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Sequestration and Recycling of β_2-Adrenergic Receptors Permit Receptor Resensitization

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SUMMARY

Stimulation of β_2-adrenergic receptors in intact cells causes, first, rapid functional uncoupling from G_s, which is triggered by receptor phosphorylation, and, second, somewhat slower sequestration of the receptors to an internal compartment. The present study addresses a possible role of sequestration in the resensitization of desensitized β_2-adrenergic receptors in human A431 cells. Exposure of these cells to isoproterenol caused rapid phosphorylation, desensitization (as assessed in adenylyl cyclase assays), and sequestration of the receptors. Subsequent removal of the agonist led to recycling of the receptors to the cell surface, dephosphorylation, and restoration of receptor function. These effects occurred without any change in the total receptor number. The rate constant of agonist-induced sequestration was 0.03/min; the rate constant of receptor recycling was 0.06/min and was not markedly altered by the presence of agonist. Blockade of sequestration with concanavalin A or 0.6 M sucrose prevented receptor dephosphorylation as well as receptor resensitization. Inhibition of protein phosphatases with calyculin A caused a similar blockade of β_2-adrenergic receptor resensitization; the effects of maximally effective concentrations of concanavalin A and calyculin A were not additive. Monensin impaired recycling of desensitized β_2-adrenergic receptors to the cell surface and also prevented receptor resensitization. We conclude that sequestration of β_2-adrenergic receptors, followed by dephosphorylation and recycling to the cell surface, may serve to restore the function of desensitized receptors.

Exposure of receptors in intact cells or tissues to agonists often causes a rapid decline in receptor responsiveness. This process is called receptor desensitization. It has been particularly well studied for the β_2-adrenergic receptor/G_s/adenylyl cyclase system, a receptor system that responds to β receptor agonists such as epinephrine or isoproterenol with the generation of cAMP (1, 2). Two types of rapid alteration in the function and disposition of β_2-adrenergic receptors are induced by agonists (3–5), i.e., functional uncoupling of the receptors from G_s and sequestration of the receptors to internal sites.

Much evidence suggests that functional uncoupling from G_s is triggered by phosphorylation of the receptors either by PKA or by one of the two known isoforms of βARK (6, 7). Whereas phosphorylation of β_2-adrenergic receptors by PKA can directly lead to uncoupling of the receptors from G_s (8, 9), phosphorylation by βARK serves to enhance the affinity of the receptors for an inhibitory protein, β-arrestin, which appears to bind to the βARK-phosphorylated receptors and thereby causes uncoupling (4, 10).

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In addition to functional uncoupling between receptors and G_s, there appears to be spatial uncoupling, which is caused by internalization of the receptors, a process usually termed receptor sequestration (11). Sequestration of β_2-adrenergic receptors was the first molecularly characterized agonist-induced alteration of β_2-adrenergic receptors. For many years, it has been defined by two criteria, i.e., the receptors become translocated to a membrane compartment that has a lower density than plasma membranes (12) and that is inaccessible to hydrophilic ligands (13). Attempts to define the intracellular localization of these receptors with antibodies have remained controversial, with some studies showing no agonist-induced loss of surface receptors and others reporting very marked reductions (14–16). More recently, experiments using antibodies directed against the receptors as well as antibodies for epitopes tagged to recombinant receptors have demonstrated that sequestered receptors are indeed translocated into the interior of cells, probably into endosomes (17).

The functional role of β_2-adrenergic receptor sequestration is not clear. Some authors proposed that it might serve to cause receptor desensitization (18, 19). This hypothesis was based on the observations that sequestration was agonist

ABBREVIATIONS: PKA, protein kinase A; βARK, β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

induced and that it moved the receptors away from G_s (which remained in the plasma membrane) and thereby abolished the capacity of the receptors to propagate a signal. However, in many cell types receptor desensitization proceeds much faster than receptor sequestration. Such kinetic differences were first noted in human astrocytoma 1321N1 cells (20). In human A431 epidermoid carcinoma cells, βARK- and PKA-mediated desensitization of β_2-adrenergic receptors occurs with half-lives of ~0.25 and ~3 min, respectively, whereas sequestration of these receptors has a half-life of ~10 min (21). Thus, sequestration mostly affects receptors that have already been uncoupled from G_s and, therefore, does not produce much additional desensitization. Furthermore, in many cell lines receptor sequestration affects only a small proportion of receptors. In A431 cells the maximal extent of sequestration is <30% of the cell surface receptors, and a similarly small extent of receptor desensitization occurs via sequestration even if PKA and βARK activities are blocked (22). This compares with receptor desensitization of 40–60% that can be triggered by the actions of either PKA or βARK (21, 22). Thus, the slow kinetics and the small extent of sequestration make it unlikely that this process plays a major role in bringing about desensitization. In fact, several studies have shown that blockade of sequestration, which can be achieved by pretreatment of cells with concanavalin A (20), does not markedly affect agonist-induced desensitization of β_2-adrenergic receptors (22–25).

Thus, it appears that the main function of sequestration cannot be to effect desensitization. Two major alternatives seem possible; (a) sequestration may serve as a first step in the process of receptor degradation that occurs in response to long term stimulation by agonists and (b) sequestration may be a pathway for the dephosphorylation, and thus resensitization, of desensitized receptors.

Mutation studies with β_2-adrenergic receptors have produced mutants that are sequestered normally but are impaired in their down-regulation (26), as well as a mutant that is not sequestered but is down-regulated normally (27); from those studies it may be concluded that sequestration and down-regulation are distinct and independent processes. However, it has been pointed out that these data are compatible with the hypothesis that sequestered receptors are subject to endosome sorting, provided that the majority of receptors are recycled to the cell surface and only a small proportion become degraded in lysosomes (17). Thus, sequestration may well be a first step in the down-regulation process.

An alternative hypothesis emerges from the observation that, after desensitization of β receptors in frog erythrocytes, sequestered receptors are phosphorylated to a lesser extent than those still residing in the plasma membrane (28). This observation may be interpreted as evidence for a receptor cycling model in which phosphorylation of the receptors by βARK leads to sequestration, sequestered receptors then become dephosphorylated and thus resensitized, and finally the receptors are recycled back to the cell surface. Although recent data suggest that sequestration occurs independently from receptor phosphorylation (22, 29), it is still possible that a major biological role of sequestration is in the dephosphorylation and subsequent recycling of desensitized receptors. Kassis *et al.* (24) and Yu *et al.* (30) addressed this hypothesis by investigating β_2-adrenergic receptor desensitization and resensitization under conditions where sequestration was blocked. Those experiments yielded contradictory results. Whereas Kassis *et al.* (24) reported that blockade of sequestration did not affect desensitization and resensitization of β_2-adrenergic receptors, Yu *et al.* (30) found impaired receptor resensitization under similar conditions. The present study was undertaken to clarify the role of receptor sequestration and to examine the effects of blockade of receptor sequestration and potential subsequent steps in the receptor recycling pathway on receptor resensitization. Preliminary results of this study have been presented earlier (31).

Materials and Methods

Cell culture. Human epidermoid carcinoma A431 cells of an individual subclone, termed A431-E3, were kindly provided by E. J. M. Helmreich, University of Würzburg. The cells were grown to approximately 90% confluence in DMEM (GIBCO) supplemented with 10% fetal calf serum (Pan Systems) and antibiotics (penicillin/streptomycin; GIBCO). They were maintained in serum-free DMEM overnight before all experiments. Unless stated otherwise, adherent cells were used. If cells were to be pretreated with concanavalin A (Sigma), they were incubated with 0.25 mg/ml concanavalin A in DMEM at 37° for 20 min and again washed twice with DMEM.

Receptor desensitization and resensitization. After the washing procedure, fresh medium containing 10 μM (−)-isoproterenol (or none, for controls) was added to adherent cells, and the cells were maintained in a CO_2 incubator at 37° for 10 min, to effect desensitization. The cells were then washed three times with ice-cold PBS and scraped in 30 ml of ice-cold 50 mM Tris·HCl, pH 7.4/150-mm Petri dish. They were disrupted with a Polytron homogenizer, and a crude membrane fraction was prepared by centrifugation of the supernatant at 50,000 × *g* for 30 min. These preparations were normalized to their protein content, as determined with the Bradford protein assay (32).

For resensitization studies, isoproterenol was removed by three rapid washes of the cells with medium (37°), fresh medium was added, and the cells were placed in an incubator for the indicated periods of time. They were harvested as described above.

The various inhibitors used in this study [10–100 nM calyculin A (Calbiochem), 100 μM monensin (Sigma), and 0.5–0.75 M sucrose (Sigma)] were present throughout the desensitization and resensitization procedures, and the procedures were timed so that all cells were exposed to the inhibitors for equal periods of times.

Determination of receptor sequestration. A431 cells were desensitized and resensitized with 10 μM (−)-isoproterenol as described above. They were then placed on ice and washed three times with ice-cold DMEM. The percentage of sequestered receptors was determined as described earlier (22). In brief, the receptor concentration was measured in the cells by binding of ^{125}I-cyanopindolol (Amersham), using 1 μM (−)-propanolol to define the total number of receptors and 0.3 μM CGP 12177 (Ciba Geigy) to define cell surface receptors. In preliminary experiments we also determined the number of sequestered receptors by sucrose density gradient centrifugation (22), to validate the results of the binding assay.

Determination of receptor phosphorylation. For phosphorylation experiments, cells were harvested with collagenase and washed twice with 10 ml of phosphate-free DMEM, with intervening centrifugations at 300 × *g* for 5 min. Cells (5×10^8) were incubated at 37° for 90 min in 20 ml of phosphate-free DMEM containing 1 mCi of [^{32}P]phosphate/tube (Amersham), to label the intracellular ATP pool. The specific radioactivity of the cellular ATP pool was determined as described by Sibley *et al.* (33) and was, on average, 150 cpm/pmol of [γ-^{32}P]ATP.

The cells were washed three times in DMEM to remove unincorporated radioactivity. They were then desensitized and resensitized as described above. At the end of the desensitization or resensitiza-

tion times, the reactions were stopped by the addition of 30 ml of ice-cold PBS, followed by three washes in ice-cold PBS. The cells were then disrupted, as described above, in 30 ml of ice-cold 5 mM Tris·HCl, pH 7.4, containing 2 mM EDTA and 10 mM sodium phosphate plus 0.15 μM okadaic acid to inhibit phosphatase activity during receptor isolation. The subsequent steps were performed as described earlier (34); the receptors were purified by affinity chromatography, and equal amounts of receptors (0.4 pmol) were electrophoresed on 12% SDS-polyacrylamide gels and visualized by autoradiography.

Adenylyl cyclase assays. Adenylyl cyclase activity in the membranes was determined as described earlier (35). Incubations contained ~50 μg of membrane protein, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 100 μM cAMP, 50 μM GTP, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 1 mg/ml bovine serum albumin, and 100 μM [α-^{32}P]ATP (0.2 μCi/tube; Amersham). [α-^{32}P]ATP was purified by anion exchange chromatography on Dowex 1X2 (chloride form) resin (36). $MgCl_2$ was added to give a free Mg^{2+} concentration in these assays of 0.8 mM, to permit detection of both heterologous (PKA-mediated) and homologous (βARK-mediated) desensitization (22, 37). Incubations were performed at 37° for 30 min. Accumulation of cAMP was linear under these conditions.

Data analysis. Quantitative data are presented as means of at least three independent experiments. The kinetics of receptor sequestration and externalization were analyzed with a model derived from the work of Koenig and Edwardson (38). It was assumed that agonist-induced sequestration of surface receptors (R_s) over time (t) occurs as an exponential process with a time constant k_1, such that $dR_s/dt = -k_1 \cdot R_s$. Likewise, externalization of sequestered receptors (R_i) was assumed to occur according to the differential equation $dR_i/dt = -k_2 \cdot R_i$. If both sequestration and externalization occur at the same time (as would normally be the case), then at steady state $dR_s/dt = dR_i/dt$. The agonist-induced sequestration process begins with an initial receptor distribution of surface (R_{s0}) and sequestered (R_{i0}) receptors. Because the total receptor number (R_t) equals $R_s + R_i$ at all time points, we can combine the two equations to $dR_i/dt = k_1R_t - (k_1 + k_2)R_i$. This can be integrated to describe the time course of agonist-induced accumulation of sequestered receptors

$$R_i(t) = R_t \cdot \frac{k_1}{k_1 + k_2} - \frac{k_1R_{s0} - k_2R_{i0}}{k_1 + k_2} \cdot e^{-(k_1+k_2)\cdot t} \quad (1)$$

The parameters of this equation were estimated by nonlinear curve-fitting, as described earlier (39).

Desensitization experiments were analyzed as described (40). Concentration-response curves for adenylylcyclase assays were normalized to the activity in the presence of 100μM forskolin. Desensitization was quantitated as the loss of signal transduction efficacy, τ, as detailed earlier (40). In brief, the concentration-response curves for control and desensitized membranes were fitted and compared using the following algorithm(41):

$$E = E_m \cdot \frac{\tau^n A^n}{(K_A + A)^n + \tau^n A^n} \quad (2)$$

with E denoting the effect, E_m the maximum possible effect, A the agonist concentration, K_A the dissociation constant of the agonist-receptor complex, and n a slope factor, which was not significantly different from 1 in all experiments presented here. τ is a parameter describing the signal transduction efficacy of the system and is estimated individually for each curve, whereas all other parameters are shared. τ_0 denotes the value under control conditions, and τ is the value for the desensitized curve. The term $(1 - \tau/\tau_0) \times 100$ is then taken as a measure of desensitization (in percentage). Intuitively, this parameter can best be described as the percentage of receptors that need to be destroyed to give an equivalent loss of receptor-stimulated adenylyl cyclase activity. The details of this quantitation procedure have been described earlier (40).

In addition, we also evaluated desensitization by quantitating the decrease of the maximal effect (E_{max}) of isoproterenol; the E_{max} values for the control (E_{max_c}) and desensitized (E_{max_d}) curves were calculated by curve fitting (42) to the Hill equation, and desensitization (in percentage) was calculated as $(1-E_{max_d}/E_{max_c}) \cdot 100$.

Results

Analysis of β_2-adrenergic receptor sequestration in A431 cells. Exposure of A431 cells to a high concentration of isoproterenol (10 μM) initiated sequestration of the β_2-adrenergic receptors, as assessed by radioligand binding (Fig. 1). The percentage of sequestered receptors increased from basal levels of <5% to about 25% within 30 min, with a half-life of about 10 min. After removal of the agonist either by washing (Fig. 1) or by addition of a saturating concentration of an antagonist such as alprenolol (data not shown), the percentage of sequestered receptors decreased with kinetics that were comparable to those of sequestration. These data indicate that agonist-induced sequestration of β_2-adrenergic receptors is a dynamic and reversible process. Pretreatment of cells with concanavalin A has been used as a means of inhibiting sequestration (20, 22). Fig. 1 shows that pretreatment of A431 cells with 0.25 mg/ml concanavalin A completely prevented β_2-adrenergic receptor sequestration in A431 cells over at least 30 min of isoproterenol exposure.

Analysis of the isoproterenol-induced sequestration kinetics with eq. 1 (described in Materials and Methods) yielded a sequestration constant (k_1) of 0.03 ± 0.005/min and an externalization constant (k_2) of 0.06 ± 0.006/min. Analysis of

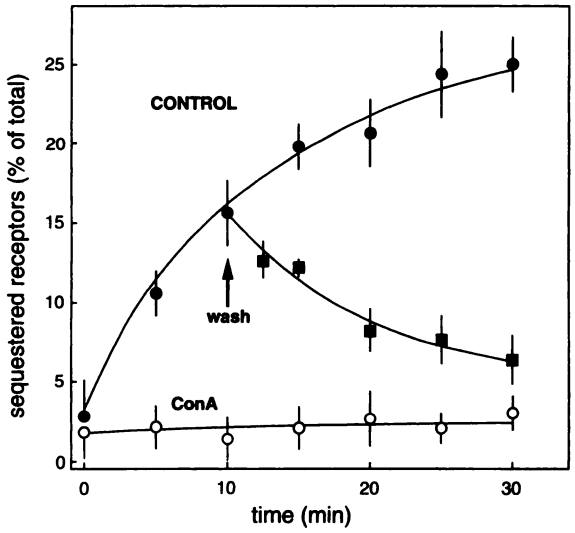

Fig. 1. Sequestration and recycling of β_2-adrenergic receptors in A431 cells in response to isoproterenol. Adherent A431 cells were either pretreated with concanavalin A (*ConA*) (○) or kept without concanavalin A (*CONTROL*) (●, ■). They were then exposed for various periods of time to 10 μM (−)-isoproterenol (●). After 10 min in the presence of (−)-isoproterenol, some plates were washed three times with medium and then incubated in the absence of (−)-isoproterenol (■). At the end of the respective incubation times, the cells were washed with ice-cold PBS, gently scraped off the plates, and collected by centrifugation. The percentage of sequestered receptors was determined by radioligand binding as described in Materials and Methods. The total number of β_2-adrenergic receptors (cell surface plus sequestered) did not change during the course of the entire experiment and amounted to 850 + 70 fmol/mg of membrane protein. Data are means and standard errors of five separate experiments.

receptor externalization after removal of the agonist yielded an externalization constant (k_2) of 0.08 ± 0.01/min.

If receptor sequestration does indeed play a role in β_2-adrenergic receptor dephosphorylation, then blockade of sequestration with concanavalin A should interfere with the dephosphorylation process that should be required for resensitization. Therefore, we measured the phosphorylation status of the β_2-adrenergic receptors in intact A431 cells in the desensitized state (10-min treatment with 10 μM isoproterenol) and after removal of the agonist (10 min of resensitization). These experiments were done by prelabeling the cellular ATP pool by preincubation of the cells in phosphate-free medium supplemented with $^{32}P_i$. The cells were then treated with isoproterenol for 10 min and (for the desensitized samples) allowed to resensitize for 10 min after removal of the agonist. The β_2-adrenergic receptors were isolated from the cell membranes, and their ^{32}P content was quantitated by autoradiography after SDS-polyacrylamide gel electrophoresis. Fig. 2 shows that in control cells, as well as in cells pretreated with concanavalin A, desensitization was accompanied by marked phosphorylation of the β_2-adrenergic receptors. In control cells, but not in concanavalin A-pretreated cells, the extent of phosphorylation was markedly reduced after removal of the agonist and a 10-min recovery period. This suggests that the receptor phosphorylation is rapidly reversed and that such dephosphorylation does not take place after pretreatment of the cells with concanavalin A, i.e., when there is no receptor sequestration.

To determine the functional consequences of this dephosphorylation, and its lack in concanavalin A-pretreated cells, we determined β_2-adrenergic receptor function in concanavalin A-pretreated and control cells. This was done by measuring the isoproterenol-stimulated adenylyl cyclase activity in membranes prepared from cells under control conditions, after 10 min of desensitization with 10 μM isoproterenol, and after various times of recovery (10, 30, and 60 min) after removal of the agonist. Fig. 3A shows the concentration-response curves for control cells, and Fig. 3B shows the curves for concanavalin A-pretreated cells. In control cells, desensitization was characterized by a decrease in maximal stimulation and a shift of the curve to the right. A curve similar to that for the desensitized state was obtained after 10 min of recovery. However, after 30 min of recovery the curve returned to the initial values, and after 60 min of recovery there was even a small increase in maximal stimulation plus a shift of the curve to the left, indicative of supersensitization. Although the pattern of desensitization was the same in concanavalin A-pretreated cells (Fig. 3B), there was no evidence of resensitization. On the contrary, the curves continued to show decreased maxima during the recovery period.

A quantitative analysis of the desensitization and resensitization patterns was done by calculating the signal transduction efficacies (τ) for the various curves by simultaneous curve-fitting to eq. 2. Fig. 4 compares the calculated desensitization values with the receptor sequestration patterns in control (Fig. 4A) and concanavalin A-pretreated (Fig. 4B) cells. In control cells, reappearance of the receptors preceded resensitization of receptor function; after a 10-min recovery period, almost 50% of the sequestered receptors had been recycled back to the cell surface but receptor function remained desensitized. After 30 min of recovery, sequestration

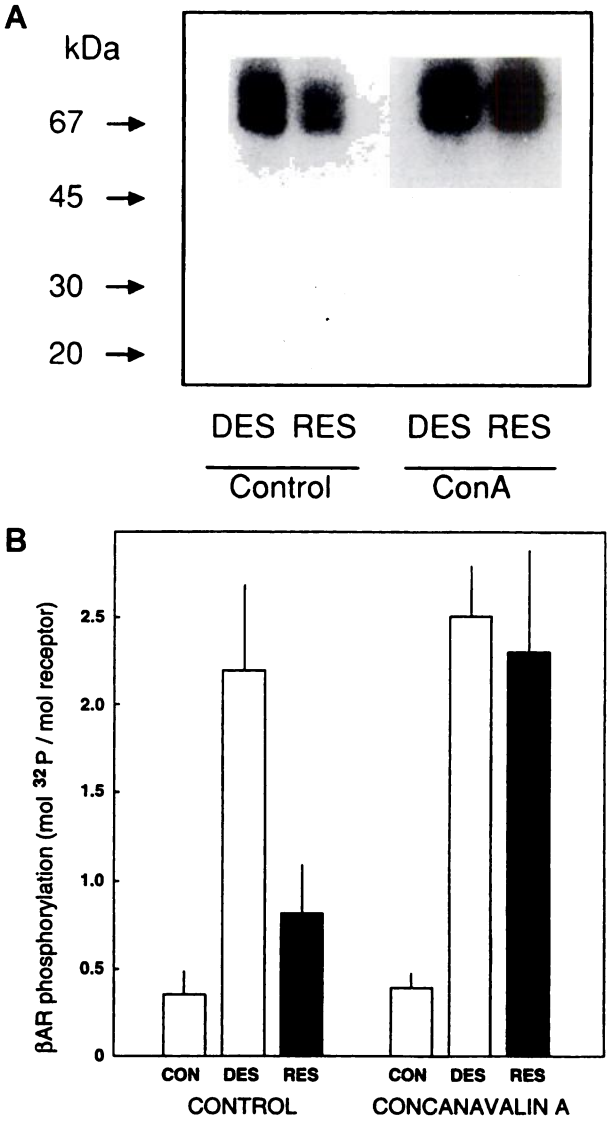

Fig. 2. Phosphorylation and dephosphorylation of β_2-adrenergic receptors in A431 cells in response to isoproterenol. A, Autoradiogram of an SDS-polyacrylamide gel showing β_2-adrenergic receptors from desensitized (*DES*) and resensitized (*RES*) A431 cells that were pretreated or not with concanavalin A. B, ^{32}P content of β_2-adrenergic receptors in A431 cells under basal (*CON*), desensitized (*DES*), and resensitized (*RES*) conditions. A431 cells were prelabeled with 1 mCi of $^{32}P_i$. The cells were then washed and either pretreated with 0.25 mg/ml concanavalin A (*ConA*) or incubated under similar conditions without concanavalin A (*Control*). The cells were washed three times and were then desensitized (*DES*) in the presence of 10 μM (−)-isoproterenol for 10 min or kept without isoproterenol as controls (*CON*). Some samples were resensitized (*RES*) by three washes and incubation for another 10 min. The reactions were stopped and β_2-adrenergic receptors were purified as described in Materials and Methods. Equal amounts of receptors (0.4 pmol/sample) were electrophoresed on 12% SDS-polyacrylamide gels, and their ^{32}P content was visualized by autoradiography and quantitated by Cerenkov counting of the excised receptor bands (B). Values in B are means and standard errors of three separate determinations.

values were not different from controls, and receptor function was almost fully restored. After 60 min of recovery, there was no further change in the percentage of sequestered receptors, but receptor function was actually increased.

In concanavalin A-pretreated cells there was no receptor sequestration and, therefore, no receptor recycling. Never-

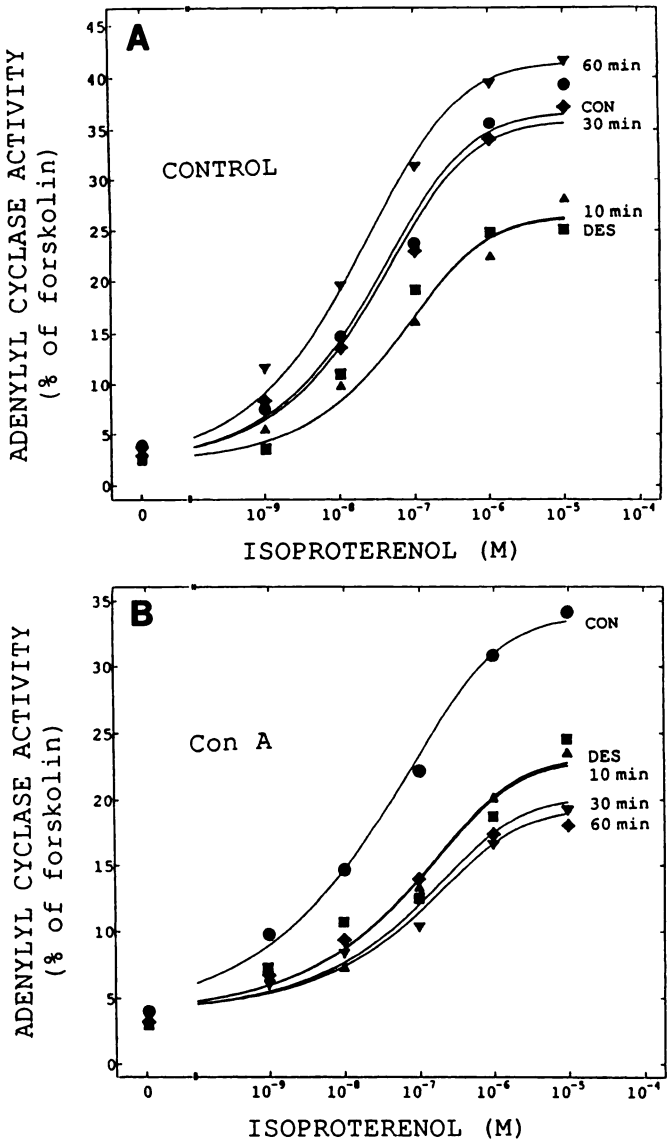

Fig. 3. Desensitization and resensitization of β_2-adrenergic receptors in A431 cells in response to isoproterenol and after removal of isoproterenol. A, Control cells; B, concanavalin A-pretreated cells. A431 cells were pretreated (or not) with 0.25 mg/ml concanavalin A and then washed. Control cells were harvested immediately (*CON*) and all other samples were desensitized for 10 min with 10 μM (−)-isoproterenol in medium (*DES*). Some samples were washed three times and then incubated in medium for various times (10 min, 30 min, and 60 min of resensitization). At the end of the respective incubation times, the cells were washed and membranes were prepared. Adenylyl cyclase activity in the membranes was determined under basal conditions, in the presence of various concentrations of (−)-isoproterenol to assess β_2-adrenergic receptor function, and in the presence of 100 μM forskolin as a reference value. Data are means of five separate experiments.

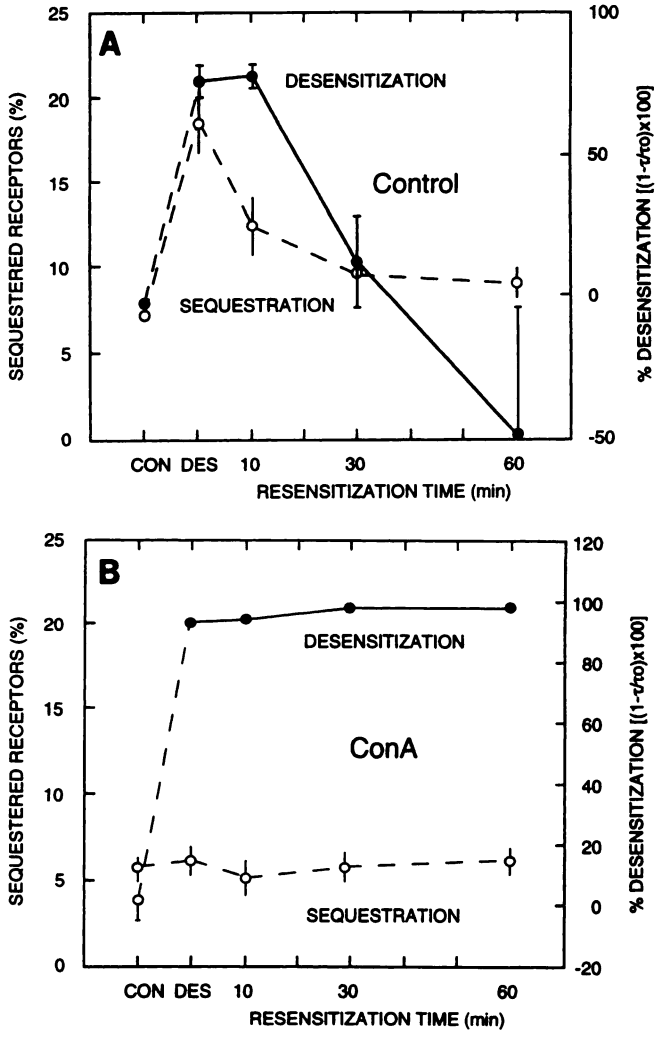

Fig. 4. Quantitation of β_2-adrenergic receptor function and sequestration in A431 cells in response to (−)-isoproterenol and after removal of (−)-isoproterenol. A, Control cells; B, concanavalin A-pretreated cells. The concentration-response data shown in Fig. 3 were analyzed by simultaneous curve-fitting as described in Materials and Methods. The signal transduction efficacy τ was calculated for all curves, including the control curve (τ_o), and the percentage of desensitization was calculated as $(1 - \tau/\tau_o) \times 100$. *Dotted lines*, percentage of sequestered receptors under conditions identical to those shown in Fig. 1. Data are means and standard errors of five separate experiments.

theless, desensitization after 10 min of isoproterenol exposure was slightly more pronounced than in control cells (91 ± 2% versus 78 ± 5% in control cells). After removal of the agonist, receptor function continued to decrease in these cells, resulting in 96 ± 2% desensitization after 60 min of recovery.

These changes in receptor distribution as well as receptor function occurred without any significant change in the total number of β_2-adrenergic receptors in the cells. Neither in control cells nor in concanavalin A-pretreated cells did we observe any alteration in receptor levels during the desensitization and resensitization processes (Fig. 1, legend). This rules out the possibility that the observed changes were related to protein degradation or synthesis and illustrates the functional nature of these changes.

Thus, concanavalin A treatment causes, at the same time, inhibition of agonist-induced receptor sequestration and inhibition of receptor dephosphorylation and resensitization after removal of the agonist. This suggests that receptor sequestration and restoration of receptor function are intimately linked.

Inhibition of β_2-adrenergic receptor sequestration with sucrose. To confirm this hypothesis, we then studied another method of inhibition of receptor sequestration. Daukas and Zigmond (43) used hyperosmolar solutions to prevent specifically receptor-mediated but not constitutive endocytosis in polymorphonuclear leukocytes, and they

reported complete inhibition of receptor-mediated endocytosis with 0.5 M sucrose. Fig. 5 shows that hyperosmolar sucrose could also inhibit sequestration of β_2-adrenergic receptors in A431 cells. Almost complete inhibition of sequestration was achieved with 0.75 M sucrose. Because the presence of 0.75 M sucrose disturbed the subsequent adenylyl cyclase assays, resensitization experiments were done in the presence of 0.6 M sucrose; under these conditions, sequestration was largely inhibited but adenylyl cyclase stimulation by isoproterenol was essentially maintained (data not shown).

The desensitization/resensitization pattern of the β_2-adrenergic receptors in the presence of 0.6 M sucrose was analyzed as described above, and the result is shown in Fig. 6. The presence of sucrose did not markedly alter the desensitization behavior. However, whereas in control cells receptor function was fully recovered after a 30-min resensitization period, there was only moderate resensitization in the presence of 0.6 M sucrose. Thus, in the presence of hyperosmolar sucrose there was again a correlation between inhibition of sequestration and inhibition of resensitization.

Inhibition of β_2-adrenergic receptor dephosphorylation with calyculin A. We then studied whether blockade at the next presumed step in the resensitization process, which is dephosphorylation of the receptors, would also prevent resensitization of receptor function. The serine and threonine phosphorylation of β_2-adrenergic receptors can be reversed by the action of intracellular phosphatases (44). Calyculin A, an inhibitor of many serine/threonine protein phosphatases, has been successfully used to inhibit thrombin receptor dephosphorylation (45). Table 1 shows that calyculin A could also prevent the dephosphorylation of β_2-adrenergic receptors. In experiments analogous to those shown in Fig. 2, calyculin A was found to prevent almost completely the receptor dephosphorylation that normally occurs after removal of isoproterenol.

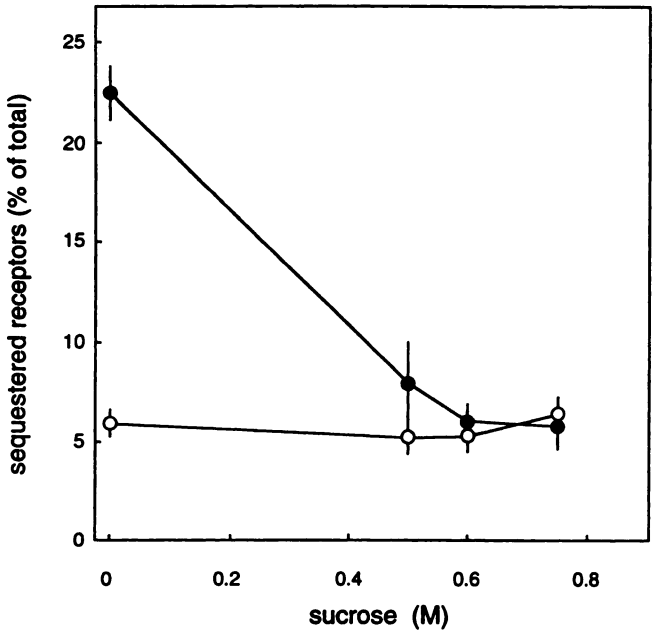

Fig. 5. Inhibition of β_2-adrenergic receptor sequestration in A431 cells by sucrose. A431 cells were exposed to 10 μM (−)-isoproterenol (●) or kept without (−)-isoproterenol (○) for 30 min, in the presence of various concentrations of sucrose. The cells were then washed with ice-cold PBS, gently scraped off the plates, and collected by centrifugation. The percentage of sequestered receptors was determined by radioligand binding as described in Materials and Methods. Data are means and standard errors of six separate experiments.

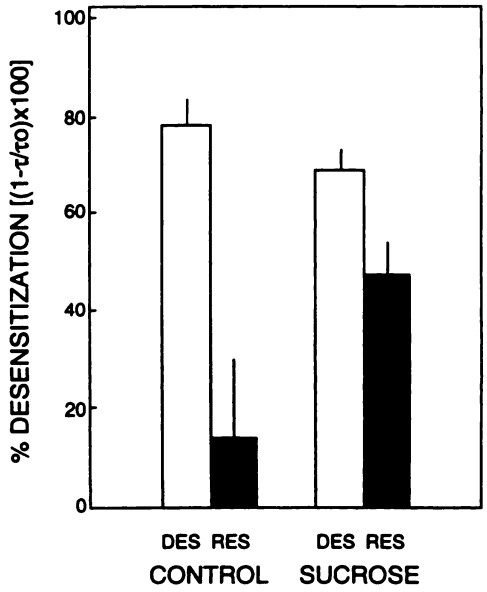

Fig. 6. Effects of 0.6 M sucrose on the desensitization and resensitization of β_2-adrenergic receptors in A431 cells. A431 cells in DMEM plus 0.6 M sucrose were desensitized for 10 min with 10 μM (−)-isoproterenol in medium and were harvested immediately (*DES*) or washed three times and then incubated in medium for 30 min (*RES*). At the end of the respective incubation times, the cells were washed with ice-cold PBS and membranes were prepared. Adenylyl cyclase assays were performed as described for Fig. 3, and desensitization was quantitated as described for Fig. 4. Data are means and standard errors of six separate experiments.

TABLE 1

Effects of calyculin A and monensin on phosphorylation and dephosphorylation of β_2-adrenergic receptors in A431 cells

A431 cells were prelabeled with 1 mCi of $^{32}P_i$. The cells were then washed three times and desensitized in the presence of 10 μM (−)-isoproterenol for 10 min or kept without isoproterenol as controls. Some samples were resensitized by three washes and incubation for another 10 min. Calyculin A (10 nM) or monensin (100 μM) was added 10 min before (−)-isoproterenol and was present throughout the experiment. The reactions were stopped and β_2-adrenergic receptors were purified as described in Materials and Methods. Equal amounts of receptors (0.4 pmol/sample) were electrophoresed on 12% SDS-polyacrylamide gels, and their ^{32}P content was visualized by autoradiography and quantitated by Cerenkov counting of the excised receptor bands, as shown in Fig. 2. Values for control conditions are from Fig. 2.

Condition	Receptor phosphorylation	
	Desensitized	Resensitized
	mol of ^{32}P/mol of receptor	
Control	2.2 ± 0.5	0.8 ± 0.3
Calyculin A	2.4 ± 0.7	2.0 ± 0.6
Monensin	2.1 ± 0.8	0.6 ± 0.4

In the desensitization/resensitization assay described above, calyculin A also prevented resensitization of β_2-adrenergic receptors (Fig. 7). Although it did not interfere with desensitization, there was no recovery from desensitization 30 min after removal of the agonist isoproterenol. In fact, as was the case with concanavalin A (Figs. 3B and 4B), receptor function was even somewhat lower after the 30 min of recovery than at the end of the desensitization period.

If we assume that sequestration, dephosphorylation, and externalization are sequential events, then it might be pos-

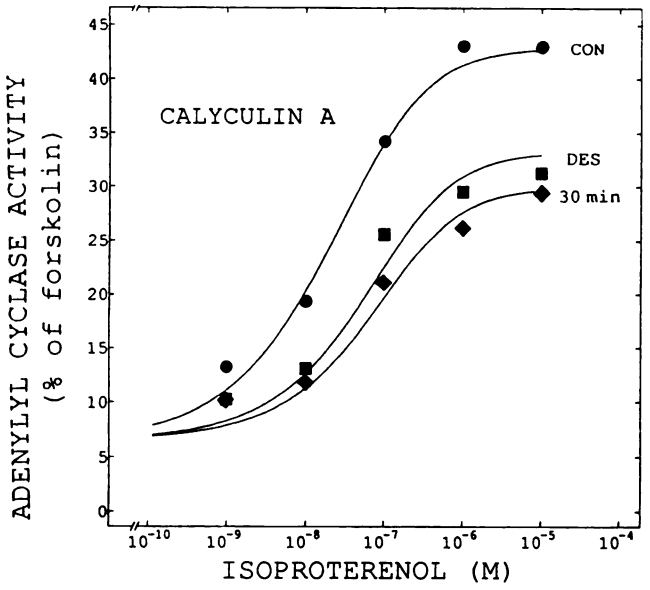

Fig. 7. Effects of calyculin A on the desensitization and resensitization of β_2-adrenergic receptors in A431 cells. A431 cells in DMEM plus 10 nM calyculin A were desensitized for 10 min with 10 μM (−)-isoproterenol in medium and were harvested immediately (*DES*) or washed three times and then incubated in medium for 30 min (*30 min*). At the end of the respective incubation times, the cells were washed with ice-cold PBS and membranes were prepared. Adenylyl cyclase assays were performed as described for Fig. 3. The calculated desensitization values are given in Table 2. Data are means of three separate experiments. *CON*, control.

sible that receptors need to be dephosphorylated before they can be recycled back to the cell surface. However, Fig. 8 shows that this was not the case. The presence of calyculin A altered neither the isoproterenol-induced sequestration of β_2-adrenergic receptors nor their return to the cell surface after removal of isoproterenol.

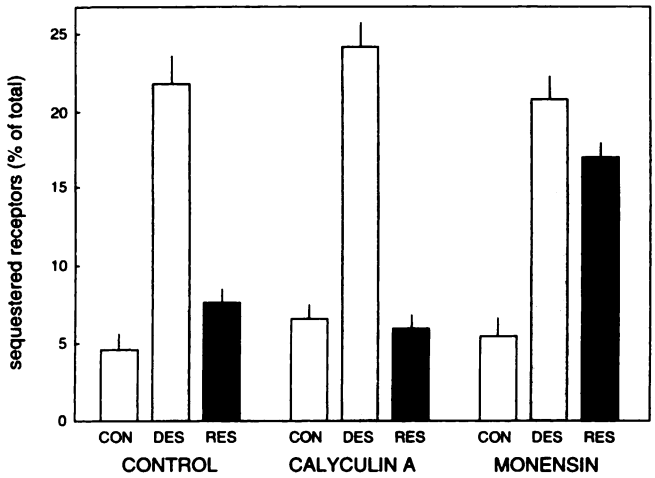

Fig. 8. Effects of calyculin A and monensin on recycling of sequestered β_2-adrenergic receptors in A431 cells. A431 cells were exposed to 10 μM (−)-isoproterenol for 30 min in the absence (control) or presence of 10 nM calyculin A or 100 μM monensin, followed by washing to remove the agonist and a 30-min recovery period in the absence or presence of calyculin A or monensin. Receptor sequestration was determined by radioligand binding before addition of (−)-isoproterenol (*CON*), after the 30-min desensitization period (*DES*), and after the 30-min recovery period (*RES*). Values are means and standard errors of five separate experiments.

Inhibition of β_2-adrenergic receptor externalization with monensin. We assumed that after dephosphorylation the receptors need to be recycled back to the cell surface for restoration of receptor function. Recycling of various types of receptors has been reported to be efficiently blocked by monensin (see, for example, Refs. 46–48). Therefore, we tested whether monensin can also prevent recycling of β_2-adrenergic receptors. Fig. 8 shows that the presence of monensin did not alter the extent of agonist-induced β_2-adrenergic receptor sequestration. However, only very few receptors reappeared on the cell surface in the presence of monensin, whereas most of them did so in the absence of monensin. Table 1 indicates that monensin did not interfere with the phosphorylation and dephosphorylation of β_2-adrenergic receptors. Thus, monensin can apparently block recycling of β_2-adrenergic receptors at a step subsequent to receptor dephosphorylation.

Such blockade of receptor recycling correlated with a lack of recovery from desensitization in the presence of monensin (Fig. 9). Again, the presence of monensin resulted not only in a lack of resensitization after removal of isoproterenol but also in some continuing desensitization.

Effects of the simultaneous presence of multiple inhibitors on receptor resensitization. Table 2 shows that the effects of the various agents that were found to block sequestration and resensitization of β_2-adrenergic receptors were not additive when each compound was present at maximally effective concentrations. Each of the compounds alone could completely prevent receptor resensitization, and the presence of two such inhibitors did not result in further impairment of receptor function. This suggests that the inhibitors do not cause impairment of receptor function by

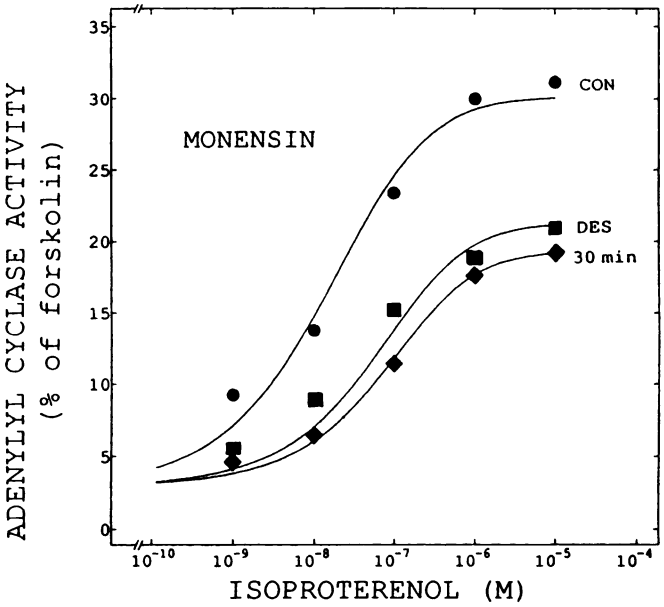

Fig. 9. Effects of monensin on the desensitization and resensitization of β_2-adrenergic receptors in A431 cells. A431 cells in DMEM plus 100 μM monensin were desensitized for 10 min with 10 μM (−)-isoproterenol in medium and were harvested immediately (*DES*) or washed three times and then incubated in medium for 30 min (*30 min*). At the end of the respective incubation times, the cells were washed with ice-cold PBS and membranes were prepared. Adenylyl cyclase assays were performed as described for Fig. 3. The calculated desensitization values are given in Table 2. Data are means of four separate experiments. *CON*, control.

TABLE 2

Effects of concanavalin A, calyculin A, and monensin on desensitization and resensitization of β_2-adrenergic receptors in A431 cells

A431 cells were exposed for 10 min to 10 μM (−)-isoproterenol in medium (desensitization), washed three times, and then incubated in medium for 30 min (resensitization). Receptor function was determined in adenylyl cyclase assays, as shown in Figs. 3, 6, and 8, and desensitization was quantitated by calculating the signal transduction efficacy as described in Materials and Methods. Concanavalin A (0.25 mg/ml) was used to pretreat the cells before the desensitization procedure; calyculin A (10 nM) and monensin (100 μM) were present throughout the desensitization and resensitization procedures.

Condition	Desensitization [$(1 - \pi/\tau_0) \times 100$]	
	Desensitized	Resensitized
	%	
Control	78 ± 5	14 ± 16
Concanavalin A	91 ± 3	95 ± 2
Calyculin A	84 ± 7	89 ± 5
Monensin	90 ± 6	92 ± 3
Concanavalin A + calyculin A	81 ± 4	86 ± 2
Concanavalin A + monensin	82 ± 7	77 ± 6

different nonspecific means. Instead, it is compatible with the hypothesis that they all interfere with the resensitization process.

Discussion

Stimulation of β_2-adrenergic receptors by agonists causes an array of effects altering receptor function, which vary widely with respect to their time frame and the agonist concentrations required. Rapid alterations are initiated within a few minutes of agonist exposure. In A431 cells, βARK- and β-arrestin-mediated uncoupling from G_s occurs in response to relatively high agonist concentrations, begins a few seconds after addition of agonists, and can decrease receptor function by as much as 60%; PKA-mediated uncoupling in the same cells is >1 order of magnitude more sensitive to agonists, occurs with a half-life of a few minutes, and can amount to 40–50% (21, 22). Sequestration has the same sensitivity as the βARK/β-arrestin-mediated process (22); it appears to be initiated immediately after addition of agonist and occurs with a half-life of about 10 min (Fig. 1). The present study investigates a possible link between agonist-induced sequestration of β_2-adrenergic receptors and the recovery of receptor function after agonist-induced desensitization. The main findings are that β_2-adrenergic receptor sequestration in A431 cells is a rapidly reversible process, which may be indicative of dynamic cycling of receptors from the cell surface to sequestered sites and back to the cell surface, and that disruption of this cycle correlates with a lack of recovery of receptor function after desensitization.

The initiation of the sequestration process appears to begin fairly rapidly after addition of the agonist. Thus, Fig. 1 shows no apparent lag period between the addition of the agonist and the onset of sequestration. Furthermore, the data could be fitted well to a monoexponential curve, indicating an immediate alteration of the kinetic parameters of sequestration and recycling. The sequestration curve shown in Fig. 1 could be analyzed with a mathematical model assuming that both sequestration and externalization occur as exponential processes. The rate constants obtained were k_1 = 0.03/min (sequestration) and k_2 = 0.06/min (externalization). After removal of isoproterenol, only externalization is assumed to occur (i.e., $k_1 \approx 0$), and an externalization constant (k_2) of 0.08/min was measured, which is in good agreement with the k_2 calculated from the sequestration curve. This suggests that externalization is the same in the absence and in the presence of agonist.

The kinetic constants indicate that more receptors participate in the sequestration/externalization cycle than might be apparent from the overall sequestration curve depicted in Fig. 1. This is because sequestered receptors are rapidly recycled back to the cell surface, with a rate constant (k_2) that is twice that of the sequestration process. However, the sequestration rate constant of 0.03/min indicates that in 30 min of agonist occupancy >60% of the cell surface receptors become sequestered. Of these 60%, however, more than half are recycled back to the cell surface within these 30 min. In other cell lines, these values are considerably higher. For example, sequestration of β_2-adrenergic receptors in S49 mouse lymphoma cells has been reported to occur with a $t_{1/2}$ of ≈1 min (49). Reappearance of the receptors on the cell surface occurred with a $t_{1/2}$ of ≈17 min (49), which is in the range of the values found here for A431 cells. The relatively low speed of sequestration in A431 cells may be related to their high levels of β_2-adrenergic receptor expression and may suggest that these expression levels exceed those of a component required for sequestration.

The inhibition of receptor externalization by monensin would be expected to result in an increase in the percentage of sequestered receptors. However, such an increase was not seen. This was most likely due to the fact that 100 μM monensin not only completely blocked receptor externalization but also reduced the rate of receptor sequestration (data not shown).

It is unclear how PKA- and βARK/β-arrestin-mediated desensitization of β_2-adrenergic receptors is reversed. The two mechanisms have different agonist sensitivities and may occur in different settings (PKA-mediated desensitization at nonsynaptic receptors and βARK/β-arrestin-mediated desensitization at synaptic receptors) (22, 50), and it is quite possible that different mechanisms to reverse desensitization exist. Furthermore, PKA-mediated desensitization appears to be a single-step process, whereas βARK/β-arrestin-mediated desensitization occurs in two steps. Consequently, recovery from the two forms of desensitization must also be different. However, in both cases the recovery must involve dephosphorylation of the receptors. Our study shows that the agonist-induced phosphorylation of β_2-adrenergic receptors, which is caused by the actions of both PKA and βARK, is rapidly reversed after removal of the agonist. Pretreatment of the cells with concanavalin A prevented sequestration and also markedly inhibited this receptor dephosphorylation process. At the same time, concanavalin A completely blocked the recovery of receptor function after removal of the agonist. The simplest explanation of these effects of concanavalin A is that dephosphorylation occurs on sequestered receptors and that, consequently, sequestration is required for recovery of receptor function (or resensitization). Similar, although less complete, inhibition of resensitization was achieved with hyperosmolar sucrose, conditions that, like pretreatment with concanavalin A, impair sequestration of β_2-adrenergic receptors.

This reasoning is supported by the observations that block-

ade of phosphatases with calyculin A also prevented resensitization of β_2-adrenergic receptors and that the effects of maximal concentrations of concanavalin A and of calyculin A were not additive. This is compatible with the hypothesis that receptor sequestration and dephosphorylation are sequential events.

If dephosphorylation occurs on sequestered receptors, then the receptors need to be recycled back to the surface before receptor function can be restored. Monensin has been used in other receptor systems to block receptor recycling. Using monensin to prevent recycling of β_2-adrenergic receptors, we observed that inhibition of receptor recycling also inhibited receptor resensitization. This finding further supports the hypothesis that resensitization involves sequestration of the receptors. However, dephosphorylation does not seem to be a requirement for receptor recycling, because inhibition of receptor dephosphorylation did not alter the reappearance of the receptors at the cell surface.

All of the inhibitors used in the present study share the property of interfering with the processes of sequestration and recycling of β_2-adrenergic receptors. Thus, the observation that all of these inhibitors impair resensitization of the receptors is most simply explained by their interference with this sequestration and recycling process. This leads us to postulate that recovery from desensitization is a multistep process; agonist occupancy of the receptors leads to rapid desensitization but at the same time initiates receptor sequestration, the first step in the resensitization process. The sequestered receptors are then dephosphorylated and subsequently recycled to the cell membrane, regaining their full function.

Interestingly, recovery of receptor function occurs a few minutes after the reappearance of receptors at the cell surface (Fig. 4A). This may suggest that the recycled receptors do not immediately regain coupling to G_s but some rearrangement in the plasma membrane is required before the recycled receptors can again fulfill their signaling function. Elucidation of the nature of the processes occurring during this lag period will require additional studies. Kinetic considerations suggest that this inactive recycled population might accumulate during receptor desensitization, because the lag period is of the same order of magnitude as the half-lives of the sequestration and externalization processes.

Our data are compatible with recent models of a cycle of receptors from the cell surface to the interior (17, 51). The effects of agonists on the receptors that may trigger the sequestration process are unknown. However, it appears that receptor phosphorylation is not linked to or required for sequestration, because (a) several β_2-adrenergic receptor mutants that are impaired in either PKA- or βARK-mediated phosphorylation are sequestered normally (29, 52) and (b) inhibition of PKA or βARK, or both, does not impair sequestration of β_2-adrenergic receptors (22).

The hypothesis that sequestration may be a step that ultimately leads to dephosphorylation of β_2-adrenergic receptors is also in line with the observation that, after exposure of frog erythrocytes to isoproterenol, sequestered receptors showed a lower degree of phosphorylation than did receptors that were still associated with the plasma membrane (28). This may indicate that dephosphorylation is initiated rapidly after sequestration of the receptors, and it is compatible with our finding that there is extensive dephosphorylation of receptors 10 min after agonist removal (Fig. 2). However, our data are in contrast to the results obtained by Kassis *et al.* (24), who found no major effects of concanavalin A pretreatment on either desensitization or resensitization of β-adrenergic receptors in A431 or C6 rat glioma cells. Those authors also observed much slower rates of receptor reappearance at the surface and of resensitization; both processes took several hours in their experiments, whereas in our experiments these processes occurred in minutes, which is a time course in agreement with other recent data (17, 51). Another possible reason for the discrepant results is that Kassis *et al.* (24) measured only the maximal responses to a single high concentration of isoproterenol. However, the maximal responsiveness of the β-adrenergic receptor/adenylyl cyclase system may not change much during desensitization when there is a significant receptor reserve (40), and only concentration-response curves allow a full appreciation of the desensitization pattern (29, 37, 40). More recent data obtained by Yu *et al.* (30) are in agreement with our findings; those authors found a lack of receptor resensitization in the presence of hyperosmolar sucrose, as well as with a sequestration-negative receptor mutant.

Fig. 10 summarizes the model that can be hypothesized from these data. Rapid desensitization is triggered by the action of βARK or PKA (or both). The reversal of this desensitization requires first that the desensitized receptors be sequestered, a process that can be inhibited by pretreatment with concanavalin A or treatment with high concentrations of sucrose. The receptors then become dephosphorylated, followed by monensin-sensitive externalization, and finally the receptors become reinserted into their functional environment. Although sequestration appears to be a requirement for receptor dephosphorylation, receptor dephosphorylation is not a requirement for externalization. However, when receptor dephosphorylation was prevented with calyculin A, the recycled receptors remained nonfunctional, as would be expected for a phosphorylated receptor. Current concepts of homologous β_2-adrenergic receptor desensitization suggest that the desensitized state of the receptor is characterized by phosphorylation of the receptor plus binding of one of the two isoforms of β-arrestin (4, 10, 50). It is unknown how and when β-arrestin is removed from the receptors. Additional studies will have to investigate the question of whether se-

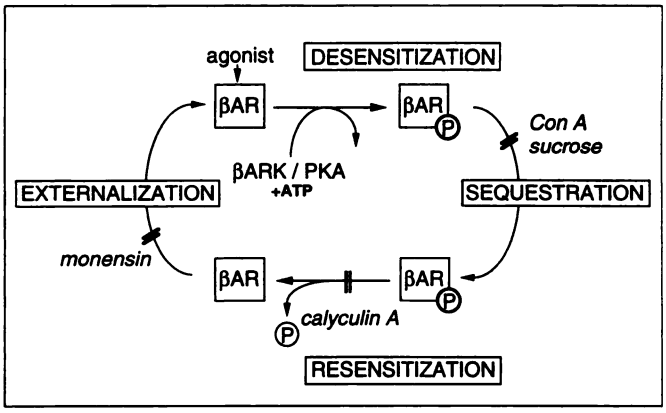

Fig. 10. Model of the desensitization, sequestration, and resensitization cycle of β_2-adrenergic receptors. *βAR*, β-adrenergic receptor; *Con A*, concanavalin A; *P*, phosphate.

questration plays a role in the separation of receptors from β-arrestin.

In conclusion, our data suggest that sequestration of β_2-adrenergic receptors in response to agonists may be a first step in the process of receptor resensitization. This hypothesis is compatible with the kinetics of sequestration, which in general are considerably slower than those of β_2-adrenergic receptor desensitization. Sequestration of β_2-adrenergic receptors may serve to enable the dephosphorylation of receptors and subsequent recycling to the cell surface, where the receptors become reintegrated into their functional context.

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