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LATERAL MOBILITY OF β-RECEPTORS INVOLVED IN ADENYLATE CYCLASE ACTIVATION

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Summary

Cationized ferritin was found to inhibit the lateral mobility of intramembrane proteins in turkey erythrocyte membranes and the activation of adenylate cyclase by the (−)-epinephrine-bound β-adrenergic receptor. It was observed that cationized ferritin has only a small direct effect on the β-receptor and on the adenylate cyclase moiety. It is concluded that the cationized ferritin-induced inhibition of the hormone-dependent cyclase activity results from the inhibition of the lateral mobility of the receptor and therefore a decrease in the bimolecular rate of interaction between the receptor and the enzyme.

Introduction

Recent studies indicate that the activation of turkey erythrocyte adenylate cyclase by β-adrenergic receptor is a bimolecular process [1] of the 'collision-coupling' type which can be described by the equation:

$$HR + E \cdot GTP \xrightarrow{k_{on}} HR \cdot E \cdot GTP \rightarrow HR + E' \cdot GTP \xrightarrow{k_{off}} E + GDP + P_i \quad (1)$$

HR is the hormone (H)-bound β-receptor (R), E · GTP the inactivate form of adenylate cyclase, E′ · GTP the activated form the enzyme and k_{on} the bimolecular rate constant of enzyme activation. The activated form E′ · GTP is obtained only when GTP occupies the GTP regulatory site and decays to E concomitantly with the hydrolysis of GTP at the GTP regulatory site [1—4]. The simultaneous presence of hormone and GTP is required for enzyme activation [1,2]. When non-hydrolyzable analogues of GTP, such as GppNHp, are used the deactivation step is blocked ($k_{off} = 0$) and all the cyclase units become

activated and remain permanently active [2,4]. The maximal level of active enzyme in the presence of saturating hormone and GTP is given by [2,4]

$$[E' \cdot GTP]_{max} = \frac{[E]_{TOTAL} \times k_{on}}{k_{on} + k_{off}} \quad (2)$$

where $[E]_{total}$ is the total enzyme concentration.

The scheme described in Eqns. 1 and 2 is the only one which can account for a number of experimental observations [1]: (a) non-cooperative hormone binding; (b) pseudo first-order kinetics of enzyme activation by hormone and GppNHp ($k_{off} = 0$), and (c) the linear dependence of the pseudo first-order rate constant on receptor concentration, namely $k_{on} = k_1[R_T]$.

These results suggest that the process of cyclase activation requires the lateral mobility of the receptor versus the cyclase moiety and that the efficiency of coupling depends on the fluidity of the membrane. Indeed, very recently it was demonstrated [5,7] that the increase of membrane fluidity causes a linear increase in the bimolecular process of cyclase activation by the hormone-bound β-receptor. This finding was interpreted to indicate that the process of cyclase activation by the hormone-bound receptor is a bimolecular process [5]. In this communication we present preliminary results of a more direct approach to study the question whether lateral mobility of the β-receptor vis-a-vis the adenylate cyclase is involved in the process of enzyme activation.

It was shown [6,7] that the fusion of two membranes requires the removal of intramembrane proteins from the regions of the two membranes which become the zone of fusion. Indeed, the inhibition of the lateral mobility of the membrane proteins in chicken erythrocytes by the binding of cationized ferritin results in an inhibition of the Ca^{2+} and ionophore-induced fusion [7]. We therefore examined the effect of cationized ferritin on the turkey erythrocyte adenylate cyclase system in parallel to its effect on the lateral mobility of turkey erythrocyte membrane proteins.

Methods

Turkey erythrocyte membranes were prepared and stored as previously described [8]. Adenylate cyclase was assayed by the method of Salomon et al. [9]. The assay was conducted either in the presence of 10 mM NaF, 0.1 mM (−)-epinephrine or 0.1 mM (−)-epinephrine plus 0.1 mM GppNHp at 37°C for 20 min. Protein was determined according to Lowry et al. [10] using bovine serum albumin as standard. Freshly purified and washed turkey erythrocyte membranes (1 mg/ml) were incubated for 5 min with (−)-epinephrine ($5 \cdot 10^{-6}$ M) at 25°C. The inclusion of (−)-epinephrine in the incubation mixture was not essential but yielded more reproducible results in repetitive experiments. This effect may be due to the protection of the β-receptor by the β-agonist. The incubation with (−)-epinephrine was followed by the addition of increasing concentrations of cationized ferritin 25—125 μg/mg protein (Miles Yeda, Rehovoth) in 50 mM Tris-HCl, 1 mM EDTA and 2 mM $MgCl_2$ and incubated for 10 min at 25°C. Control membranes were incubated for the same length of time in the absence of cationized ferritin. The incubation mixtures

were diluted to 12 ml and centrifuged for 10 min at 20 000 × *g* followed by resuspension of the pellet and centrifugation. This step was performed in order to remove unbound cationized ferritin. Bound cationized ferritin does not dissociate from the membrane when incubated at 37°C for as long as 45 min. This could be assessed by the finding that cold-induced clustering remains to be inhibited subsequent to this prolonged incubation.

Subsequently, the membranes were assayed in direct binding experiments using ^{125}I-labeled hydroxybenzylpindolol as described earlier [11,12] and for their adenylate cyclase activity.

Samples were subjected to freeze-etching, shadow casting and electron microscopy. Protein was determined for each incubation mixture.

Freeze-etching and electron microscopy. These were conducted as follows: Membranes were fixed by addition of glutaraldehyde (Ladd Research Industries, Inc., Burlington, VT) to a final concentration of 1% (v/v) and then freeze-fracturing was performed as previously described [6]. Micrographs were obtained on a Philips electron microscope EM 300 operating at 80 kV.

Samples from the control membranes as well as from the ferritin-treated membranes were assayed for adenylate cyclase activity in the presence of NaF, (−)-epinephrine with GTP and (−)-epinephrine with GppNHp.

Results

The inhibition of protein mobility by cationized ferritin

Fig. 1b shows that the intramembrane particles of isolated turkey erythrocyte membranes are capable of undergoing the thermotropic separation (cold-induced clustering) when transferred to 4°C for 10 min. Fig. 1c shows that cationized ferritin (100 μg/mg membranes) strongly inhibits this cold-induced clustering of intramembrane particles.

These results indicate that ferritin inhibits the lateral mobility of turkey erythrocyte membrane proteins, as reflected by the inhibition of the cold-induced clustering of membrane proteins, similarly to the cationized ferritin-induced inhibition of protein mobility within the chicken erythrocyte membranes [6,7].

Titration of turkey erythrocyte membranes with increasing concentrations of cationized ferritin revealed (data not shown) that 25 μg/mg membranes had no inhibitory effect on the cold-induced clustering of intramembrane particles; 50 μg/mg induced about 50% inhibition were full inhibition occurs with 100 μg/mg.

The inhibition of (−)-epinephrine-dependent adenylate cyclase activity

Treatment with cationized ferritin induces a large decrease in the (−)-epinephrine-dependent adenylate cyclase activity and a much smaller decrease in both the NaF-stimulated activity and the (−)-epinephrine plus GppNHp-dependent activity (Fig. 2). The reduction in (−)-epinephrine-dependent activity is not due to a reduction of the receptor affinity towards the agonist which exhibits a dissociation constant of $6.0 \pm 1.0 \cdot 10^{-6}$ M towards the β-receptor in the presence and in the absence of ferritin. At 100 μg ferritin/mg membrane protein 80% of the (−)-epinephrine-dependent activity is inhibited, whereas

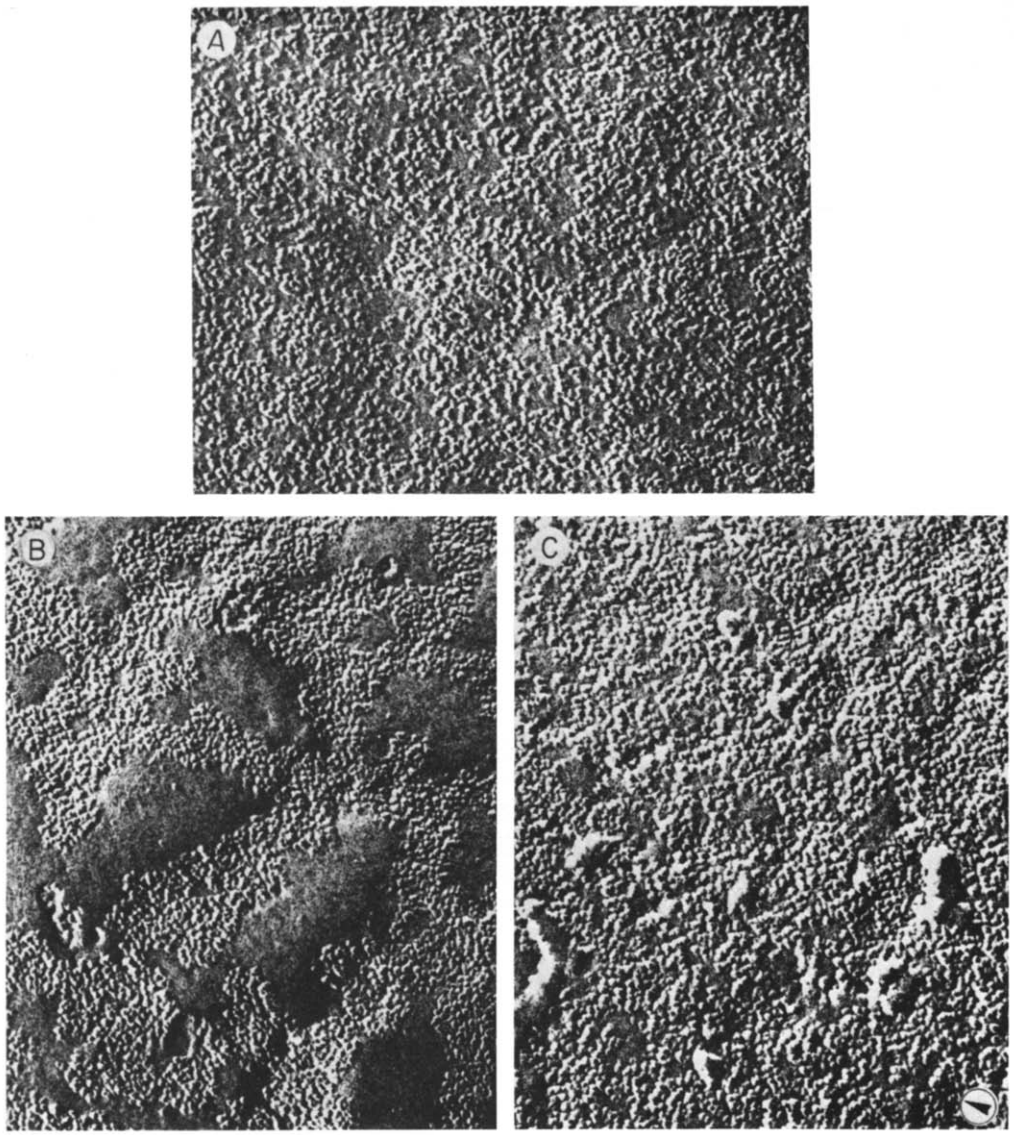

Fig. 1. Cold-induced clustering of intramembrane particles in turkey erythrocyte plasma membranes and its inhibition by cationized ferritin. Turkey erythrocyte plasma membranes were prepared and the membrane suspension in Tris/$MgCl_2$/EDTA buffer (1 mg/ml) equilibrated for 10 min at 25°C then freeze-fractured and shadow-casted subsequent to the following treatments: (A) Control membranes at 25°C without any treatment with cationized ferritin. (B) The membranes after incubation at 4°C for 10 min. Note large smooth areas between aggregated intramembrane particles. (C) Cationized ferritin-treated membranes at 25°C, incubated at 4°C for 10 min. No aggregation of intramembrane particles could be detected. The membranes were first incubated with cationized ferritin (100 μg/ml) at 25°C for 10 min, then washed once with the Tris/$MgCl_2$/EDTA buffer and transferred to 4°C. (A—C) P, fracture phases; final magnification; ×75 000. The encircled arrowhead indicates the direction of shadow.

the NaF, and the (−)-epinephrine plus GppNHp-stimulated activities are inhibited by only 20%. It can be seen that the progressive inhibition of lateral mobility of intramembrane particles as a function of increasing cationized

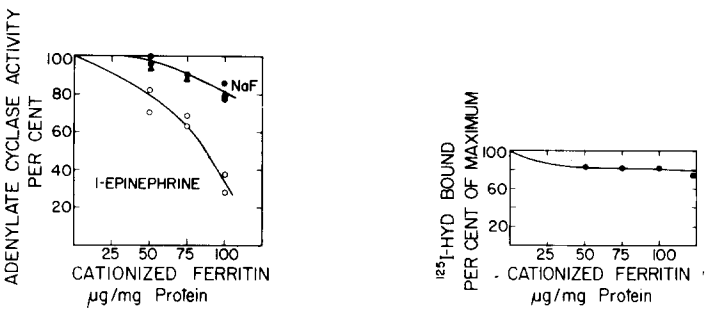

Fig. 2. The inhibitory effect of cationized ferritin on the adenylate cyclase activity of turkey erythrocytes stimulated by (—)-epinephrine, NaF and (—)-epinephrine in the presence of GppNHp. Turkey erythrocyte membranes were treated by ferritin and then assayed in the presence of 0.1 mM (—)-epinephrine (○———○) and 10 mM NaF (●———●) and 0.1 mM (—)-epinephrine plus 0.1 mM GppNHp (▲———▲).

Fig. 3. The inhibition of ^{125}I-labeled (±)hydroxybenzylpindolol binding to turkey erythrocyte membranes by cationized ferritin. The binding of ^{125}I-labeled (±)hydroxybenzylpindolol (^{125}I-HYD) was assayed as previously described [11] using the method of Maguire et al. [12]. The experimental details are published elsewhere [1].

ferritin concentration is paralleled by the progressive inhibition of the (—)-epinephrine-dependent adenylate cyclase activity (Fig. 2).

The absence of an effect of cationized ferritin on the β-receptor

The effect of cationized ferritin on the hormone-dependent activity does not seem to result from the direct effect of ferritin on the β-receptors themselves since the inhibition ^{125}I-labeled hydroxybenzylpindolol binding to the β-adrenergic receptors by cationized ferritin, amounts to no more than 15—20% (Fig. 3). Also, the affinity constant for ^{125}I-labeled hydroxybenzylpindolol remains unchanged subsequent to treatment with increased concentrations of cationized ferritin. The value for the β-receptor ^{125}I-labeled hydroxybenzylpindolol dissociation constant was found to be $1.1 \pm 0.2 \cdot 10^{-10}$ M, identical to the value reported by us previously [1]. Furthermore, the (—)-epinephrine dose-response curves of adenylate cyclase in the presence of cationized ferritin concentrations reveals an apparent dissociation constant for the hormone of $K_D = 6.0 \pm 1.0 \cdot 10^{-6}$ M. This value is identical to apparent dissociation constant for (—)-epinephrine exhibited by untreated turkey erythrocyte membranes.

Discussion

The strong inhibition of the (—)-epinephrine-dependent cyclase activity by cationized ferritin probably results from the slowing down of the rate of collision between the hormone-bound receptor and the enzyme, k_{on} (Eqn. 1).

F^- activate the adenylate cyclase by a receptor-independent mechanism [13] and thus the small effect (Fig. 2) of cationized ferritin on the NaF-dependent activity most probably reflects a direct effect on the adenylate cyclase enzyme. Furthermore, the small inhibitory effect of cationized ferritin on the GppNHp plus epinephrine activity is similar to the inhibition of the NaF-stimulated activity. Since in the presence of saturating (—)-epinephrine and saturating

GppNHp all the catalytic units become activated [1] the effect of cationized ferritin measured under these conditions most probably measures the direct effect of cationized ferritin on the catalytic efficiency of the cyclase system. Therefore, it is not surprising that the cationized ferritin effect is similar for the NaF and the (−)-epinephrine plus GppNHp mode of activation.

In conclusion, the present study shows that cationized ferritin, which restricts severely the lateral mobility of membrane proteins, also inhibits strongly the bimolecular activation process of adenylate cyclase by the hormone-bound β-receptors, in turkey erythrocytes. These findings correlate well with other lines of investigation which indicate that the coupling between β-receptors and adenylate cyclase in turkey erythrocyte membranes is a bimolecular process and requires the relative lateral movement of the two components.

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