Human A431 and rat glioma C6 cells exposed to isoproterenol underwent a time- and dose-dependent loss of isoproterenol-stimulated adenylate cyclase activity. Desensitization was accompanied by sequestration of β-adrenergic receptors, which became less accessible to the hydrophilic antagonist [3H]-labeled 4-(3-tert-butylamino-2-hydroxypropoxy)benzimidazole-2-one hydrochloride ([3H]CGP-12177) and redistributed from the heavier density plasma membrane fraction to a lighter density membrane fraction. Prior treatment of the cells with concanavalin A or phenylarsine oxide blocked sequestration of the receptors but not desensitization of the agonist-stimulated adenylate cyclase. The membranes from such pretreated cells were exposed to alkali to inactivate adenylate cyclase, and the receptors were transferred to a foreign adenylate cyclase by membrane fusion with polyethylene glycol. β receptors from desensitized cells exhibited a reduced ability to maximally stimulate the foreign adenylate cyclase, but remained accessible to [3H]CGP-12177 in the fused membranes. When isoproterenol-treated cells were washed free of agonist, there was a time-dependent recovery of agonist responsiveness and [3H]CGP-12177-binding sites. Using the fusion technique, the receptors recovered their functional activity in the resensitized cells. In concanavalin A-treated cells, desensitization and resensitization appeared to occur in the absence of receptor sequestration. Finally, membranes from desensitized cells pretreated with concanavalin A were fused with polyethylene glycol and assayed for agonist-stimulated adenylate cyclase. There was no reversal of the desensitized state. Thus, the primary, essential step in the desensitization process is a reduction in functional activity of the β-adrenergic receptor. In contrast, sequestration of the receptors is not a prerequisite, but a secondary event during desensitization.

The initial event in the stimulation of adenylate cyclase by β-adrenergic agonists is their binding to specific receptor on the cell surface. Following activation of the enzyme, there is often a rapid attenuation of the response. This phenomenon of desensitization has been extensively studied in mammalian cells (1-6). It is generally agreed that the receptors become "uncoupled" from the regulatory component (N,) of adenylate cyclase and exhibit a lower affinity for agonist without any change in N,. The receptors also appear to undergo a sequestration process. When lysates from desensitized cells are analyzed by sucrose density gradient centrifugation, the β-adrenergic receptors redistribute from the heavier density plasma membrane fraction to a lighter density membrane fraction which is depleted of N, (7-12). In addition, the receptors become less accessible to hydrophilic agonists and antagonists (13-17). Thus, there is both functional and physical uncoupling of the receptors from N,.

Using membrane fusion techniques, we recently demonstrated that during desensitization, the functional activity of the mammalian β-adrenergic receptor is reduced (6, 10). In contrast, Strasser and co-workers have reported that purified receptors from control and desensitized cells were functionally equivalent (18) and that fusion of intact, desensitized cells with polyethylene glycol caused resensitization (19). They proposed that sequestration of the receptors plays a functionally significant role in the desensitization process. Although we used conditions where loss of total receptors as measured by binding of a hydrophobic antagonist was minimal and controlled for, we did not assess whether receptor sequestration had an effect on receptor function (6, 10). In the present paper, we present evidence that desensitization can occur in the apparent absence of receptor sequestration and is accompanied by loss of receptor function.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

In the present study, we observed that cells exposed to a β-adrenergic agonist underwent a time-dependent loss of agonist-stimulated adenylate cyclase activity and a sequestration of their β-adrenergic receptors. Similar results were obtained with both human A431 cells and rat glioma C6 cells which have predominantly β2- and β1-subtype receptors, respectively.

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†Portions of this paper (including "Experimental Procedures," "Results," Figs. 1-5, and Tables I-VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1160, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

1 The abbreviations used are: N, stimulatory regulatory component of adenylate cyclase; C, catalytic component of adenylate cyclase; ConA, concanavalin A; CGP-12177, 4-(3-tert-butylamino-2-hydroxypropoxy)benzimidazole-2-one hydrochloride; ISO, isoproterenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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We were not able to temporally separate desensitization from receptor sequestration. Others have reported that desensitization occurs slightly more rapidly than receptor sequestration (8). Changes in time, temperature, and agonist concentration may optimize such differences. In order to avoid a possible complex analysis, we decided to use ConA and phenylarsine oxide, agents which are known to inhibit receptor redistribution and sequestration (8, 10, 12, 16, 22). This approach had an additional advantage as it is still unclear whether the sequestered receptors are internalized in endocytic vesicles (14, 16, 22, 23) or remain associated with the plasma membrane, possibly in a distinct domain (17, 26), and what is their functional state. In this regard, we fused the lighter density membranes from desensitized cells with appropriate acceptor membranes and obtained isoproterenol-stimulated adenylate cyclase activity. Because of the limitation of obtaining sufficient amounts of "light" receptors from control cells, we are unable to determine whether receptors in these membranes are fully functional. When we used the heavier density membrane fractions, we found that the receptors from desensitized cells were less functional than their control counterparts (data not shown). It is clear that prior treatment of the cells with either ConA or phenylarsine oxide does not prevent agonist-mediated desensitization but does inhibit receptor sequestration. In particular, the receptors from the desensitized cells remained accessible to the hydrophilic antagonist [3H]CGP-12177, which is regarded as a reliable probe for internalization or sequestration of β-adrenergic receptors (14, 17, 23). When we prepared crude membranes from the pretreated cells, we were able to recover the bulk of the receptors in such membranes from desensitized cells. Using these membranes and a fusion technique (6, 10), we examined what happened to the functional activity of the receptors in the absence of sequestration. In agreement with our previous studies (6, 10), a reduction in β-adrenergic receptor function accompanied the desensitization process. Furthermore, fusion of the donor and acceptor membranes with polyethylene glycol did not interfere with the accessibility of the receptors to the hydrophilic antagonist.

We were also able to demonstrate that receptor function was restored when the intact cells were allowed to resensitize in the absence of agonist. Reappearance of sequestered receptors as measured by [3H]CGP-12177 also occurred and paralleled the recovery of agonist-stimulated adenylate cyclase activity. Using cells pretreated with ConA, desensitization and resensitization with a corresponding modulation of receptor function occurred in the absence of receptor sequestration. Although resensitization was observed in intact cells, we were unable to resensitize membranes prepared from desensitized cells by membrane fusion. We used membranes from cells pretreated with ConA to ensure complete recovery of the receptors and fused the membranes with polyethylene glycol.

In this regard, our results differ from those of Strasser and co-workers (18, 19). They fused intact desensitized S49 cells with polyethylene glycol (and phospholipids at 30 °C) and observed resensitization (19). As S49 cells have the necessary machinery to become resensitized in the absence of agonist (27), it is possible that polyethylene glycol-induced fusion of the intact cells brings the desensitized receptors in contact with such machinery. They also fused affinity-purified β-adrenergic receptors from control and desensitized rat lung with Xenopus laevis erythrocytes and observed no differences in receptor function (18). Again, fusion of the receptors with intact cells might promote resensitization. Finally, they fused membranes from desensitized rat lung with polyethylene glycol (18). They were unable to measure agonist-stimulated adenylate cyclase activity but did assay the affinity of the receptors for agonist by competition binding. They found that after fusion, the percentage of receptors in the high affinity state was increased to a level similar to that found in control membranes. We found that such changes in agonist affinity were small compared to the large reduction in maximal isoproterenol-stimulated adenylate cyclase activity following desensitization, but the shifts in affinity before and after fusion were comparable (see "Results").

In their initial report on β-adrenergic receptor redistribution, Harden et al. (7) observed that the receptors in the heavier density membrane fractions from control and desensitized human astrocytoma cells exhibited similar affinities for agonist based on competition binding studies, and the affinities were similarly reduced in the presence of GTP. The receptors in the lighter density fractions had similar low affinities for agonist in the presence and absence of GTP (7). Strasser et al. (9) also found that the receptors in the light membrane fraction from control and desensitized rat lung tissue were in a low affinity, guanine nucleotide-insensitive state. In contrast to the observations of Harden et al. (7), the receptors in the heavy membrane fraction from desensitized tissue also were in this same low affinity state, whereas those from control tissue were in a high affinity, guanine nucleotide-sensitive state (9, 18). More recently, Toews et al. (16) performed agonist competition binding studies on intact astrocytoma cells. They found that pretreatment of the cells with ConA impaired the conversion of the receptors from a high affinity to a low affinity state during desensitization. Clark et al. (11) observed that when they desensitized S49 cells with a suboptimal concentration of agonist, there was no apparent receptor redistribution but a 1.8-fold increase in the Kᵦᵦ for agonist-stimulated adenylate cyclase. This compares to a 3.7-fold shift when cells were desensitized with a high dose of agonist and receptor redistribution was maximum. Thus, the relationship between redistribution of receptors and their affinity for agonists during desensitization remains unclear.

It is most probably that both functional uncoupling of the receptor from N, and reduced accessibility of the hydrophilic agonist to the receptor contribute to this effect.

In the present paper, we have emphasized that desensitization occurs in the absence of receptor sequestration. We have described previously that under different circumstances, receptor sequestration alone can account for desensitization (10). When rat glioma C6 cells were exposed to phorbol esters, which activate protein kinase C, the cells underwent an apparently homologous desensitization of their isoproterenol-stimulated adenylate cyclase. There was a redistribution of β-adrenergic receptors into a lighter density membrane fraction, and this process was blocked in cells pretreated with ConA. In contrast to agonist-mediated desensitization, ConA pretreatment prevented the desensitization induced by phorbol esters. Furthermore, cells desensitized by phorbol esters exhibited no reduction in receptor function. Finally, fusion of membranes from phorbol ester-desensitized cells with poly-

[5]Although these results agree with our previous studies (6, 10), they do not address the issue of receptor sequestration. From our own results as well as those of Toews et al. (16), some of the receptors in the heavier density membranes from desensitized cells are inaccessible to [3H]CGP-12177.
Desensitization of Mammalian β Receptors

ethyleneglycol resulted in complete recovery of isoproterenol-stimulated adenylyl cyclase activity.

The last observation is particular salient to our present studies. It clearly indicates that the desensitized state can be reversed by membrane fusion as long as the receptors remain fully functional. Thus, phorbol ester-mediated desensitization appears to involve a sequestration of functional receptors into a membrane compartment physically separated from N, (10). In contrast, agonist-mediated desensitization appears to involve a functional change in the receptors which impairs their ability to couple to N,: in addition, the receptors become sequestered away from N,. Fusion of the original membranes with polyethylene glycol or during the transfer of the receptors to a foreign adenylyl cyclase will not reverse the impairment. It is important to point out that Strasser et al. (18) did not rule out the possibility of an early functional alteration in β-adrenergic receptors during desensitization. They suggested that they may have chosen conditions where receptor function but not sequestration already had been reversed.

The underlying biochemical basis for desensitization in mammalian cells remains obscure. Phosphorylation of β-adrenergic receptors during desensitization of avian and amphibian erythrocytes has been demonstrated (3). We speculated that in mammalian cells, phosphorylation of the receptors by a receptor-specific protein kinase may be the signal for both functional and physical uncoupling of the receptors from N,. In the present study, we clearly demonstrate that a reduction in receptor function can occur in the absence of receptor sequestration and that the former appears to be the key event in agonist-mediated homologous desensitization of mammalian cells.

Acknowledgment—We thank Trudy Kohout for growing the cells.

REFERENCES


SUPPLEMENT TO

DESENSITIZATION OF THE β-ADRENERGIC RECEPTOR-COUPLED ADENYLYL CYCLASE IN CULTURED HUMAN CELLS: RECEPTOR SEQUESTRATION VERSUS RECEPTOR FUNCTION

Shouki Kassis, Marjot Simons, Molly Sullivan and Peter H. Fishman

EXPERIMENTAL PROCEDURES

Materials—[35S]Carnosol was obtained from Sigma and penicillin G from Eurofins (167, 283). [3H]-CYP-21377 (31 nM) was from Amersham Corp. The sources of all other reagents used have been reported previously (16, 10, 23, 26).

Cell Culture—Rat glial cells were cultured as described previously (21). A431 cells, obtained from Dr. M. K. Risier, National Cancer Institute, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For experiments, C6 cell cultures were confluent and A431 cell cultures were 60% confluent. The levels of β-adrenergic receptors in A431 cells were very sensitive to cell density, since subconfluent cultures contained 90-100 fmol/mg protein whereas those of confluent cultures contained 30-50 fmol/mg protein. Cells were treated with isoproterenol and [3H]-CYP-21377 as described previously (10, 37). Cells were treated with 0.5 mM phosphatase/cysteine (50 mg/ml) stock solution in dimethyl sulfoxide at a final concentration of 1 mM in PBS (pH 7.2) for 30 min. After washing, cells were washed and lysed as described before (27) except the lysate solution was 1 mM Triton X-100, 250 mM NaCl, 10 mM Tris, and 2 mM ethylenediaminetetraacetic acid.

Binding of [35S]Carnosol—Binding of [35S]Carnosol to lysates and membranes was done in an isoproterenol solution. Briefly, lysates or membranes were diluted in 0.5 ml of 20 mM Hepes (pH 7.4), 120 mM NaCl, 5 mM MgCl, and 2.4 mM [35S]Carnosol for 1 h at 30°C. The complexes were divided with 4 ml of cold 10% TCA containing 1.0 mM phoshatase buffer (pH 7.4) containing 1.0 mM MgCl and filtered under vacuum on GF/A glass fiber filters using a Branden Type II filter. 24-cell harvester was used for receptor binding analysis. The filters were washed twice with the same buffer and placed in counting vials containing 10 ml of Beckman Ready-Scint. After shaking the vials at room temperature for 30 min, they were counted in a Beckman model 1260 liquid scintillation counter using automatic quantitation correction. Counting efficiency was 40-50% and values in dpm were used to calculate fold of ligand bound. Nonspecific binding was determined in the presence of 2 μM [3H]-CYP-21377 and was routinely less than 20% of the total binding.

Cell Lysates—Cell lysates were analyzed by tumor necrosis factor alpha-specific centrifugation (31). Cell plasma membranes were prepared from cell lysates by differential centrifugation.
Desensitization of Mammalian β Receptors

Effect of pretreatment of cells with cold and phenylarsine oxide on desensitization of agonist-stimulated adenylate cyclase and sequestration of β-adrenergic receptors.

Table 1: Effect of pretreatment of cells with cold and phenylarsine oxide on desensitization of agonist-stimulated adenylate cyclase and sequestration of β-adrenergic receptors.

| Pretreatment | [125I]iodopindanol binding | [3H]CGP-17177 bound | [3H]CGP-17177 bound
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>83.1±7</td>
<td>83.7±4.6</td>
<td>84.2±9</td>
</tr>
<tr>
<td>ConA</td>
<td>47.1±7</td>
<td>60.8±7.8</td>
<td>95.2±8</td>
</tr>
<tr>
<td>Iso-OMP</td>
<td>95.6±5.3</td>
<td>94.5±12.3</td>
<td>92.0±12</td>
</tr>
<tr>
<td>PAO</td>
<td>85.7±3.7</td>
<td>49.7±2</td>
<td>230.7±18</td>
</tr>
<tr>
<td>Phenylarsine</td>
<td>73.5±2.3</td>
<td>30.2±2</td>
<td>93.7±3</td>
</tr>
</tbody>
</table>

When the binding observed after isoproterenol treatment is expressed as a percentage of that observed in cells not exposed to the agonist, the values for [125I]iodopindanol binding were: 57.8±1.6 (None), 99.7±4.4 (ConA) and 92.5±2.0 (PAO). For [3H]CGP-17177 bound, the corresponding values were: 88.4±3.9 (None), 90.5±1.1 (ConA) and 84.4±2.5 (PAO).

β-adrenergic cyclase activity was detected after treatment with ConA and PAO. Similar results were obtained when cells were exposed to only 0.1 M PAO at 30°C or to 100 μM PAO for 10 min at 25°C or for 20 min at 4°C. In the latter treatments, the cells were washed prior to being desensitized.

Table 2: Effect of pretreatment of cells with cold and phenylarsine oxide on desensitization of β-adrenergic receptors to a higher particulate fraction.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[3H]iodopindanol binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>84.6±2.5</td>
</tr>
<tr>
<td>ConA</td>
<td>93.7±4.0</td>
</tr>
<tr>
<td>PAO</td>
<td>90.3±2.1</td>
</tr>
</tbody>
</table>

The values represent the percentage of total receptors in both pellets recovered in the high speed particulate fraction and are the mean ± range for two separate experiments.

Table 3: Effect of desensitization of cultured cells on β-adrenergic receptors.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[3H]iodopindanol binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>84.6±2.5</td>
</tr>
<tr>
<td>ConA</td>
<td>93.7±4.0</td>
</tr>
<tr>
<td>PAO</td>
<td>90.3±2.1</td>
</tr>
</tbody>
</table>
Desensitization of Mammalian β Receptors

TABLE IV
Desensitization of β-Adrenergic receptors from Mammalian cells by Fusion of Acceptor Membranes

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fusion</th>
<th>[3H]-CGP-12177 Binding</th>
<th>Net β-Adrenergic-stimulated Adenylate Cyclase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>before</td>
<td>87.2</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>88.2</td>
<td>58.9</td>
</tr>
<tr>
<td>C6</td>
<td>before</td>
<td>109</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>57.1</td>
<td>40.7</td>
</tr>
</tbody>
</table>

Similar results were obtained with cells treated with phenylarsine oxide except we were unable to detect adenylate cyclase activity in C6 cells before fusion as noted above. After fusion, we were able to measure 35S-stimulated adenylate cyclase activity when donor membranes were from desensitized C6 cells, the activity was 85% of that obtained with donor membranes from control cells. Thus, the effect of this in function on β-adrenergic receptors from phenylarsine oxide-treated cells was the same as for receptor cells not pretreated with the agent (see Table III). As the donor membranes only provide the β-adrenergic receptors, these results support our previous conclusion that the functional activity of the receptors is abrogated during agonist stimulation.

We also explored the effect of desensitization on the affinity of the β-adrenergic receptors for the agonist. We measured the half-maximal concentration of isoproterenol required either to stimulate adenylate cyclase (K_{D1}) or to inhibit tritiated N-ethylmaleimide binding (K_{D2}). There was considerable variation in the absolute values for K_{D1} and K_{D2} amongst different experiments as well as in the magnitude of the shifts in agonist affinity. The values observed before and after fusion, however, were always comparable. Shifts of 1.5-3.0-fold in both parameters of agonist affinity were observed after desensitization and were maintained after fusion. Thus in a typical experiment with C6 cells pretreated with isoproterenol and then incubated with the cells, the K_{D1} values were 18 and 37 nM for control and desensitized, respectively, after fusion. The corresponding values were 83 and 60 nM. Based on our previous results (15), it appears that pretreatment of the cells with agents that reduce receptor redistribution and sequestration also reduce the magnitude of the shifts in agonist affinity. Similar observations have been made by others on different cell lines (11,16) as well as in the present study (data not shown).

Reassembly - After A431 cells were washed free of isoproterenol and reincubated in fresh medium, there was a time-dependent recovery of agonist-stimulated adenylate cyclase activity as well as a reappearance of [3H]-CGP-12177 binding sites (Fig. 3A). Similar results were obtained with T4 cells (Fig. 3B). Reassembly was essentially complete in both cell lines by 2 h. Furthermore, using the membrane fusion technique, β-adrenergic receptor function was restored in the reassembled cells (Fig. 3C). As shown in Table V, prior treatment of the cells with isoproterenol had no effect on either desensitization or reassembly. Receptors in membranes prepared from untreated cells pretreated with isoproterenol and desensitized by fusion to acceptor membranes in the absence of isoproterenol were not reassembled even though they were desensitized. Similar results were obtained after pretreatment of the cells with isoproterenol for 1 h. In contrast, pretreatment of the cells with isoproterenol for 30 min resulted in increased adenylate cyclase activity. As the desensitized state in intact A431 cells is relatively stable, we explored the possibility that the fusion protocol by adding physoprophages and increasing the temperature to 37°C did not reverse the desensitization (data not shown).

Figure 3. Time dependence of resensitization of adenylate cyclase activity and reappearance of [3H]-CGP-12177 binding sites after fusion of A431 and C6 cells. A431 (A) and C6 (B) cells were incubated with 10−6 M isoproterenol for 2 h. Some of the cells were washed extensively and reincubated in fresh medium for the indicated times. After all of the cells were lysed, the lysates were assayed for [3H]-CGP-12177 binding (A) and net [3H]-CGP-12177-stimulated adenylate cyclase activity (B) as described in the legend to Figure 1. (C) Membranes were prepared from control (open bars), desensitized (closed bars) and resensitized (cross-hatched bars) cells, treated with alkali and fused with acceptor membranes as described in Methods. The fused membranes were assayed for net [3H]-CGP-12177-stimulated adenylate cyclase activity. Activity prior to alkali treatment and fusion is given as per cent of control at 52 and 90 min for desensitized and resensitized A431 cells, respectively.

TABLE V
Effect of Pretreatment of cultured cells with agonist on desensitization and resensitization

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>State</th>
<th>[3H]-CGP-12177 Binding</th>
<th>Net [3H]-CGP-12177-stimulated activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>control</td>
<td>268</td>
<td>3095</td>
</tr>
<tr>
<td></td>
<td>desensitized</td>
<td>232</td>
<td>709</td>
</tr>
<tr>
<td></td>
<td>resensitized</td>
<td>253</td>
<td>903</td>
</tr>
<tr>
<td>C6</td>
<td>control</td>
<td>216</td>
<td>786</td>
</tr>
<tr>
<td></td>
<td>desensitized</td>
<td>216</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>resensitized</td>
<td>216</td>
<td>432</td>
</tr>
</tbody>
</table>

TABLE VI
Effect of fusion of membranes from desensitized, nondesensitized cells on agonist-stimulated adenylate cyclase activity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>State</th>
<th>[3H]-CGP-12177 Binding</th>
<th>Net [3H]-CGP-12177-stimulated activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>control</td>
<td>85</td>
<td>775</td>
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<td></td>
<td>desensitized</td>
<td>216</td>
<td>786</td>
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<tr>
<td></td>
<td>resensitized</td>
<td>216</td>
<td>788</td>
</tr>
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</table>

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