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β-Agonist- and prostaglandin E_1-induced translocation of the β-adrenergic receptor kinase: Evidence that the kinase may act on multiple adenylate cyclase-coupled receptors

(desensitization/receptor uncoupling/down-regulation)

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ABSTRACT β-Adrenergic receptor kinase (β-AR kinase) is a cytosolic enzyme that phosphorylates the β-adrenergic receptor only when it is occupied by an agonist [Benovic, J. Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. USA* 83, 2797–2801.] It may be crucially involved in the processes that lead to homologous or agonist-specific desensitization of the receptor. Stimulation of DDT_1MF-2 hamster smooth muscle cells or S49 mouse lymphoma cells with a β-agonist leads to translocation of 80–90% of the β-AR kinase activity from the cytosol to the plasma membrane. The translocation process is quite rapid, is concurrent with receptor phosphorylation, and precedes receptor desensitization and sequestration. It is also transient, since much of the activity returns to the cytosol as the receptors become sequestered. Stimulation of β-AR kinase translocation is a receptor-mediated event, since the β-antagonist propranolol blocks the effect of agonist. In the kin^- mutant of the S49 cells (lacks cAMP-dependent protein kinase), prostaglandin E_1, which provokes homologous desensitization of its own receptor, is at least as effective as isoproterenol in promoting β-AR kinase translocation to the plasma membrane. However, in the DDT_1MF-2 cells, which contain α_1-adrenergic receptors coupled to phosphatidylinositol turnover, the α_1-agonist phenylephrine is ineffective. These results suggest (*i*) that the first step in homologous desensitization of the β-adrenergic receptor may be an agonist-promoted translocation of β-AR kinase from cytosol to plasma membrane and (*ii*) that β-AR kinase may represent a more general adenylate cyclase-coupled receptor kinase that participates in regulating the function of many such receptors.

Desensitization, the tendency of biological responses to wane over time despite the continuous presence of a stimulus of constant intensity, is widely observed in eukaryotic and even prokaryotic systems (1–5). In the case of the receptor-coupled adenylate cyclase system, several forms of this process are known. Homologous or "agonist-specific" desensitization refers to a process whereby exposure to one type of agonist is followed by refractoriness only to that class of agonist but not to agonists that work through distinct receptors (5). This form of desensitization is characterized by functional "uncoupling" of the receptors and their sequestration by internalization within the cell. It is not cAMP-mediated (6).

Recently it has been demonstrated that homologous desensitization of the β-adrenergic receptor-coupled adenylate cyclase is associated with non-cAMP-mediated phosphorylation of the receptors (7, 8). Moreover, a cAMP-independent kinase, termed the β-adrenergic receptor kinase (β-AR kinase) (1) has been identified and partially purified from the cytosolic fraction of the kin^- mutant of S49 cells [lacks cAMP-dependent protein kinase (9, 10)] and other tissues. This kinase stoichiometrically phosphorylates only the agonist-occupied form of the pure β-adrenergic receptor.

The discovery of β-AR kinase may provide the key link in explaining the biochemical mechanisms that underlie homologous desensitization of adenylate cyclase-coupled receptors. However, important questions about its biology remain to be answered. Two of the most salient are as follows: (*i*) How does a cytosolic enzyme function to modify a plasma membrane receptor? (*ii*) Is the specificity and activity of β-AR kinase limited to the β-adrenergic receptor or might it include other types of adrenergic and/or adenylate cyclase-coupled receptors? We report here that agonist stimulation of β-adrenergic receptors or PGE_1 (prostaglandin E_1) receptors leads to rapid and reversible translocation of β-AR kinase from cytosol to plasma membrane.

MATERIALS AND METHODS

Cells. DDT_1MF-2 smooth muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 20 mM Hepes. S49 lymphoma cells deficient in cAMP-dependent protein kinase (kin^-) (clone 24.6.1) were grown in DMEM with 10% horse serum. The phenotype of kin^- cells was verified, in order to exclude revertants (11), by cAMP-dependent protein kinase determination (12).

***In Situ* Receptor Phosphorylation.** Incubations of intact cells with carrier-free $[^{32}P]P_i$ to label the intracellular ATP pool, ATP determination, and purification of the β-adrenergic receptors from cell plasma membranes by affinity chromatography were performed as described (8).

Preparation of Cell Fractions for Assay of β-AR Kinase. Cells were incubated in serum-free DMEM at 37°C in the presence or absence of agonists as indicated. Cells were sedimented by centrifugation (800 × *g* for 5 min) and then lysed in 2 volumes of 10 mM Tris/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/leupeptin (5 μg/ml)/pepstatin (5 μg/ml) at pH 7.4, using a Polytron tissue disruptor (Brinkmann) at full speed (3 times, 30 sec each). Unbroken cells and cell nuclei were sedimented at 800 × *g* for 3 min and discarded. The plasma membranes were then sedimented at 48,000 × *g* for 20 min. The supernatant, representing the cytosolic fraction, was spun at 150,000 × *g* for 60 min. Preparations were stable at 4°C for at least 4

Abbreviations: β-AR kinase, β-adrenergic receptor kinase; PGE_1, prostaglandin E_1; kin^-, cAMP-dependent protein kinase-deficient.
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weeks. Initially, the β-AR kinase was extracted with 0.5% Triton X-100 in the same buffer and isolated in the soluble fraction after centrifugation (150,000 × g for 60 min). In later experiments, we discovered that we were able to use the plasma membranes directly (Fig. 4). Both methods yielded similar amounts of receptor kinase.

Kinase Assay. To determine the β-AR kinase activity, we used a modified procedure as published previously (1). As substrate, we used β-adrenergic receptors from hamster lung, affinity-purified on alprenolol-Sepharose columns (13). To avoid detergents in the incubation, we first reconstituted the β-adrenergic receptors into phospholipid vesicles as described (1, 14). The reconstituted receptor (3–5 pmol) was incubated in 25 mM Tris, pH 7.4/10 mM NaCl/1.5 mM EDTA/1 mM EGTA/5 mM $MgCl_2$/0.1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/leupeptin (5 μg/ml)/pepstatin (5 μg/ml) containing 5 mM NaF and 50 μM Na_3VO_4 as phosphatase inhibitors, in the presence of 50 μM [γ-^{32}P]ATP (15–25 cpm/fmol), 0.1 mM (−)-isoproterenol, and the appropriate kinase preparation, for 20 min at 30°C in a total volume of 100 μl. The reaction was stopped by adding 1 ml of ice-cold 100 mM NaCl/10 mM Tris/2% (wt/vol) digitonin, pH 7.2. After this incubation, further purification of the β-adrenergic receptor was necessary, since with these crude kinase preparations other, non-agonist-dependent kinases were found to phosphorylate contaminating substrates (1). This was accomplished by sequential wheat germ agglutinin-Sepharose and alprenolol-Sepharose affinity chromatography.

Equal amounts of receptor were then electrophoresed in $NaDodSO_4$/polyacrylamide gels (15). After autoradiography, the receptor bands were excised and radioactivity was measured in a Packard scintillation counter with ≈100% counting efficiency. Phosphate incorporation into the receptor was estimated based on the specific activity of [γ-^{32}P]ATP in the incubation assay (1). In some experiments the relative amounts of β-AR kinase activity in different preparations were compared by performing laser densitometry of the phosphorylated receptor bands.

Adenylate Cyclase Assays. Desensitization was verified by measuring the adenylate cyclase activity in plasma membranes (16).

[^{3}H]CGP-12177 Binding. Whole cell β-adrenergic receptor binding assays with [^{3}H]CGP-12177 were as described (8, 17).

RESULTS

Fig. 1 *A* and *B* present the time course of β-agonist-induced homologous desensitization in DDT_1MF-2 cells. After exposure to the β-agonist isoproterenol, the responsiveness of the enzyme to isoproterenol stimulation rapidly falls (Fig. 1*A*). $t_{1/2}$ for this process is ≈15 min, with maximal (≈60%) desensitization reached at ≈40 min. Adenylate cyclase activities stimulated by PGE_1, NaF, and forskolin are unaffected. Over precisely the same time course, ≈40% of the β-adrenergic receptors are sequestered away from the cell surface, as assessed by binding of the hydrophilic β-adrenergic ligand [^{3}H]CGP-12177 (Fig. 1*B*). During this time period (60 min) there is no change in the total number of cellular β-adrenergic receptors measured by the binding of the hydrophobic ligand [^{125}I]iodocyanopindolol to whole cells or broken-cell preparations (data not shown).

Fig. 1*C* displays the phosphate incorporation into the β-adrenergic receptor at each time point during the desensitization process. In control cells, the incorporation is very low. During desensitization, phosphate incorporation rapidly rises to a peak by 20 min and then gradually declines. Thus the receptor phosphorylation event appears to slightly precede receptor uncoupling and sequestration. We have obtained similar results with S49 lymphoma cells (8), although

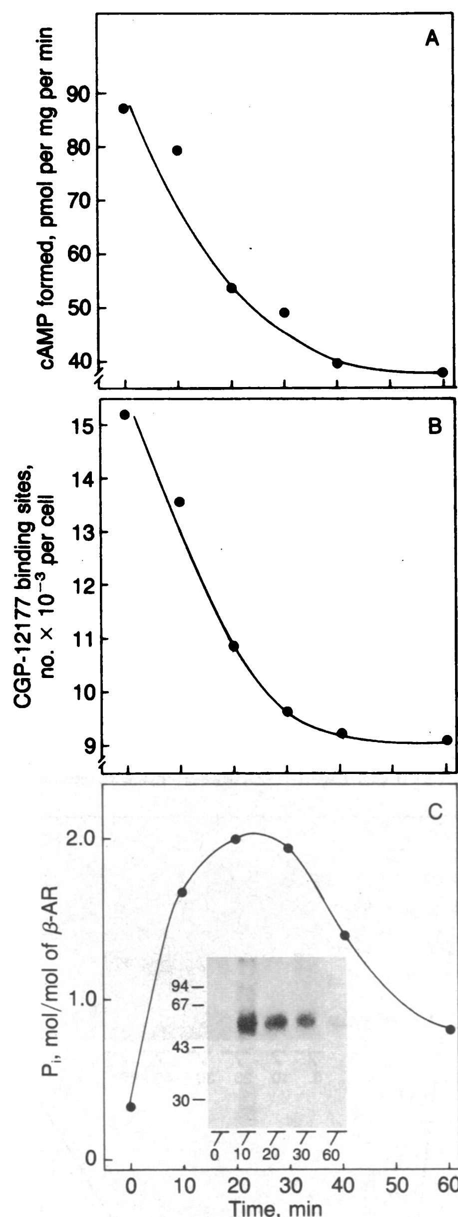

FIG. 1. Desensitization of the β-adrenergic receptor-coupled adenylate cyclase in DDT_1MF-2 cells. Cells were incubated in the presence of 10 μM (−)-isoproterenol for 10–60 min. Control cells were incubated in the absence of the drug for 10–60 min. (*A*) Isoproterenol (100 μM)-stimulated adenylate cyclase activity in plasma membranes. The means of four sets of experiments with triplicate determinations are shown. (*B*) Maximal number of specific β-receptor binding sites at the cell surface. Whole cells were incubated for 16 hr at 4°C with various concentrations of [^{3}H]CGP-12177 (0.05–4 nM). Nonspecific binding was determined in the presence of 10 μM alprenolol. Total number of receptor binding sites was determined by computer-assisted analysis of saturation isotherms (18). Data shown are the means of four sets of experiments with duplicate determinations. (*C*) Time course of β-agonist-promoted phosphorylation of the β-adrenergic receptor (βAR) in DDT_1MF-2 cells. DDT_1MF-2 cells were prelabeled with carrier-free [^{32}P]P_i (0.3 mCi/ml; 1 Ci = 37 GBq). (*Inset*) Autoradiogram after $NaDodSO_4$/polyacrylamide gel electrophoresis of the affinity-purified β-adrenergic receptors at various times during desensitization (see *Materials and Methods*). Relative mobilities and apparent molecular weights ($M_r \times 10^{-3}$) of standard proteins are shown at left. Data are representative of four experiments. The stoichiometry of receptor phosphorylation, determined from the mean of four experiments, is plotted in the graph.

all of these processes occur more rapidly in that cell line. We speculate that dephosphorylation of receptors in a sequestered intracellular compartment might account for the decline in receptor phosphate content during the later phase of the desensitization process (19).

To assess the subcellular localization of the β-AR kinase during the time course of desensitization in these cells, we measured the activity of the enzyme in the cytosol (150,000 × g supernatant) and in a detergent extract of the plasma membranes at various times after exposure to isoproterenol. Prior to exposure to the agonist, β-AR kinase activity was primarily found in the cytosol, with very little activity demonstrable in the membranes (Figs. 2 and 3). In the absence of an agonist, β-AR kinase activity did not change during incubation. Exposure to a β-agonist resulted in a rapid and marked fall in cytosolic β-AR kinase activity (by ≈90% at 10–20 min), which gradually returned to 80–100% of control by 60 min (Fig. 2). This was closely mirrored by a reciprocal 5- to 10-fold rise in β-AR kinase activity extractable from the plasma membranes, which peaks at 20 min and then gradually returns toward normal between 30 and 60 min (Fig. 3). In three experiments, ≈40% of the β-AR kinase activity that was lost from the cytosol was recovered in the membrane fractions. The β-adrenergic antagonist propranolol blocked the (−)-isoproterenol effect (data not shown).

DDT_1MF-2 cells, derived from hamster vas deferens smooth muscle cells (20), are a rich source of α_1-adrenergic receptors coupled to the hydrolysis of polyphosphoinositides (21). Each cell contains ≈60,000 α_1-adrenergic receptors vs.

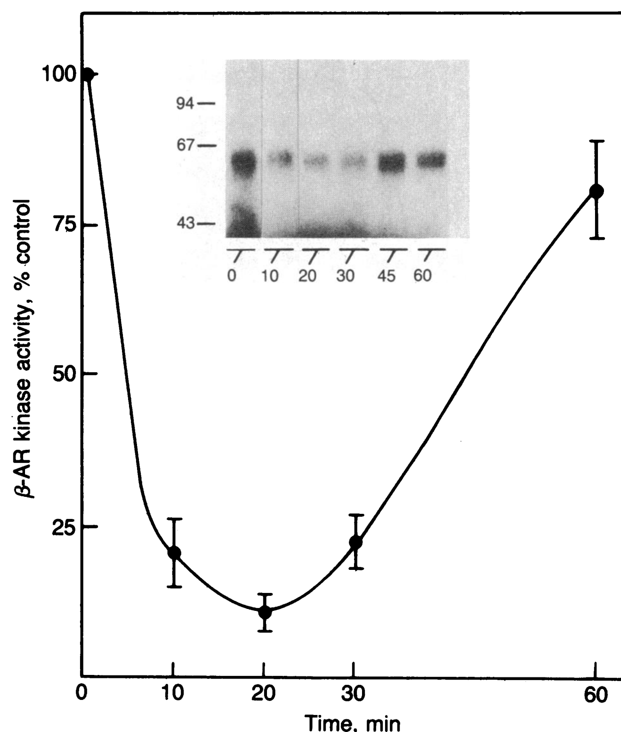

FIG. 2. Disappearance of the receptor kinase activity from the cytosol during desensitization. DDT_1MF-2 cells were incubated at 37°C in the presence of isoproterenol (10 μM) for 10–60 min. Controls were incubated for 60 min. Receptor kinase activity remaining in the cytosol was measured by incubating with reconstituted pure hamster lung β-adrenergic receptor, isoproterenol (100 μM), and [γ-^{32}P]ATP (15–25 cpm/fmol) for 20 min at 30°C. β-Adrenergic receptor kinase activities (normalized as % of control) in the cytosol from control (0 time) or desensitized (10–60 min) cells are shown. Values are the means ± SEM from four experiments. (*Inset*) Autoradiogram of affinity-purified β-adrenergic receptors phosphorylated by receptor kinase isolated from the cytosol of control or desensitized cells.

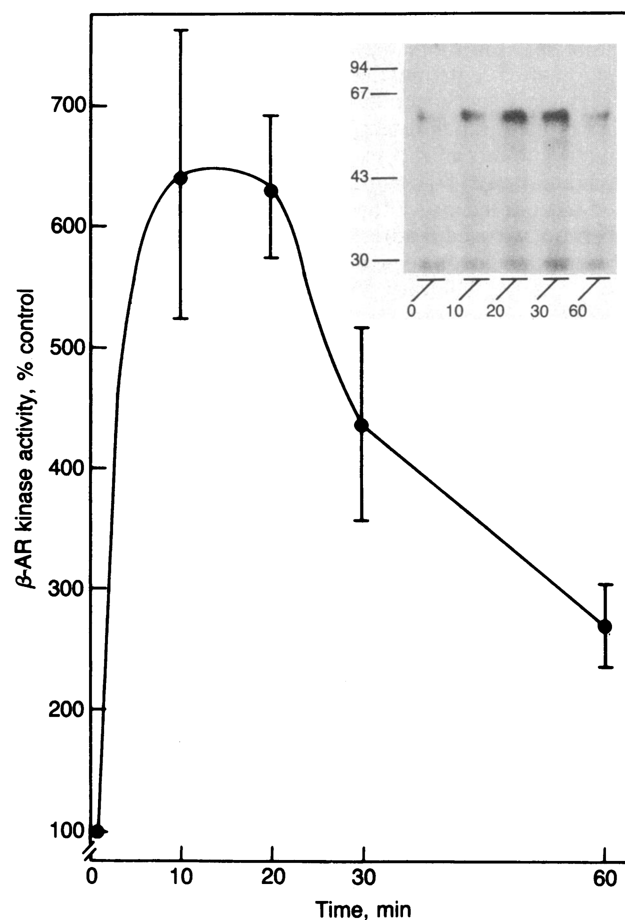

FIG. 3. Appearance of the receptor kinase activity in the plasma membrane fraction during desensitization. DDT_1MF-2 cells were incubated as for Fig. 2. The receptor kinase solubilized from the plasma membranes was assayed using reconstituted hamster lung β-adrenergic receptor, isoproterenol (100 μM), and [γ-^{32}P]ATP. (*Inset*) Autoradiogram of affinity-purified receptors phosphorylated by receptor kinase isolated from the plasma membranes of control (0 time) or desensitized cells. Data are representative of four experiments.

15,000–25,000 β_2-adrenergic receptors (21, 22). Stimulation of the cells with the full α_1-agonist phenylephrine (10 μM for 20 min) did not result in a decline in cytosolic β-AR kinase activity (data not shown). This suggests that β-AR kinase does not interact with α_1-adrenergic receptors and that its translocation is not activated by protein kinase C.

Since DDT_1MF-2 cells do not contain adenylate cyclase-stimulatory receptors other than the β-adrenergic receptor (of which we are aware), we next studied the S49 lymphoma cell, and in particular its kin$^-$ mutant. When these cells are incubated with isoproterenol (10 μM) or PGE_1 (1 μM) for 15 min, each hormone induces homologous desensitization to its own actions, but not to the actions of the other agonist (data not shown). Fig. 4 shows the results obtained when β-AR kinase activity was measured in cytosol and plasma membrane fractions of cells homologously desensitized with either PGE_1 or isoproterenol. PGE_1 was at least as effective as isoproterenol in leading to a decrease in cytosolic and an increase in plasma membrane β-AR kinase activity. In the experiment shown, isoproterenol promoted a 75% and PGE_1 an 85% fall in cytosolic β-AR kinase activity. Conversely, the β-agonist promoted an ≈7.5-fold increase and PGE_1 a 12-fold increase in plasma membrane β-AR kinase activity. Thus, homologous desensitization of the PGE_1 receptor is associated with translocation of β-AR kinase, just as is desensiti-

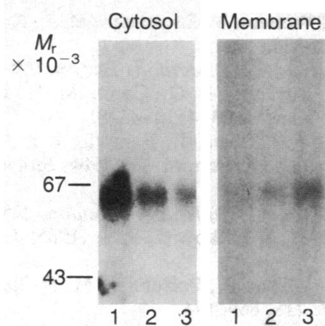

FIG. 4. Translocation of receptor kinase activity from the cytosol to the plasma membranes during isoproterenol- or PGE_1-induced desensitization in kin⁻ S49 lymphoma cells. Cells were incubated for 15 min at 37°C with no drug, with 10 μM isoproterenol, or with 1 μM PGE_1. The receptor kinase activity from the cytosol and the plasma membranes was determined utilizing pure reconstituted β-adrenergic receptor as substrate. Autoradiogram obtained after $NaDodSO_4$/polyacrylamide gel electrophoresis of the affinity-purified phosphorylated receptors is shown. The experiment was replicated three times with comparable results. Lanes: 1, control cells; 2, isoproterenol-desensitized cells; 3, PGE_1-desensitized cells.

zation of the β-adrenergic receptor, and cAMP-dependent protein kinase is not involved.

DISCUSSION

Recent evidence indicates that phosphorylation of the β-adrenergic receptor by the cAMP-dependent protein kinase is involved in some forms of heterologous desensitization (23–26), whereas phosphorylation by the cAMP-independent β-AR kinase may be related to homologous desensitization (1). Here we report that stimulation of cells with a β-agonist leads to rapid translocation of the β-AR kinase from cytosol to plasma membranes. That this physical translocation of the enzyme, rather than a simple inactivation, explains the loss of enzyme activity from the cytosolic fraction after stimulation with agonist is documented by the reciprocal appearance of the enzyme in the plasma membrane fraction. The mechanism by which agonist occupancy of the β-adrenergic receptor triggers movement of the kinase to the membrane can only be conjectured. One possibility is the generation of a second messenger. However, this would presumably not be cAMP working through its usual effector, the cAMP-dependent protein kinase, since the translocation occurs in kin⁻ cells, which lack this enzyme. Also, there is no precedent for messengers other than cAMP coupled to β-adrenergic receptors. A more likely explanation is that agonist occupancy induces conformational changes in the receptor that expose the sites of phosphorylation recognized by β-AR kinase. The enzyme then binds to its substrate, the conformationally altered receptor, presumably at the inner surface of the plasma membrane.

The agonist-induced translocation of β-AR kinase from cytosol to membranes bears an obvious analogy to the translocation of protein kinase C induced by phorbol esters and several agonists whose actions are mediated via the hydrolysis of polyphosphoinositides (27–31). Like that translocation, the movement of β-AR kinase to the membrane reverses and much of the kinase returns to the cytosol even in the continued presence of agonist. This might be due to the sequestration of the receptors which occurs during the desensitization process, thus releasing β-AR kinase back to the cell interior.

The agonist-promoted translocation of β-AR kinase is quite consistent with its proposed role in the homologous desensitization of the receptor. First, and most obviously, the kinase must catalyze the phosphorylation of a substrate (β-adrenergic receptor) that is located in the plasma membrane. Moreover, the time course of the translocation is quite compatible with that of other parameters of the homologous-desensitization pathway in the cells. The sequence of events appears to be β-AR kinase translocation, receptor phosphorylation, and then receptor desensitization and sequestration. The late decline in the level of receptor phosphorylation while desensitization persists, also observed in S49 cells (8), is likely due to dephosphorylation and resensitization of the receptors in the sequestered compartment (18). Thus, at this point in the desensitization time course (e.g., 60 min in DDT_1MF-2 cells), the desensitized state is presumably maintained by virtue of the receptor sequestration per se (16). These findings are depicted schematically in the model shown in Fig. 5.

Can β-AR kinase phosphorylate any receptors other than the β-adrenergic receptor? The problem in approaching this question is the current unavailability of purified preparations of other adenylate cyclase-coupled receptors. While admittedly somewhat indirect, our results suggest that the PGE_1 receptor may also be a substrate for β-AR kinase. Thus, PGE_1, which causes homologous desensitization of its own receptor but which does not induce phosphorylation of the β-adrenergic receptor (7), is at least as effective as isoproterenol in promoting the translocation event. Stimulation of α_1 receptors coupled to the phosphatidylinositol cycle rather than adenylate cyclase did not lead to translocation of β-AR kinase.

Is it possible, then, that β-AR kinase is in fact a general

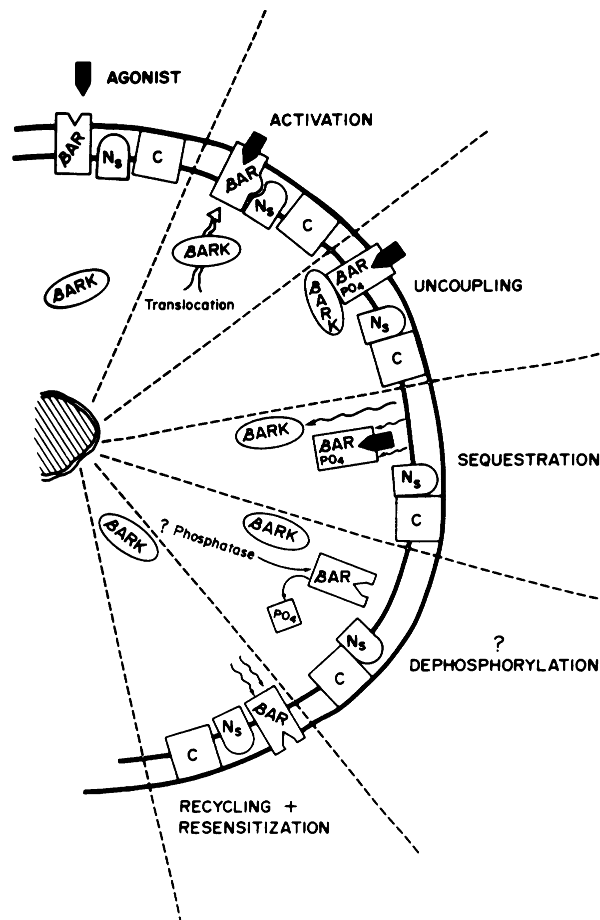

FIG. 5. Schematic representation of the molecular mechanisms of homologous desensitization. βAR, β-adrenergic receptor; βARK, β-AR kinase; N_s, stimulatory guanine nucleotide-binding regulatory protein; C, catalytic unit of adenylate cyclase.

"adenylate cyclase-coupled receptor" kinase? Several considerations make this hypothesis an attractive one. All such receptors couple to the same stimulatory guanine nucleotide-binding regulatory proteins (32). Thus, they presumably share a common structural domain involved in such coupling. Moreover, such a domain would likely be a site of crucial conformational modification in response to agonist occupancy of the receptor and hence a likely site of phosphorylation by β-AR kinase. There is also no inherent contradiction between the concept of a general adenylate cyclase-coupled receptor kinase and the notion of homologous or agonist-specific desensitization. This is so because the kinase will only phosphorylate a receptor if it is occupied by its agonist. Other adenylate cyclase-coupled receptors that are not being stimulated would presumably not be substrates for the kinase.

It is worth reemphasizing the analogies between β-AR kinase and rhodopsin kinase (33-36). The latter enzyme phosphorylates the retinal "light receptor" rhodopsin, in a light ("agonist")-dependent fashion leading to light adaptation ("desensitization"). Perhaps rhodopsin kinase and β-AR kinase represent related members of a more general family of receptor kinases that play important roles in regulating receptor function. The functional similarities between the β-adrenergic receptor and rhodopsin systems are even more striking in light of the recent cloning and analysis of the gene and cDNA for the β-adrenergic receptor, showing striking structural homologies between these two types of receptors (37).

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