THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 10, Issue of May 25, pp. 6410-6414, 1983
Printed in U.S.A.

Functional Integrity of Desensitized β-Adrenergic Receptors

INTERNALIZED RECEPTORS RECONSTITUTE CATECHOLAMINE-STIMULATED ADENYLATE CYCLASE ACTIVITY*

(Received for publication, December 27, 1982)

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The adenylate cyclase-coupled β_2-adrenergic receptor of the frog erythrocyte has served as a useful model system for elucidating the mechanisms of catecholamine-induced desensitization. In this system, it has been previously demonstrated that agonist-induced refractoriness is associated with sequestration of the β-adrenergic receptors in vesicles away from the cell surface and from their effector unit, the adenylate cyclase system (Stadel, J. M., Strulovici, B., Nambi, P., Lavin, T. N., Briggs, M. M., Caron, M. G., and Lefkowitz, R. J. (1983) *J. Biol. Chem.* 258, 3032–3038). These internalized β-adrenergic receptors appear to be structurally intact as assessed by photoaffinity labeling, but their functional status has previously been unknown.

In the present studies, we sought to assess the functionality of the sequestered vesicular receptors by fusing them to *Xenopus laevis* erythrocytes. This cell is suitable for such studies, since it has almost no detectable β-adrenergic receptor or catecholamine-sensitive adenylate cyclase, but contains prostaglandin E_1-stimulable adenylate cyclase. Fusion of β-adrenergic receptor-containing vesicles from desensitized frog erythrocytes with *X. laevis* erythrocytes results in a 30-fold stimulation of the hybrid adenylate cyclase by the β-adrenergic agonist isoproterenol. This effect was entirely blocked by the β-antagonist propranolol. The catecholamine-sensitive adenylate cyclase activity established in the vesicle-*Xenopus* hybrids showed the characteristic agonist potency series of the donor frog erythrocyte β_2-adrenergic receptor. Fusion of vesicles from desensitized frog erythrocytes in which the β-adrenergic receptors had been inactivated with the group specific reagent dicyclohexylcarbodiimide, or of vesicles derived from control frog erythrocytes, which contain low amounts of β-adrenergic receptor, did not establish catecholamine-sensitive adenylate cyclase activity in the hybrids.

These data demonstrate that β-adrenergic receptors internalized during desensitization retain their functionality when recoupled to an adenylate cyclase system from a different source. The functional uncoupling of these receptors during desensitization is thus more likely due to their sequestration away from the other components of the adenylate cyclase than to any alterations in the receptors themselves.

Prolonged exposure of target cells to hormone or drugs often results in a reduction in responsiveness of the cells to rechallenge with the same hormone. This process, termed "desensitization" or "refractoriness," has been reported to occur in systems in which the biological response is mediated by cAMP (1, 2), as well as in those in which hormonal responses are independent of this cyclic nucleotide (3, 4).

Desensitization has been extensively investigated in tissues containing receptors coupled to adenylate cyclase. Studies of catecholamine-induced desensitization in frog erythrocytes (5–8) and certain cultured cell lines (9, 10), utilizing radioligands specific for the β-adrenergic receptor, have indicated that an "uncoupling" of the receptors from the adenylate cyclase complex and a "down regulation" or decrease in the concentration of β-adrenergic receptors in the plasma membranes of desensitized cells occur.

Recently, Harden *et al.* (11) have reported that in 1321N1 astrocytoma cells uncoupled receptors produced during desensitization accumulate in a subpopulation of "light" membranes which show altered sedimentation properties on sucrose gradients.

In recent years, numerous studies have documented the hormone-promoted internalization of plasma membrane receptors. Diverse mechanisms for such phenomena have been uncovered including pathways involving so called "bristle-coated pits" (12, 13) and other endocytotic vesicles (14). Moreover, the fate of the internalized receptors may vary widely. Thus, in some cases, internalized receptors are degraded via a chloroquine-sensitive process (15), while in others the receptors appear to recycle to the cell surface (16, 17). Potential roles for these receptor-internalization pathways include regulation of the number or function of receptors at the cell surface and/or delivery of hormone to intracellular sites of action.

Until recently, relatively little has been known of the fate of the down-regulated β-adrenergic receptors after their agonist-promoted loss from the cell surface. Using the frog erythrocyte model system (1, 7, 8), it has been shown that prolonged exposure to the β-agonist isoproterenol promotes internalization of the β-adrenergic receptors. The down-regulated receptors appear to be sequestered in cytosolic vesicles which are devoid of the other components of the adenylate cyclase system, *i.e.* the N[1] and C (1). When the internalized receptors were "tagged" covalently with the photoaffinity reagent ^{125}I-*p*-azidobenzylcarazolol (18), they appeared *structurally* unchanged in comparison with β-adrenergic receptors derived

* This work was supported by Grants HL16037 and HL20339 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: N, nucleotide regulatory component; PGE_1, prostaglandin E_1; DCCD, dicyclohexylcarbodiimide.

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from control cells, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In the present studies, we have investigated the functional integrity of these internalized receptors. To approach this question, we developed a fusion assay system for measuring the ability of these "internalized"[2] receptors to stimulate C by implanting them into a cell which is devoid of β-adrenergic receptors, but which contains the other elements of the adenylate cyclase system. The recipient cell chosen for this fusion assay is the *Xenpous laevis* erythrocyte, which contains PGE_1 but not catecholamine-stimulable adenylate cyclase. The results provide the first insights into the functional status of a plasma membrane receptor after it has been translocated within the cell.

EXPERIMENTAL PROCEDURES

Southern grass frogs (*Rana pipiens*) and *X. laevis* were obtained from Nasco, Fort Atkinson, WI. Throughout this manuscript the term "frog" will be used to refer to *R. pipiens*.

Desensitization of Frog Erythrocytes—Frog erythrocytes were incubated in the presence or absence of 10 μM (−)-isoproterenol for 3 h at room temperature as previously described (5, 6).

Preparation of Vesicles—Vesicles from control and desensitized frog erythrocytes were prepared essentially as described (1) with minor modifications. In brief, the procedure was as follows. Frog erythrocytes were subjected to hypotonic lysis and cell lysates were centrifuged at 40,000 × *g* for 10 min to pellet the plasma membranes and nuclei. The supernatant was centrifuged at 158,000 × *g* for 1 h in a Beckman 45 Ti rotor using a Sorvall OTD 75B ultracentrifuge. From the pellet obtained from this high speed centrifugation, only the halo was taken, resuspended, and spun again at 158,000 × *g* for an additional hour, while the hard core of the pellet was discarded. The pellet obtained was resuspended in 75 mM Tris-HCl (pH 7.6), 12.5 mM $MgCl_2$, 1.5 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol to give a protein concentration of 1 mg/ml and stored under liquid N_2. Equal amounts of protein were recovered in the pellets from control and desensitized cell supernatants. This procedure yields a specific activity of receptors of approximately 3000 fmol of β-adrenergic receptor/mg of protein for the vesicles derived from desensitized erythrocytes.

Preparation of X. laevis Particulate Fraction—Membrane preparations have been performed by a freeze-thaw technique as previously described (19).

Radioligand Binding Assays—Saturation curves were performed as previously described (1). Specific binding was defined as the amount of the total binding of ^{125}I-cyanopindolol competed for by 10^{-4} M (−)-isoproterenol. Data analysis was carried out using nonlinear least squares computer programs as previously described (20, 21).

Treatment of Vesicles with DCCD—Vesicles derived from desensitized frog erythrocytes were exposed to 100 μM DCCD for 30 min at 25 °C as previously described (1).

Vesicle-Cell Fusion Technique—Washed *X. laevis* erythrocytes were prepared as previously described for frog erythrocytes (5, 6). Vesicles derived from control or desensitized frog erythrocytes were spun down at 158,000 × *g* for 1 h and 2 × 10^7 packed *X. laevis* erythrocytes were added to the pellets. Phospholipids (200 μg of lecithin, 10 μg of lysolecithin) were added to the mixture. After 5 min of incubation at 10 °C, $MgCl_2$ was added to 10 mM, and the incubation continued for 5 min. The tubes were placed in a 30 °C water bath, and the fusion with polyethylene glycol was performed as described by Schramm (22), Eimerl *et al.* (23), and Korner *et al.* (24) except that the fusion buffer contained 95 mM NaCl, 5 mM KCl, 4.8 mM $MgCl_2$, and 10 mM Tris to which 5 mM glucose and 2 mM ATP were added fresh for each experiment. The pH was adjusted to 7.5 at 30 °C. Membranes from the hybrids were prepared as described (19). Different fusion experiments were performed at least three times, and the results of representative experiments are reported.

Adenylate Cyclase Assay—The adenylate cyclase assay was performed as previously described (25, 26). Incubations were carried out for 30 min at 30 °C. Basal activity was determined in the presence of 0.1 mM GTP. GTP was also present when assays were performed

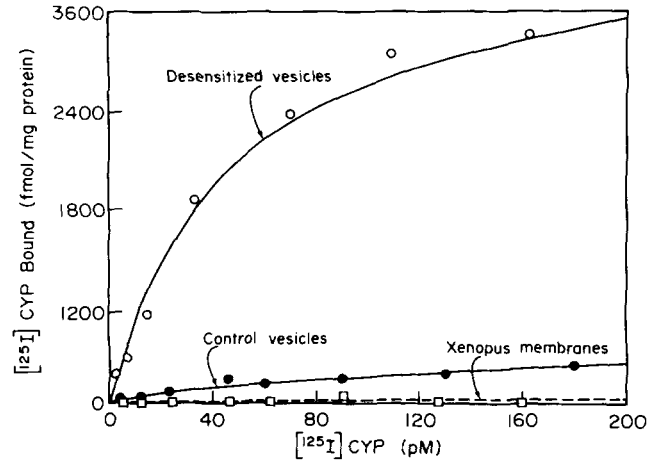

FIG. 1. **Saturation curves of ^{125}I-cyanopindolol binding to vesicles prepared from control and desensitized frog erythrocytes and to plasma membranes from *X. laevis* erythrocytes.** Frog erythrocytes were incubated either with (*Desensitized*) or without (*Control*) 10^{-4} M isoproterenol for 3 h, and vesicles isolated as described under "Experimental Procedures." The amount of β-adrenergic receptors was determined (see above) using ^{125}I-cyanopindolol in both the vesicles and in plasma membranes prepared from *X. laevis* erythrocytes. Curves were analyzed by computer modeling procedures (20, 21). Nonspecific binding was defined with 10^{-4} M isoproterenol. The *data points* represent the means of triplicate determinations from three separate experiments.

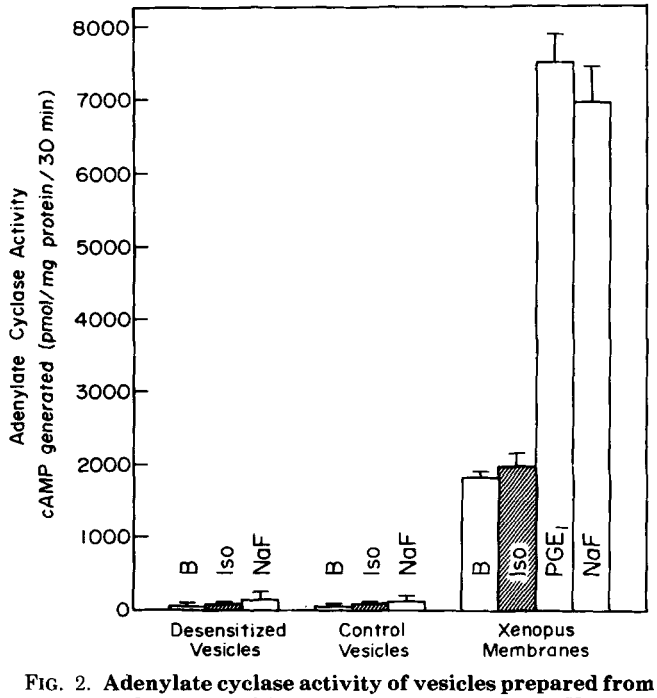

FIG. 2. **Adenylate cyclase activity of vesicles prepared from control and desensitized frog erythrocytes and from *X. laevis* erythrocyte plasma membranes.** Adenylate cyclase activity was assayed as described under "Experimental Procedures" utilizing 20 μg of protein/assay for vesicles and 5 μg of protein/assay for *Xenopus* membranes. The results are the mean ± S.E. of the results from four experiments each determined in triplicate and are represented as pmol of cAMP/mg of protein/30 min. *B*, basal; *Iso*, stimulated by 5 × 10^{-5} M isoproterenol; *NaF*, stimulated by 10 mM NaF.

[2] We have previously demonstrated that the down-regulated β-adrenergic receptors in frog erythrocytes are removed from the cell surface as assessed by their inaccessibility to reagents such as DCCD which ordinarily inactivate β-adrenergic receptors at the cell surface (1). These sequestered receptors are found in a light vesicle fraction of the cell after lysis and differential centrifugation. Although the precise location of these receptors within the intact cell has not been rigorously determined, we refer to them here as internalized.

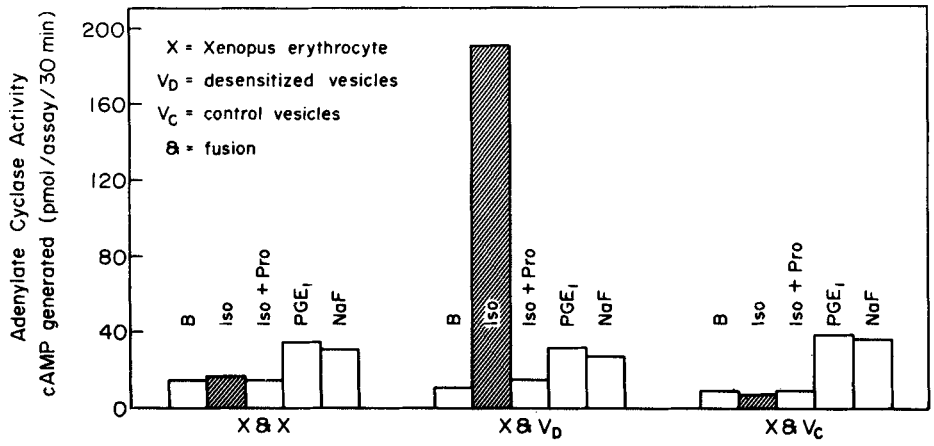

FIG. 3. **Fusion of *X. laevis* erythrocytes with vesicles prepared from control and desensitized frog erythrocytes.** Vesicles from control and desensitized frog erythrocytes containing equal amounts of protein (0.8 mg) and 0.180 and 2 pmol of β-adrenergic receptor, respectively, were fused with 2×10^7 *X. laevis* erythrocytes as described under "Experimental Procedures." Hybrid membranes were prepared by a freeze-thaw technique as previously described (19), and basal and hormone-stimulated adenylate cyclase activity was measured as described under "Experimental Procedures." In each experiment, *X. laevis* erythrocytes alone were carried through the same fusion procedure and basal and isoproterenol-stimulated adenylate cyclase activity was measured in the membranes. The data are the means of triplicate determinations from a representative experiment which was repeated three times with comparable results. *B*, basal; *Iso*, 5×10^{-5} M isoproterenol; *Iso + Pro*, 5×10^{-5} M isoproterenol plus 5×10^{-5} M propranolol; *PGE_1*, 3×10^{-6} M prostaglandin E_1; *NaF*, 10 mM NaF.

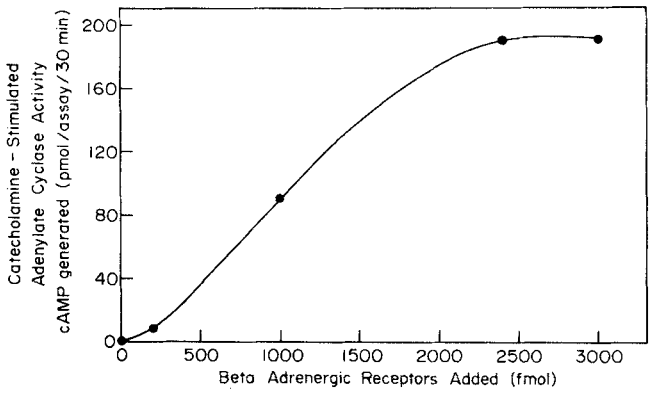

FIG. 4. **Adenylate cyclase activity of *X. laevis* erythrocyte membranes as a function of the amount of vesicular β-adrenergic receptors added to the fusion system.** For each point, the indicated amount of β-adrenergic receptors from vesicles prepared from desensitized frog erythrocytes was fused with 5×10^7 *X. laevis* erythrocytes. The specific activity of the desensitized vesicle preparation was 2800 fmol of β-adrenergic receptor/mg of protein. Basal and isoproterenol-stimulated adenylate cyclase activity in the hybrid membranes was measured as described under "Experimental Procedures." The basal adenylate cyclase activity was 7 pmol/30 min and was subtracted from each point. The data represent triplicate determinations from a representative experiment which was repeated four times with similar results.

with isoproterenol (5×10^{-5} M), propranolol (5×10^{-5} M), or PGE_1 (3×10^{-6} M).

Protein was determined by the method of Lowry (27).

RESULTS

The β-adrenergic receptor binding characteristics and adenylate cyclase activities of the internalized vesicles from control and desensitized frog erythrocytes and of the acceptor *X. laevis* erythrocyte membranes are summarized in Figs. 1 and 2. The density of receptors in the vesicles from desensitized frog erythrocytes is ~3600 fmol/mg of protein. Nonetheless, as reported previously (1), catecholamine- and F^--sensitive adenylate cyclase are barely detectable (Fig. 2). Although the protein yield of vesicles prepared from control erythrocytes is the same as that from desensitized cells, these contain <1/10th as many β-adrenergic receptors (Fig. 1). These findings are consistent with the notion that the vesicles prepared from desensitized frog erythrocytes provide a rich source of β-adrenergic receptor. Membranes from *X. laevis* erythrocytes contain PGE_1- and F^--sensitive adenylate cyclase, however these cells contain negligible quantities of β-adrenergic receptor and catecholamine-sensitive adenylate cyclase (Figs. 1 and 2).

In order to assess the functionality of the desensitized frog erythrocyte β-adrenergic receptors present in the vesicles, we developed a fusion technique utilizing *X. laevis* erythrocyte membranes as a source of adenylate cyclase components (see "Experimental Procedures"). This technique was patterned after methods previously described by Schramm (22), Eimerl *et al.* (23), and Korner *et al.* (24). Fusion of vesicles from desensitized frog erythrocytes with *X. laevis* erythrocytes results in establishment of a markedly (30–40-fold) catecholamine-sensitive adenylate cyclase in the hybrid membranes (Fig. 3). The isoproterenol stimulation is entirely blocked by the β-adrenergic receptor antagonist propranolol. The PGE_1- and F^--sensitive adenylate cyclase activities of the acceptor *X. laevis* erythrocytes are unaffected by the fusion procedure.

The level of catecholamine-sensitive adenylate cyclase is related to the amount of β-adrenergic receptor added to the fusion system over an ~10-fold range of R (Fig. 4). Furthermore, if vesicles from control frog erythrocytes, which possess much lower amounts of β-adrenergic receptor, are used as the R donor, the adenylate cyclase activity of the resulting hybrids is not catecholamine-sensitive (Fig. 3).

Two independent lines of evidence indicate that the catecholamine responsiveness of the adenylate cyclase in the fused hybrids is mediated by the β-adrenergic receptors donated by the vesicles from desensitized frog erythrocytes. First, if the β-adrenergic receptors present in the vesicles are inactivated by the group-specific reagent DCCD prior to their fusion to *Xenopus* erythrocytes, the resultant hybrids possess no isoproterenol-stimulated adenylate cyclase (Fig. 5). Second, catecholamine-stimulated adenylate cyclase in the hybrids shows the characteristic β_2-adrenergic specificity typical of the frog erythrocyte system (Fig. 6).

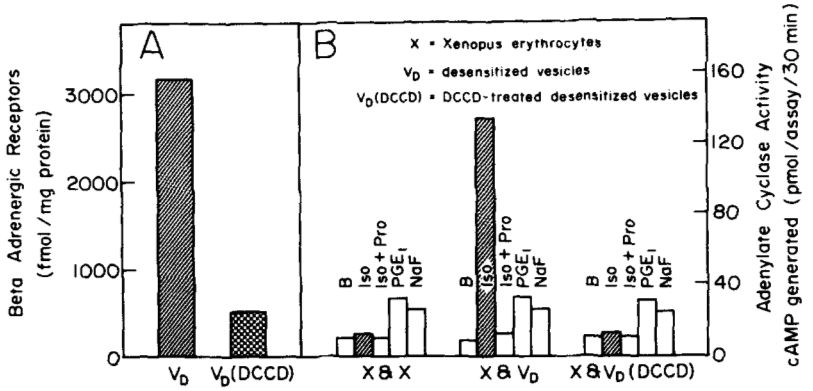

FIG. 5. **Effect of DCCD treatment on β-adrenergic receptor binding and reconstitutive activity of vesicles prepared from desensitized frog erythrocytes.** Vesicles prepared from desensitized frog erythrocytes were incubated with 100 μM DCCD for 30 min at 25 °C as previously described (1). *A*, β-adrenergic receptors were assayed by ^{125}I-cyanopindolol (50 pM) binding. Vesicles from desensitized frog erythrocytes (V_D = 3000 fmol of β-adrenergic receptor equivalent to ~1 mg of protein) or vesicles which were DCCD-treated before undergoing the fusion procedure (V_D(DCCD) = 400 fmol of β-adrenergic receptor/1 mg of protein) were fused with 2×10^7 *X. laevis* erythrocytes. *B*, adenylate cyclase activity of the hybrids was measured as described under "Experimental Procedures." Data represent the mean of duplicate (binding) or triplicate (adenylate cyclase) determinations from a representative experiment. This experiment was replicated twice more with comparable results. See legend to Fig. 3 for identification of abbreviations.

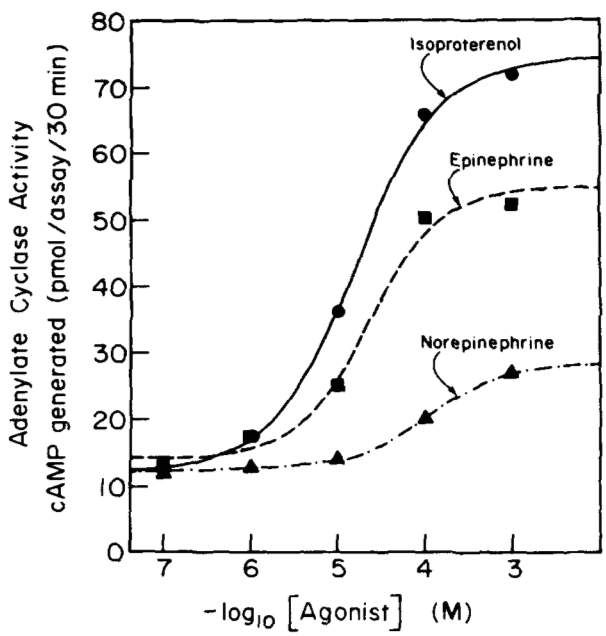

FIG. 6. **Dose-response curves for agonist stimulation of adenylate cyclase in hybrid cell membranes.** Membranes were prepared from hybrids resulting from the fusion of 2.4 pmol (0.8–1 mg of protein) of β-adrenergic receptors and 2×10^7 *X. laevis* erythrocytes. Data shown are the average of two experiments determined in triplicate.

DISCUSSION

Most previous work on internalization of hormone receptors has been performed with polypeptide hormones such as insulin (Refs. 15 and 17; for review see Ref. 28) for which the biochemical effectors are as yet poorly understood. An important question not previously amenable to direct experimental investigation has been the functional status of the internalized receptors. In the present work, we have been able, for the first time, to assess the functional status of a hormone receptor after it has been sequestered away from the cell surface. This became possible by choosing to work with a hormone receptor which is closely coupled to a well defined biochemical effector, specifically the adenylate cyclase-coupled β-adrenergic receptor of the frog erythrocyte. It has been previously demonstrated (1) that when these cells are exposed to agonists, ~50% of their surface receptors are translocated to cytosolic vesicles which are devoid of the effector nucleotide regulatory protein and catalytic units.

The fusion system which we have used to establish the functionality of the internalized β-adrenergic receptors is a modification of the membrane-cell fusion technique originally developed by Schramm (22), Eimerl *et al.* (23), and Korner *et al.* (24). Since the receptor-containing vesicles are devoid of nucleotide regulatory protein and catalytic unit (1), it was not necessary to treat them prior to fusion with reagents to inactivate these components. The use of the *Xenopus* erythrocyte as an acceptor for the β-adrenergic receptor is a novel feature of the present work. Whereas most amphibian and avian erythrocytes contain a full complement of β-adrenergic receptors, surprisingly, these erythrocytes are virtually devoid of β-adrenergic receptor and catecholamine-sensitive adenylate cyclase. Moreover, the cells are easily accessible and there is no requirement for tissue culture facilities. This new system has obvious applications as an acceptor for other membrane-bound, solubilized, or even purified receptors. The presence of an endogenous PGE_1 receptor provides an internal control to assess the overall functionality of the system after the fusion manipulations.

The present findings are in accord with our previous observations (1), utilizing photoaffinity-labeling techniques, that the desensitized receptors are not altered structurally. These findings also correlate with work *in vivo* (29) and *in vitro* (30) which has shown that frog erythrocytes can recover their full complement of β-adrenergic receptors in the plasma membranes once agonist is removed, even in the presence of cycloheximide which blocks protein synthesis (30). Collectively, these data suggest that the frog erythrocyte β-adrenergic receptors internalized during desensitization are not processed and are likely recycled to the cell surface. However, the mechanisms by which agonists promote the translocation of the receptors from the plasma membrane to the vesicular compartment remain to be elucidated.

Previous work has demonstrated that frog erythrocyte β-adrenergic receptors which are internalized within vesicles appeared uncoupled as assessed by their inability to form a high affinity agonist receptor complex. This complex, which

is necessary for agonist-stimulated adenylate cyclase, appears to consist of hormone, R, and N in a "ternary" complex (21, 31). Although the demonstrated absence of N from the vesicles provides an obvious explanation for the inability of R to form RN complexes, functional alterations in R might also exist. The present work establishes the functional integrity of these receptors which, within the intracellular vesicles appear totally uncoupled from their normal effectors. A major thrust of the present work, then, is the clear delineation of a specific mechanism of cellular desensitization—physical sequestration of a *functionally normal receptor* away from the effector units with which it normally interacts.

Acknowledgments—We wish to acknowledge the assistance of Dr. Catherine Strader in the development of the fusion technique. We would like to thank Dr. Marc Caron and Thomas Michel for valuable suggestions during the preparation of this manuscript. We would also like to thank Donna Addison for excellent secretarial assistance.

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