indicated, 0.100 ml aliquots were withdrawn into 'stopping solution' and cyclic AMP isolated [12].

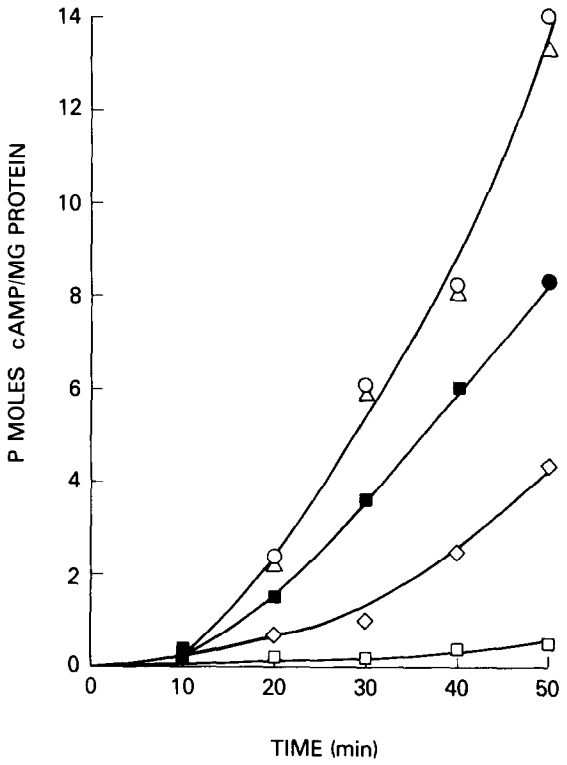

Fig.2. Human erythrocyte and turkey erythrocyte membranes were mixed (6.6:1) and assayed as in fig.1, except that Gpp(NH)p was: none (□); 10^{-7} M (◇); 10^{-6} M (■); 10^{-5} M (△); or 10^{-4} M (○).

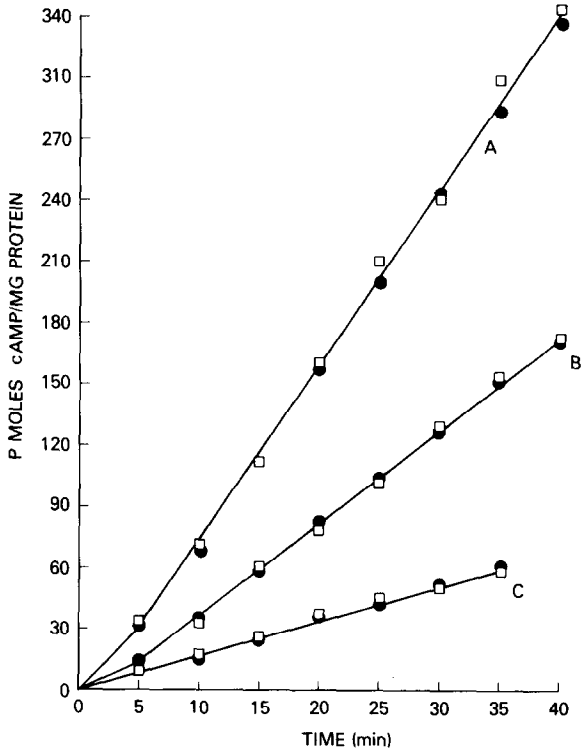

Fig.3. Human and turkey erythrocyte membranes were combined and assayed as in fig.1. Saturating concentrations of both donor membrane and the various ligands were used: (A) activity in the presence of 0.05 mM isoproterenol and 0.1 mM GTP; (B) assay in the presence of 10 mM sodium fluoride; (C) assay in the presence of 0.05 mM isoproterenol and 0.1 mM Gpp(NH)p; (●) activity of the turkey erythrocyte membrane alone; (□) activity of the turkey erythrocyte combined with the human erythrocyte membrane.

Table 1
Adenylate cyclase activity [pmol . (15 min)$^{-1}$. (mg)$^{-1}$]

	Donor membranes pretreated with NAD	Donor membranes pretreated with NAD and cholera toxin
Turkey erythrocyte	2.7 ± 0.3	2.8 ± 0.3
Human erythrocyte	0.1 ± 0.2	0.1 ± 0.4
Turkey erythrocyte + human erythrocyte	4.0 ± 0.4	9.0 ± 0.7

Adenylate cyclase was assayed in the presence of 10^{-5} M GTP. Steady state activities were determined from time curves as in other experiments. The donor human erythrocyte membrane was incubated without or with cholera toxin (64 μg/mg) as in [10]

human erythrocyte membrane. The question of the stoichiometry of the turkey erythrocyte system can be analyzed by extending the experiments outlined earlier to include ligands which activate the acceptor turkey erythrocyte cyclase. These ligands are isoproterenol + GTP, fluoride, and isoproterenol + Gpp(NH)p. In none of these cases did the addition of a saturating concentration of human erythrocyte membrane to the acceptor turkey erythrocyte membrane alter the levels of activation seen with these ligands for the acceptor membrane alone (fig.3). Of particular interest in this regard was the inability of the added N unit to improve the levels of activation with isoproterenol and GTP which in the avian erythrocyte systems (in contrast to other cyclase systems) is well below the levels of activity seen with either fluoride or isoproterenol and Gpp(NH)p.

4. Discussion

This study shows that the catalytic unit in the turkey erythrocyte membrane is available for functional linkage with N component from human erythrocyte membranes. Although the details of the nature of this linkage are unclear, the units interact in a manner which confers the ability of Gpp(NH)p to stimulate the turkey erythrocyte C unit. We have demonstrated, using cholera toxin treatment, that the linkage is through the exogenously added N component.

The maximal extent of the activation by the donor N unit under optimal conditions is much less than the activity observed in the presence of hormone. Two possible explanations for this difference are:

(1) Compared to cyc^-, the turkey erythrocyte C unit may exhibit a different geometry which makes it less accessible to the donor N unit. That is, if the C unit is already linked to the endogenous N component, the donor N would be incapable of full activation. Alternatively, the vesicles obtained from lysis of the turkey erythrocytes may exhibit 'sidedness', making the C units less available for interaction with the donor N component. A comparison of the results in [7] using detergent in complementation studies, with those in [10] where it was not, suggests that one role for the detergent may be to loosen the membrane organization and enhance subunit interaction. The studies presented here clearly required a minimally perturbed system – therefore, no detergent was used.

(2) The C unit may exist in multiple forms of which only a fraction would be available for interacting with endogenous or exogenous N units. If all the C units are located in NC complexes, then it would follow that the C unit is multivalent, i.e., capable of accepting more than one N unit. An obligatory role for such multivalent complexes in the functioning of the adenylate cyclase systems has been proposed in [17,18].

A most interesting result was that the exogenous N unit did not influence the ability of the adrenergic receptor to modify the endogenous N unit. If the receptor were uncoupled from N, this result is not expected. However, this result is consistent with recent target size analysis studies which suggest that the β-adrenergic receptor is prelinked to the N and C components in the turkey erythrocyte membrane adenylate cyclase [19]. If the endogenous N and R are indeed precoupled, no effect of added N on stimulation by isoproterenol plus GTP would be expected – as is indeed the case. Thus, the inability of exogenous N to modify the hormone-promoted exchange of tightly-bound GDP with free Gpp(NH)p becomes understandable in terms of the structure of the enzyme.

These studies also address the stoichiometry of N relative to C in the acceptor, turkey erythrocyte membrane. Several lines of experimentation have demonstrated that the fluoride response requires the presence of both the N and the C units assembled in a structurally linked complex [1,2,10,19,20]. The relative stoichiometry between these units can therefore be probed by examining the level of the fluoride response under conditions where the N unit is known to functionally link with C. Thus if $N < C$, then the addition of exogenous N would lead to an enhancement of the fluoride response. However, as shown in fig.3 there is no enhancement of the fluoride response. Thus the turkey erythrocyte adenylate cyclase system must contain sufficient N units to couple with all available C units.

The relative amounts of N component and C unit in the turkey erythrocyte was also approached from a consideration of the role of guanine nucleotide exchange in activation of the enzyme. We have shown that a good functional test of the level of GDP binding in the turkey erythrocyte adenylate cyclase is the degree of activation observed with Gpp(NH)p relative to that observed with isoproterenol and Gpp(NH)p [6]. As shown in fig.1, 2 and table 1, the added level of activation achieved with Gpp(NH)p is a result of coupling of exogenous N unit with acceptor C and not due to a repair of the endogenous N unit. Maximal activation in the acceptor system still required the presence of hormone (fig.3). As the level of activation with isoproterenol and Gpp(NH)p is neither enhanced nor impaired by the addition of the exogenous N unit, one would conclude that neither the tight binding of GDP nor promotion of its exchange with Gpp(NH)p is influenced by the addition of exogenous N unit. It also follows that the level of activation with isoproterenol and GTP should be unaltered by this addition.

These types of experiments are useful in determining the functional stoichiometry of adenylate cyclase systems, can monitor structural features of the enzyme in its membrane bound form, and also allow for the separation of events at the catalytic unit from events between the unit and receptor in the acceptor system.

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