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Topographic separation of adenylate cyclase and hormone receptors in the plasma membrane of toad erythrocyte ghosts

(membrane vesicles/β-receptor–enzyme coupling/mobile receptor hypothesis)

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ABSTRACT **Brief sonication of whole erythrocyte plasma membranes (ghosts) from toads at 4° does not inactivate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] or destroy the receptor binding properties of hydroxybenzylpindolol or insulin. The hormonal (but not the fluoride-induced) stimulation of this enzyme is, however, lost. Fractionation of the small, resealed membrane fragments (vesicles) on discontinuous sucrose gradients results in the separation of vesicle populations differing grossly in size and protein composition. In addition, the distribution of the β-adrenergic receptor, an insulin binding site, and adenylate cyclase among these vesicle fractions differs. The pattern of distribution of these functional structures can be altered differentially by manipulations of the ghosts before sonication. For example, brief preincubation with isoproterenol leads to a change in the relative distribution of β-receptor (but not adenylate cyclase) among the various vesicle fractions; this effect is not obtained with β-receptor antagonists, which block the isoproterenol effect. Exposure of the ghosts to different temperatures, changes in the divalent cation composition of the medium, or the addition of ATP also leads to changes in the distribution of surface markers of the subsequently formed vesicles. The results indicate gross asymmetries in the distribution of protein components within the plane of the membrane and raise important questions regarding the manner whereby functionally related and coupled components, such as hormone receptors and adenylate cyclase, interact.**

The fluid mosaic model of cell membranes (1) has been widely accepted and discussed (2–7), particularly with respect to the restricted orientation of phospholipids, glycolipids, and glycoproteins. Less is known, however, about the asymmetry of distribution of various components in the plane of the membrane. Obvious morphologic evidence of planar asymmetry exists in specialized areas such as microvilli (8, 9), synapses, and junctional cell–cell contact zones, as well as in oriented cells such as hepatocytes and intestinal epithelial cells. Other indications of membrane microheterogeneity are the nonuniform binding of certain ligands to cells [melanocyte-stimulating hormone (10), ferritin-labeled lipoprotein (11), the clustering of intramembranous structures (12–14), and the patched and capped distribution of receptors for lectins and antibodies (15–18) and cholera toxin (19, 20)].

Despite these advances, very little is known about the consequences of such asymmetries on the dynamics of interactions between functionally related components (e.g., enzymes and substrates, hormone receptors and ion channels or enzymes). The present study addresses this problem by examining the distribution of various surface components in small, resealed membrane fragments derived from whole erythrocyte ghost plasma membranes. In addition to finding gross differences in the composition of such vesicles, we have surprisingly found that the β-adrenergic receptor that directly modulates adenylate cyclase, and which has, therefore, been presumed to be physically linked to the enzyme, is physically separated from the enzyme. These findings raise fundamental questions regarding the mechanism by which hormone receptors are "coupled" to this and perhaps other membrane components.

Abbreviations: HYP, hydroxybenzylpindolol; Gpp(NH)p, 5′-guanylylimino-diphosphonate; cAMP, adenosine 3′:5′-cyclic monophosphate.

MATERIALS AND METHODS

^{125}I-Labeled hydroxybenzylpindolol (^{125}I-HYP, 2200 Ci/mmol), was generously prepared (21) by D. B. Bylund, The Johns Hopkins Department of Pharmacology. ^{125}I-Labeled insulin (^{125}I-insulin) (300 cpm/pg) was prepared as described earlier (22). [α-^{32}P]ATP (300–700 cpm/pmol) was synthesized and purified on a DEAE-cellulose column eluted with a triethylamine/formic acid gradient (23, 24).

Female *Bufus marinus* erythrocyte ghosts were prepared as described (23), from isotonic saline-washed cells, by hypotonic lysis using cold 5 mM Tris·HCl, pH 8.0, containing 0.1 mM $CaCl_2$ and 20 μg of micrococcal nuclease (Worthington) per ml of packed cells. Ghosts were separated from nuclei and nucleated ghosts by differential centrifugation, pelleted at 4° for 15 min at 40,000 × *g* after $MgCl_2$ (0.7 mM) was added, and washed twice with cold 25 mM Tris·HCl, pH 8.0. After each centrifugation, the fluffy upper layer of the pellet was saved while the gelatinous, firmer lower part was discarded. By phase contrast microscopy, contamination with nucleated ghosts and free nuclei was less than 5%; it was not possible to obtain a fraction of nuclei sufficiently free from plasma membrane to test the binding properties of the nuclei *per se*. Toad erythrocytes were used unless otherwise specified. Erythrocytes from Sprague–Dawley rats were similarly lysed, but without micrococcal nuclease. The ghosts from 1 ml of packed red cells were finally suspended in 1.3 ml of 25 mM Tris·HCl, pH 8.0, in 12 × 75 mm polystyrene tubes and sonicated at 4° by delivering eight 1-sec shocks (power setting 60) from a Fisher Sonic Dismembrator. The vesicles were fractionated on discontinuous sucrose gradients centrifuged at 27,000 rpm for 120 min at 4° in an SW-27 rotor. From two to up to six fractions were obtained, depending on the goals of a particular experiment. The interphase fractions and pellet were collected, washed, and suspended in 25 mM Tris·HCl, pH 8.0 (0.25–1 mg of protein per ml).

Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] was assayed at 30° for 7 min as described (23, 24) with 150 μM ATP, 1 to 2 × 10^6 cpm of α-^{32}P, 7 mM $MgCl_2$, 7 mM phospho*enol*pyruvate, 20 μg of pyruvate kinase/ml, 10 mM aminophylline, and 30–150 μg of membrane protein in 0.1 ml of 50 mM Tris·HCl, pH 8.0. Routinely, 12 mM NaF was added to activate cyclase in vesicle fractions. Specific binding of ^{125}I-HYP was measured at saturation (5 nM) using 10–30 μg of membrane protein in the presence and absence of 1 μM propranolol, which when added 10 min prior to ^{125}I-HYP was sufficient to saturate all specific binding sites. After 30 min at 30°, the membrane-bound ^{125}I-HYP was separated from un-

Table 1. Effect of sonication on adenylate cyclase activity and binding of ^{125}I-HYP

	Membrane preparation	
	Ghosts	Vesicles
Adenylate cyclase activity*		
No additions	15	11
Gpp(NH)p†	122	92
Gpp(NH)p + isoproterenol†	500	150
Gpp(NH)p + isoproterenol‡	1540	1230
NaF†	1000	600
NaF‡	1340	1120
^{125}I-HYP binding§		
HYP added before sonication	0.15	0.14
HYP added after sonication	—	0.10

* Cyclase activity (pmol of cAMP/mg of protein per min). Washed ghosts were incubated (30°, 7 min) in 25 mM Tris·HCl, pH 8, with no additions or in the presence of either NaF (12 mM) plus $MgCl_2$ (4 mM) or Gpp(NH)p (10^{-5} M), isoproterenol (10^{-4} M), $MgCl_2$ (7 mM), phospho*enol*pyruvate (7 mM), and pyruvate kinase (20 μg/ml). The samples were washed with 20 volumes of cold buffer, resuspended, and, for vesicle preparation, sonicated at 4° prior to cyclase assay as described in *Materials and Methods*.

† Ghosts were treated in the absence of additions; activating reagents [10^{-5} M Gpp(NH)p with or without 10^{-4} M isoproterenol; 12 mM NaF] were added only to the cyclase assay.

‡ Ghosts were preactivated by treatment with reagents and washed before sonication and/or cyclase assay; activating reagents were not added to the final cyclase assay.

§ ^{125}I-HYP binding (pmol/mg of protein). Ghosts were incubated for 30 min at 30° without or with ^{125}I-HYP as for the routine measurements of binding (see *Materials and Methods*). After cooling to 4°, aliquots were sonicated and either passed through Sephadex G-50 columns (HYP added before sonication) for the measurement of specific binding or assayed for binding by the routine procedure (HYP added after sonication).

bound ^{125}I-HYP by applying the membrane suspension (0.1 ml) to a Sephadex G-50, medium column (0.5 × 8 cm) and eluting at 4° with 25 mM Tris·HCl, pH 8, at approximately 1.2 ml/min; the void volume contained more than 95% of the protein applied. This method, which proved superior to filtration or microfuge techniques since there is no loss of very small vesicles, was also used for measurements of binding to intact ghosts (results the same as with glass filter filtration) and for the measurement of ^{125}I-insulin binding (Sephadex G-150 columns). The low temperature (4°) reduced the dissociation rate of ^{125}I-HYP such that at 30 min less than 20% of the bound label dissociated from the membranes. Values reported in the figures and tables for cyclase activity and ^{125}I-HYP binding represent the mean of triplicate determinations, which agreed within 10%. Polyacrylamide disc-gel electrophoresis was performed essentially as described (25).

RESULTS

Effects of Sonication on Adenylate Cyclase and β-Adrenergic Receptor. Sonication of ghosts causes a modest inactivation (25–30%) of adenylate cyclase, as indicated by basal activities and activities stimulated by 5′-guanylylimino-diphosphonate [Gpp(NH)p] (26–28) and NaF (Table 1). Importantly, after sonication, the isoproterenol potentiation of Gpp(NH)p stimulation, an index of hormonal sensitivity, is reduced by more than 80%. It is thus striking that preactivation of ghosts by isoproterenol plus Gpp(NH)p prior to sonication preserves the hormone-augmented activity. Activation by NaF, while affected somewhat by sonication, provides the most sensitive indicator of adenylate cyclase in vesicles prepared from ghosts that had not been pretreated by reagents [Gpp(NH)p or isoproterenol] prior to vesiculation. Fluoride-stimulated activity was, therefore, used in subsequent experiments to detect adenylate cyclase in fractionated vesicle preparations.

Table 2. Changes in distribution of β-receptors in vesicles by preincubating ghosts with isoproterenol

	Binding of ^{125}I-HYP (interphase/pellet)
No additions	3.07 ± 0.23
Isoproterenol	15 ± 0.81
^{125}I-HYP preincubation*	
Alone	7.41 ± 0.43
Followed by isoproterenol	6.75 ± 0.35

Washed ghosts were incubated at 30° with or without ^{125}I-HYP (10 pmol/mg of protein per ml). After 30 min, each sample was divided into two equal portions, isoproterenol (5×10^{-4} M) was added to one portion, and the incubation was continued for 3 min more. Samples were sonicated and applied on a sucrose gradient (8%/38%). For samples preincubated with ^{125}I-HYP, gradient solutions contained this radioactive ligand at an equivalent concentration (10 pmol/ml) to prevent dissociation of the ligand from the membranes. Interphase and pellet fractions were quickly resuspended at 4°, and those preincubated with ^{125}I-HYP (10 nM) were promptly passed over Sephadex G-50 while other samples were used for the standard binding assay. Samples were run in triplicate. Calculations of ratios (interphase/pellet) are based on total binding activity.

* The increased "binding" here represents mainly that trapped in inside-out vesicles (see *text*).

Sonication of ghosts does not damage the β-adrenergic recognition site, as measured by the binding of ^{125}I-HYP (Table 1). When binding is measured after sonication of ghosts, there is an apparent loss of approximately 30% of the binding activity. However, if ghosts are preincubated with ^{125}I-HYP, no loss of specifically bound radioactivity is observed. Similar results are obtained if the binding of ^{125}I-labeled wheat germ agglutinin (29, 30) to vesicles is measured with or without preincubation of the ghosts with the lectin. The results suggest that, as described previously (30), sonication leads to the formation of inside-out vesicles in which the internalized receptors are no longer accessible to exogenously added ligand. Experiments (Table 2) indicate that the prelabeled vesicles, some of which are presumably inside-out, can be recovered in the interphase fraction of a discontinuous (8%/38%) sucrose gradient.

Fractionation of Vesicles and Distribution of Adenylate Cyclase and β-Receptor. The size of vesicles produced by sonication depends on the volume of the suspension, the concentration of ghosts, and the number of sonic shocks delivered. The sonication conditions selected (*Materials and Methods*) minimize the inactivation of adenylate cyclase while yielding a sufficiently heterogeneous population of vesicles for subsequent fractionation. On the gradients, more than 90% of the protein and receptor and more than 75% of the adenylate cyclase activity can be recovered.

Electron microscopy (31) and electrophoretic analysis reveal marked differences in vesicle size and protein content of the interphase fractions from discontinuous gradients. The three interphase fractions of a representative gradient (8%/28%, 28%/33%, and 33%/38% wt/vol sucrose) contained relatively homogeneously sized vesicles of approximate diameter 0.1 μm, 0.5 μm, and 1 μm, respectively; the pellet contained small numbers of nuclei amongst stacked membranes 2–3 μm in diameter, simulating a lamellated structure. From the size of vesicles produced, it can be estimated that each cell gives rise to about 4×10^4 vesicles. Remarkable amongst the many qualitative and quantitative differences in protein composition of the fractions was the presence only in the small vesicle

Table 3. Adenylate cyclase activity (pmol of cAMP/mg of protein per min) of two major fractions obtained from membrane vesicles*

	Vesicle fraction	
	Interphase	Pellet
No additions	11	2.5
Gpp(NH)p†	58	6
Gpp(NH)p + isoproterenol†	110	8
Gpp(NH)p + isoproterenol‡	1100	90
NaF†	650	55
NaF‡	1020	110

* Ghosts, treated and sonicated as in Table 1, were fractionated on an 8%/38% discontinuous sucrose gradient. Values represent the means of triplicate measurements, which agreed within 10%.
†,‡ See these symbols, Table 1.

fraction (8%/28%) of a major periodic-acid-Schiff-staining component and the absence in this fraction and the pellet of two major closely migrating components of high molecular weight. The results indicate a highly asymmetric distribution of proteins in the vesicle fractions, and thus in the erythrocyte ghost membranes from which they were derived.

Fractionation of vesicles, from ghosts preactivated either by Gpp(NH)p plus isoproterenol or by fluoride (as in Table 1) or from untreated ghosts, on a two-step (8%/38%) discontinuous sucrose gradient yields an interphase fraction with about a 10-fold higher specific activity of adenylate cyclase than the pellet (Table 3). Since, in all cases, there was twice as much protein in the interphase fraction as in the pellet (values not shown), the ratio of total cyclase activity (interphase/pellet) is about 20:1 from either untreated or preactivated ghosts.

Striking differences in the distribution of adenylate cyclase and β-receptors are observed after fractionation of vesicles on a six-step discontinuous gradient (Fig. 1*A*). The ratio of enzymatic activity to HYP binding (pmol of cAMP per min/pmol of HYP bound) was chosen to emphasize the differences in membrane distribution in this and other figures. The enzyme is increased dramatically in fraction 35%/40%, while ^{125}I-HYP binding shows less dramatic differences. The two lighter fractions as well as the pellet are relatively enriched in β-receptors compared to enzyme, while fraction 35%/40% shows a reversed relative enrichment. The ratio of enzyme activity to binding gradually increases from the lightest to the 35%/40% fraction, and falls abruptly in the pellet (Fig. 1*B*). If the ghosts are labeled with ^{125}I-HYP before sonication, a very similar pattern of receptor distribution is obtained despite increased binding in the lighter fractions, indicating that the sidedness of the vesicles does not basically alter the fractionation pattern. A similar type of differential enzyme–receptor enrichment occurs when rat erythrocytes are used instead of toad cells.

Isoproterenol-Induced Changes in Receptor and Cyclase Distribution. In a two-step (8%/38%) sucrose gradient, the enrichment of receptors in the lighter vesicle fractions of the six-step gradient (Fig. 1) is accentuated in the single interphase fraction (Table 2); there is three times as much ^{125}I-HYP binding in the interphase fraction as in the pellet. This enrichment in the interphase fraction is rendered more apparent (7-fold enrichment in the interphase) when ghosts are preincubated with ^{125}I-HYP prior to sonication and vesicle fractionation; the additional ^{125}I-HYP binding in the interphase is most likely present in inside-out vesicles as discussed above.

The distribution of receptors between the interphase fraction and the pellet is strikingly affected by pretreatment of ghosts with (−)-isoproterenol, which causes a further 5-fold increase in the receptor ratio (interphase/pellet) relative to untreated

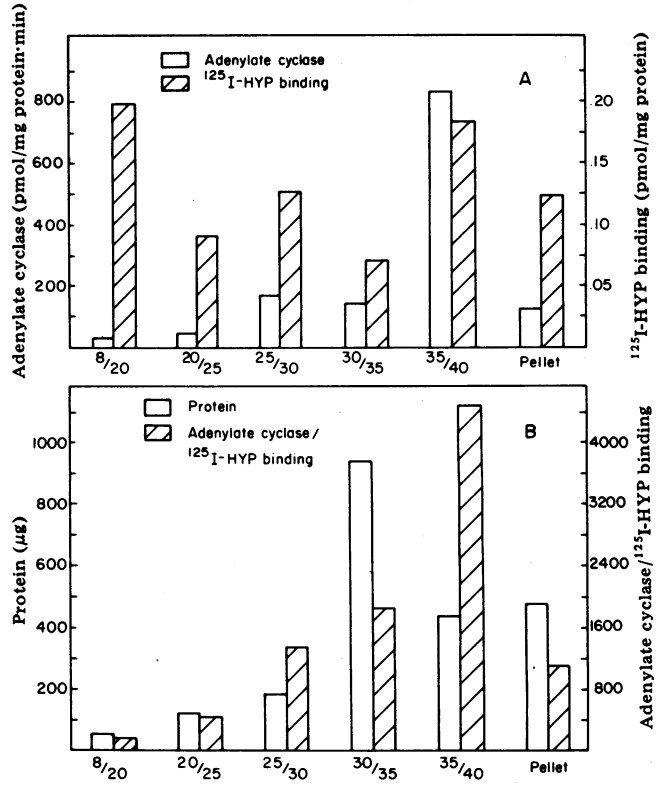

FIG. 1. Differences in the distribution of adenylate cyclase activity and ^{125}I-HYP binding among different ghost vesicle membranes. The chilled ghosts were sonicated and fractionated on a six-step sucrose gradient (8%/20%/25%/30%/35%/40%). The interphase fractions and pellet were collected separately and washed.

ghosts (Table 2). The effect is not observed with (+)-isoproterenol, is concentration-dependent between 10^{-6} and 5×10^{-4} M (−)-isoproterenol, and occurs after preincubation with hormone at 30° but not 4°. In ghosts preincubated with ^{125}I-HYP, a specific β-adrenergic blocking agent, the addition of isoproterenol has no effect on the distribution of receptor. It can be concluded that the membrane changes leading to a redistribution of receptor in the gradient relate to the agonist occupation of the β-receptor.

More extensive fractionation of the sonicated ghosts indicates that pretreatment with (−)-isoproterenol causes changes primarily in the distribution of β-receptors and does not appreciably affect the adenylate cyclase distribution (Fig. 2*A*); the distribution of protein was not affected (data not shown). Isoproterenol pretreatment markedly decreases the receptor content of the pellet fraction with an increase in fractions of lower density (Fig. 2*B*). The large differences between fractions in the ratio of enzymatic activity to β-receptor, either in the presence or absence of isoproterenol, are also apparent (Fig. 2*C*). In addition, after isoproterenol treatment there was, compared to control membranes, a net loss in the pellet fraction of from 20 to 35% of the ^{125}I-HYP binding, which could not be accounted for by the binding in other fractions.

Changes in Cyclase and Receptor Distribution by Divalent Cations, Temperature, and ATP. Preincubation of ghosts with $MgCl_2$ or $CaCl_2$ prior to sonication also causes distinct changes in the adenylate cyclase and β-receptor distribution on a four-step discontinuous gradient (Fig. 3). Alternatively, warming ghosts (30°, 4 min) before sonication and fractionation causes a decrease of protein and β-receptor content of the pellet, about a 2-fold increase in the enzyme/receptor ratio in the lighter two interphase fractions (8%/28% and 28%/33%), and a decrease in the two dense fractions (33%/38% and pellet). In

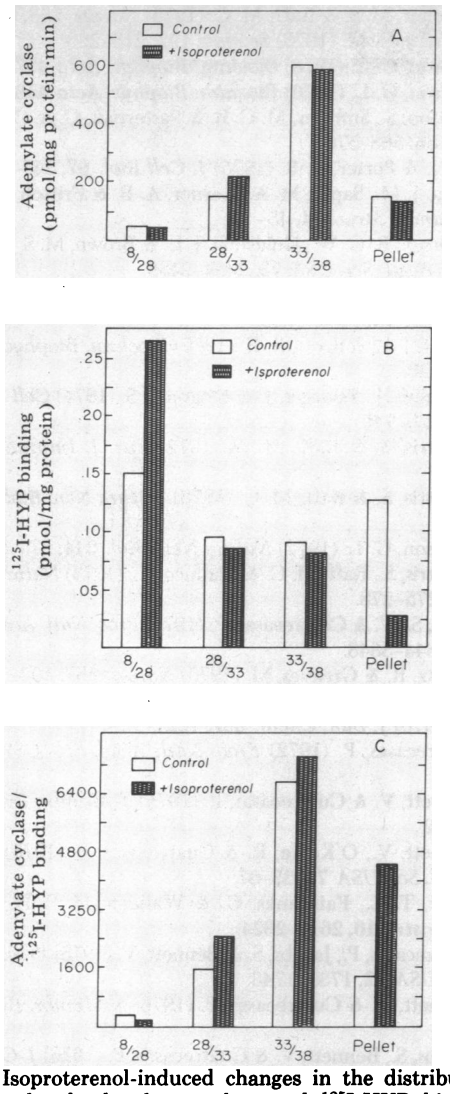

FIG. 2. Isoproterenol-induced changes in the distribution of fluoride-stimulated adenylate cyclase and ^{125}I-HYP binding of membrane vesicles. Washed, warmed (30°, 3 min) ghosts were incubated with or without isoproterenol (5×10^{-5} M) at 30° for 3 min, sonicated, and immediately applied at 4° on a four-step sucrose gradient (8%/28%/33%/38%). The fractions were washed three times before assay.

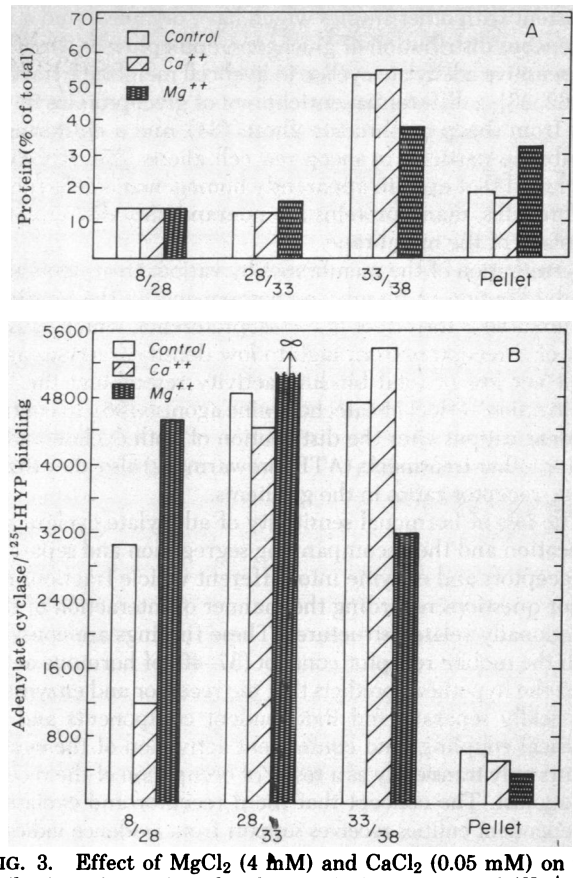

FIG. 3. Effect of $MgCl_2$ (4 mM) and $CaCl_2$ (0.05 mM) on the distribution of protein, adenylate cyclase activity, and ^{125}I-HYP binding of isolated membrane vesicles. Washed ghosts were incubated with the respective ions at 30° for 4 min, sonicated, and directly applied to a four-step sucrose gradient (8%/28%/33%/38%); appreciable cyclase activity, but no detectable binding of ^{125}I-HYP, was present in fraction 28%/33% from Mg^{2+}-treated ghosts, so as to yield a value of infinity for the ratio of enzymatic activity to binding.

contrast, pretreatment with 1 mM ATP (5 min at 30° in the presence of 7 mM $MgCl_2$, 20 μg/ml of pyruvate kinase, and 7 mM phospho*enol*pyruvate, in a 1.5-ml final volume) prior to sonication (five times for 1 sec each, 30°) leads to a 1.5-fold increase in the cyclase/receptor ratio at the 33%/38% interphase (data not shown).

Distribution of Binding of ^{125}I-Insulin. After sonication of ghosts by the routine procedure (*Materials and Methods*), essentially all of the specific binding of ^{125}I-insulin was recovered in the interphase fraction of a two-step discontinuous (8%/38%) sucrose gradient; little if any specific binding of insulin was recovered in the pellet, in contrast to the recovery of up to 25% of the total specific binding of ^{125}I-HYP in the pellet fraction under similar conditions (Table 3). Less severe sonication of ghosts (from 8 ml rather than 1 ml of packed cells, sonicated eight times for 1 sec each in 3 ml of buffer rather than in 1.3 ml) leads to an increase in the amount of binding of both ^{125}I-insulin and ^{125}I-HYP recovered in the pellet of the same discontinuous gradient. Approximately 70% of the ^{125}I-HYP binding and 40% of the ^{125}I-insulin binding are recovered in the pellet; equal amounts of protein were present in the interphase and pellet fractions. The differences in the relative distribution of binding of insulin and HYP suggest that, in the intact ghost, the binding sites are present in different environments with different susceptibilities to vesicle formation upon sonication.

DISCUSSION

If the various membrane constituents were randomly and homogeneously distributed in the plane of the toad erythrocyte membrane, all vesicle subpopulations generated by sonication would be expected to possess similar ratios of membrane components. Given the number and size range of the vesicles, it could be anticipated that only the absolute amounts of constituents might differ among subfractions. The present study, demonstrating vesicle subpopulations differentially enriched in β-receptor, in adenylate cyclase, and in several other protein constituents, argues against such a homogeneous distribution. Rather, the evidence suggests that the components are present in heterogeneous membrane environments which permit their preferential inclusion in vesicle subpopulations that are separable on the basis of density. The rapidity (seconds) of sonication, a procedure that is unlikely to induce artifactual biochemical heterogeneity amongst vesicles, may be an advantage in the instantaneous production of fragments that reflect underlying differences in membrane microenvironments. Although the introduction of an artifact of sonication cannot be ruled out (e.g., dissociation of membrane enzyme complexes), the reproducibility of the resulting subpopulations argues in favor of a difference in the original membrane microenvironments of the various components. The present observations are

consistent with other studies which have demonstrated a heterogeneous distribution of glucagon-, epinephrine-, and fluoride-sensitive adenylate cyclase in liver cell membrane fractions (29, 32, 33), a differential enrichment of glycoproteins in vesicles from sheep erythrocyte ghosts (34), and a clustering of membrane particles in sheep red cell ghosts (35). It may be concluded that even in apparently homogeneous cells such as erythrocytes, many proteins are nonrandomly distributed in the plane of the membrane.

Perturbation of the membranes by various treatments alters the distribution of membrane components in the gradients. Remarkable is the effect of (−)-isoproterenol, which causes a shift of β-receptors from high to low density fractions and a small net loss of total binding activity resembling the "desensitization" effect of catecholamine agonists (36). In contrast, divalent cations alter the distribution of both cyclase and receptor; other treatments (ATP, prewarming) also alter the enzyme/receptor ratios in the gradients.

The loss of hormonal sensitivity of adenylate cyclase after sonication and the accompanying segregation and separation of receptors and enzyme into different vesicle fractions raise major questions regarding the manner of interaction of these functionally related structures. These findings are consistent with the mobile receptor concept (37–40) of hormone action, since the hypothesis predicts that the receptor and enzyme are physically separate and independent components and that physical coupling (and consequent activation of the cyclase) occurs only transiently as a result of occupation of the receptor by agonist. The concept that the β-receptor and cyclase are independent entities receives support from evidence indicating that the two constituents are products of different genes (41, 42) and that these components may vary independently during maturation of the rat erythrocyte (43). The present study implies that these structures exist, at least in part, in different membrane microenvironments. It is noteworthy, however, that in the presence of the agonist isoproterenol there was no segregation of receptor and cyclase into a particular membrane fraction as might be predicted by the mobile receptor hypothesis. There may be technical reasons why such a segregation was not observed (e.g., disruption of the spectrin network during ghost preparation, inability to detect all receptor moieties because of a "desensitization" phenomenon). Nonetheless, an alternative to the mobile receptor hypothesis may also be seriously considered. Possibly, hormone-mediated specific information may be conveyed over "distances" by some physical-structural membrane alteration. Alternatively, in response to certain hormones, earlier, yet unrecognized chemical signals or "messengers" may be generated which in turn modify the activity of adenylate cyclase and, perhaps simultaneously, other membrane functions. The latter possibility, if correct, would lead to a reevaluation of the widely accepted "*first* messenger" role ascribed to cAMP.

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