Alanine mutagenesis scanning of the intracellular portion of the human muscarinic cholinergic M1 receptor was performed to identify domains mediating agonist-induced receptor sequestration. Using these multiple alanine point mutants of H1, we had previously identified several receptor domains in the intracellular loops i-3 that play a role in coupling to phosphatidylinositol turnover, most notably, a lipophilic residue, Leu-131, in the conserved i2 loop domain DRYXXXVXL (Moro, O., Lameh, J., Hogger, P., and Sadée, W. (1993) J. Biol. Chem. 268, 6862-6865). We now demonstrate that alanine substitutions in three of these domains, i.e. middle of the i2 loop and both junctions of the i3 loop, also result in defective sequestration (loss of surface receptor sites accessible to a polar tracer) in transfected human kidney U293 cells. The i2 loop was studied further by single point mutations. The strongest impairment of sequestration occurred with mutant L131A which was also highly defective in phosphatidylinositol (PI) coupling. Substitution of Leu-131 with several distinct amino acids indicated that a bulky lipophilic residue is required for sequestration in this position, as shown for coupling to PI turnover. Further, the double point mutation, V127A/L131A, which almost completely suppressed both sequestration and coupling of H1. In the β2 adrenoceptor, alanine substitution of the i2 residue Phe-138, equivalent to Leu-131 in H1, also resulted in impaired coupling to adenylyl cyclase and sequestration, indicating a general role for this conserved i2 loop residue in both processes. The combined results show that the multi-site domain involved in signal transduction of H1 is similar to and overlaps with that involved in sequestration. However, three H1 mutants that were moderately deficient in stimulating PI turnover displayed normal sequestration defects, from nearly normal to complete, and no single i3 loop domain could be identified that was consistently associated with strongly defective sequestration (1). We therefore proposed that the i3 loop rich domains, possibly upon phosphorylation, regulates sequestration by affecting the i3 loop conformation. Thus, the i3 loop is thought to act as a molecular switch, permissive for sequestration only in a conformation that enables access of any sequestration factor to the requisite domains elsewhere on the receptor. Similar results were obtained previously with the β2 adrenoceptor. Whereas a small domain in the COOH tail containing Ser and Thr residues was shown to affect coupling and sequestration (6), truncation mutations indicated that the COOH tail is not required per se (5). Further, truncation of the extended COOH tail of the avian β2 receptor unmask its ability to internalize (8). These results support the hypothesis that the overall conformation of the highly variable i3 loops and COOH tails plays a permissive role for receptor function. It further implies that the receptor domains directly mediating sequestration remain unknown.

Because both G protein activation and sequestration are dependent upon agonist stimulation, several studies have addressed the relationship between these two processes. Earlier mutational analysis of the β2 receptor demonstrated that structural features involved in receptor activation are also essential to receptor sequestration, suggesting that G protein activation and sequestration depend on the same receptor domains (9). However, several mutants of H1 and H3 were able to stimulate fully PI turnover, but were completely resistant to sequestration (1, 2, 10). Conversely, an H1/β2 adrenoceptor chimera had lost the ability to couple to G proteins, but yet continued to undergo sequestration (11). In this study (11), the i3' loop region 222-229 located near the 5'-junction of β2 was either deleted or replaced with the equivalent sequence 220-230 of H1. Whereas deletion of domain 222-229 abolished both Gi coupling and sequestration, introduction of the H1 domain 220-230 restored sequestration but not Gi coupling (11) suggesting a specific role for the H1 domain 220-230 domain in sequestration. However, deletion of 215-231 from the H1 receptor had no
effect on sequestration (1), arguing against a direct role of this domain. Further, \( \beta_2 \) receptors of S49 cells that are functionally uncoupled from adenylyl cyclase because of genetic lesions of \( \gamma_2 \) still were able to internalize normally (12, 13). These results support distinct and independent mechanisms for G protein activation in signal transduction and sequestration.

To identify the receptor domains involved in coupling to PI turnover, we performed alanine mutagenesis scanning covering the intracellular portion of Hm1 (14). Residues shown by earlier point and deletion mutations not to affect coupling and sequestration (see Fig. 1) (1, 2, 10, 15) were excluded, and the remaining receptor portions were analyzed by distributing 2-4 alanine mutations per construct throughout loops i1-3 and the COOH tail (mutants 1-9). The i1 and i2 loops and both junctions of the i3 loop were found to play a role in G protein signal transduction of Hm1 (14). Further, a bulky lipophilic amino acid in the conserved i2 domain DRYXXVXXPL (where L is any lipophilic amino acid) was shown to play a key role in G protein coupling of Hm1, Hm3, and the \( \beta_2 \) adrenoceptor (14). In the present study, a similar expanded set of point mutants was used to compare domains involved in signal transduction and sequestration. Alanine substitution in three domains, i.e., both junctions of the i3 loop, and most notably, the conserved i2 domain, were found to affect sequestration, suggesting the hypothesis that overlapping multi-site domains mediate both signal transduction and sequestration.

**EXPERIMENTAL PROCEDURES**

**Materials**—\([^3H]NMS\) (specific activity 80 Ci/mmol), \([^3H]QNB\) (specific activity 40 Ci/mmol), and \([^3H]CGP12177\) (specific activity 46 Ci/mmol) were obtained from Amersham Corp. and DuPont NEN. All other reagents were of analytical grade quality. Restriction enzymes were from either Boehringer Mannheim or Life Technologies, Inc.

**Construction of Vectors Expressing Hm1 Point Mutants**—The construction of Hm1 in vector pSG5 was described previously (1, 2), having EcoRI and BamHI restriction sites at the 5' and 3' ends, respectively. The point mutations were introduced using the “unique site elimination” method (USE, TransGenic™ site-directed mutagenesis kit, Clonetech) (see Ref. 14). All mutants were sequenced before further use.

**Construction of \( \beta_2 \) Adrenoceptor Mutant**—The hamster \( \beta_2 \) adrenoceptor expression vector (pCDM8) was provided by Dr. Diane Barber in the Department of Stomatology and Surgery, University of California, San Francisco. The point mutation F139A was introduced using the unique site elimination method.

**Transfection of Human Embryonal Kidney Cells (U293)**—The cells were transfected with use of the calcium phosphate precipitation method as previously described (1, 2). Transient expression yielded ~900 fmol/mg of protein for Hm1, and ~4000 fmol/mg of protein for \( \beta_2 \).

**Receptor Binding and Sequestration**—These procedures were described previously (1, 2, 10). Briefly, the transfected cells were replated onto 12-well cell culture dishes and allowed to attach overnight. In the case of Hm1, the cells were then incubated with or without 1 mM carbachol for up to 2 h. For the \( \beta_2 \) adrenoceptor, 1 \muM isoproterenol was used. After drug treatment, the cells were washed three times with ice-cold phosphate-buffered saline to remove residual carbachol or isoproterenol and incubated in an isotonic buffer containing 1.5 mm \([^3H]NMS\) or 3 nM \([^3H]CGP12177\) at 12 °C (to prevent receptor recycling) for 90 min. At the end of the incubation, the cells were filtered through glass-fiber filters (SS 32), followed by three rapid rinses with phosphate-buffered saline. Six replicate independent samples were assayed for each data point unless noted otherwise. To ascertain that loss of surface \([^3H]NMS\) binding was not paralleled by a loss of total receptor binding in the cells, \([^3H]QNB\) binding was assayed similarly in Hm1 transfected cells. There was no detectable loss of \([^3H]QNB\) binding after 2 h of carbachol (1 mM) treatment (1, 2). This result indicates that, during the observation period, no measurable down-regulation of Hm1 occurred in U293 cells.

**RESULT AND DISCUSSION**

A series of nine scanning mutants with multiple alanine substitutions (nos. 1-9) (Fig. 1) was tested for receptor expression, carbachol-induced PI turnover (14), and sequestration. Two of the scanning mutants (3 and 9) failed to yield measurable expression, possibly because of folding defects. The expression levels and PI-coupling efficiency of mutants with detectable receptor yield were previously reported (14), and these results are reproduced in Table I for comparison with the sequestration values. Several mutants showed defective coupling to PI turnover (mutants 1, 4, 5, 6, and 7; below 60% coupling efficiency compared to wild-type Hm1, at 1 mM carbachol), and we proposed a multi-site domain of Hm1 to be involved in G protein coupling (14).
Sequestration of Muscarinic Receptors

Table I

Effect of mutations of Hm1 on [3H]NMS binding and carbachol-induced activation of PI turnover and sequestration

| Mutant           | [3H]NMS binding | Stimulation of [3H]IP release | Cell surface receptor after carbachol treatment |
|------------------|-----------------|------------------------------|-----------------------------------------------|
|                  | fmol/mg of protein | %                           | %                                             |
| Hm1 wild-type    | 920 ± 236 (19)   | 100 ± 7                      | 55 ± 7 (38)                                  |
| 1 K131A/Y133A/K136A | 617 ± 342 (12)   | 52 ± 11                      | 47 ± 6 (12)                                  |
| 2 K87A/T55A/V59A | 203 ± 40 (12)    | 99 ± 54                      | 54 ± 6 (12)                                  |
| 3 Y124A/S126A/V127A/T128A | Not detectable | 76 ± 4 (3)                   | 66 ± 12 (10)                                 |
| D122N*           | 267 ± 45 (9)     | 100 ± 11                     | 40 ± 8 (15)                                  |
| F125A            | 356 ± 117 (15)   | 48 ± 6                       | 54 ± 7 (15)                                  |
| V127A/L131A*     | 350 ± 125 (30)   | 8 ± 6 (4)                    | 95 ± 15 (32)                                 |
| P130A            | 931 ± 157 (6)    | 96 ± 29                      | 62 ± 7 (6)                                   |
| 4 L131A/Y133A/K136A | 356 ± 40 (17)   | 15 ± 7                       | 80 ± 7 (17)                                  |
| L131A            | 541 ± 189 (18)   | 14 ± 5                       | 77 ± 12 (18)                                 |
| L131N            | 919 ± 93 (5)     | 15 ± 2                       | 81 ± 5 (6)                                   |
| L131D            | 676 ± 121 (11)   | 10 ± 12                      | 78 ± 7 (9)                                   |
| L131F            | 1160 ± 256 (6)   | 84 ± 7                       | 53 ± 4 (5)                                   |
| L131M            | 508 ± 43 (6)     | 60 ± 14                      | 65 ± 7 (6)                                   |
| Y133A            | 282 ± 4 (2)      | 71 ± 24                      | 51 ± 2 (12)                                  |
| K136A            | 676 ± 54 (4)     | 94 ± 7                       | 50 ± 3 (4)                                   |
| 5 R137A/T138A/R140A | 175 ± 10 (6)    | 59 ± 26                      | 42 ± 2 (6)                                   |
| 6 W209A/I211A/Y212A | 678 ± 170 (14)  | 34 ± 12                      | 72 ± 8 (14)                                  |
| 7 E294A/K362A/T366A | 439 ± 121 (14)  | 39 ± 6                       | 65 ± 7 (14)                                  |
| 8 K423A/R426A    | 762 ± 348 (7)    | 89 ± 6                       | 50 ± 5 (5)                                   |
| 9 F425A/D427A/F429A | Not detectable |                             |                                               |

* The expression yield is given for the sequestration experiments. For expression in the PI turnover experiments, see Moro et al. (14).

Numbers in parentheses = number of data points.

* Mutants not included with data of Moro et al. (14).

To test whether any of these mutated residues also play a role in carbachol induced sequestration of Hm1, we studied the sequestration behavior of each construct, in comparison to the wild-type receptor serving as a control. The polar tracer [3H]NMS was used to label Hm1 receptor sites accessible on the surface of intact U293 monolayers, and the carbachol induced loss of tracer binding was taken as a measure of sequestration (1, 2, 10). This procedure does not distinguish between sequestration at the surface and internalization into the cell. Muscarinic receptor localization by immunohistochemistry in our laboratory and elsewhere (16) indicates that agonist stimulation causes rapid receptor clustering at the surface and true internalization. We use the term sequestration here to account for both processes.

Sequestration of Alanine Scanning Mutants—Three of the nine alanine scanning mutants with multiple substitutions (nos. 4, 6, and 7) were defective in carbachol induced sequestration (Table I). Each of these mutants was also strongly defective (by 70–85%) in stimulating PI turnover Table I (14). On the other hand, mutants 1 and 5 were defective only in PI coupling but not internalization (Table I). These results implicate domains of the i2 loop and both junctions of the i3 loop in receptor sequestration.

Analysis of i2 Loop Residue Leu-131—The marked coupling and sequestration defect of the triple alanine mutant 4 suggested a role of the i2 loop. As previously shown for PI coupling (14), the sequestration defect of mutant 4 was again solely attributable to alanine substitution of Leu-131, whereas alanine substitution of the other two amino acids failed to affect coupling and sequestration (Table I). Substitution of L131 with alanine did not affect carbachol affinity (14). Residue Leu-131 is located at the 3' end of the consensus motif DRYXXVXXPL (Fig. 1), where L is leucine or any other bulky lipophilic amino acids, such as I, V, M, F in 64 out of 70 mammalian GPCR sequences listed in (17). Substitution with polar amino acids (D, N) led to both defective coupling (14) and sequestration, whereas substitution with F, as found in the equivalent position of the β2 receptor, resulted in normal functions (Table I). On the other hand, L-131 substitution with M, present in the equivalent position of the thrombin receptor, caused moderately impaired coupling (14), and it also moderately impaired agonist induced sequestration (Table I). Because of these parallel changes of coupling efficiency and sequestration behavior, we propose that a bulky lipophilic residue in the position equivalent to Leu-131 in Hm1 plays a key role in both processes.

Residue Phe-139 in the β2 Adrenoceptor—To evaluate the general importance of the lipophilic residue L-131 of Hm1 in the sequestration of other G protein coupled receptors, we also tested the equivalent F139A mutation in the p2 receptor. Alanine substitution of Phe-139 did not affect binding affinity to carbachol treatment (14), and it also moderately impaired agonist induced sequestration (Table I). Because of these parallel changes of binding affinity and sequestration behavior, we propose that a bulky lipophilic residue in the position equivalent to Leu-131 in Hm1 plays a key role in both processes.

Other Residues in the i2 Loop Motif DRYXXVXXPL of Hm1—Because the quadruple mutant 3, scanning the 5' junction of the i2 loop, did not yield measurable tracer binding, single alanine substitutions were introduced throughout the conserved motif DRYXXVXXPL. No effect on coupling and sequestration was observed by substituting the highly conserved P130 with alanine (Table I). Since alanine occurs naturally in lieu of proline in a few GPCRs, e.g., the o2 adrenoceptors (17), alanine in this position appears to be permissive for receptor function.

As residue Val-127 is also very highly conserved (only V or I are found in 67 out of 70 mammalian GPCR sequences listed in Ref. 17), the single point mutant V127A was constructed.

2 J. Arden, M. S. Shockley, J. Lameh, and W. Sadée, unpublished results.
Sequestration of Muscarinic Receptors

TABLE II
Effect of F139A point mutation of the β2 adrenoreceptor on expression, agonist-induced stimulation of cAMP production, and sequestration

|            | [3H]GTP12177 | Stimulation of cAMP production (%) | Cell surface receptors after isoproterenol pretreatment (%) |
|------------|--------------|-----------------------------------|--------------------------------------------------------|
| β2 AR wild-type | 4300 ± 451 (n=6) | 100 | 69 ± 4 |
| F139A      | 4100 ± 303 (n=6)  | 25 ± 4 | 58 ± 4 |

* Numbers in parentheses = number of data points.

Whereas PI coupling was measurably impaired (14), any effect on sequestration was absent or too small for detection (Table I). To test further the role of V127A, the double mutant V127A/L131A was newly constructed. A profound defect in both coupling and sequestration (Table I) demonstrates that this portion of the conserved i2 loop motif is essential for both processes. The fact that the V127A mutation alone did not measurably change internalization suggests that conformational changes of i2 caused by alanine substitution at this site contribute to the deficiency.

The less conserved residue Phe-125 was also exchanged without effect on PI turnover (14), but with even stronger sequestration than observed with the wild-type receptor (Table I). The reason for this enhanced internalization remains unknown, but it emphasizes the relevance of the i2 loop in sequestration.

Residue Asp-122 was previously shown to play a role in Hm1 mediated stimulation of PI turnover (18). Thus, mutation D122N decreased carbachol potency, although maximum effects on PI turnover were barely affected (18). We therefore tested the response of D122N to 1 μM carbachol (18). As seen in Table I, both stimulation of PI turnover and sequestration of D122N were moderately impaired in comparison to wild-type Hm1, and similar data were obtained at 100 μM carbachol (not shown). Hence, mutations throughout domain DRYXXVXXPL affected coupling and sequestration, indicating its relevance to both processes.

Time Course of Internalization—To test whether the Hm1 mutants displayed changed kinetics of sequestration/internalization-recycling, we measured the time course of carbachol induced loss of [3H]NMS binding sites from the cell surface (Fig. 2). The double mutant V127A/L131A did not measurably sequester at any time point. Each of the defective mutants tested rapidly sequestered over the initial 30 minutes, but only to an intermediate level. Because of the rather low degree of sequestration, we were unable to measure accurately initial sequestration rates for the mutants. Nevertheless, the time to reach equilibrium was not prolonged compared to wild-type Hm1. This result suggests that the rate of sequestration is reduced, rather than the rate of recycling, which would be expected to delay equilibrium between surface accessible and sequestered receptor sites. This result differs from the slower equilibration of Hm1 sequestration observed previously with several i3 loop deletions, e.g. d247–304 (1). The different kinetics suggests different mechanisms affecting the sequestration behavior of the point mutations studied here and the i3 loop deletion mutations, consistent with the distinct function proposed for the large i3 loop in regulating sequestration.

Comparison of Signal Transduction and Sequestration—The processes of signal transduction and sequestration maybe related or independent of each other. Fig. 3 shows a plot comparing degree of sequestration and coupling efficiency to PI turnover for Hm1 and the mutants listed in Table I. There is a striking correlation between coupling to PI turnover and sequestration, indicating that both functions require very similar domains. However, three mutants, i.e. scanning mutants 1 (i1 loop) and 5 (5' junction of i2), and V127A, are measurably defective only in stimulating PI turnover, but not in sequestration. As the observed changes in coupling efficiency are rather small, one could argue that a threshold activation of second messenger systems is necessary for sequestration, but not for signal transduction.
effects of mutations of the large Hml i3 loop on sequestration (1) cause conformational changes, rather than disrupt direct binding to target molecules. One cannot exclude the possibility that the alanine point mutation-derived second messengers. Since both processes are dependent upon agonist stimulation, a common mechanisms of signal transduction via G proteins and also subserves other receptor functions such as sequestration. This hypothesis can account for previous results obtained with mutagenesis experiments, where selected mutations may either affect coupling, or sequestration, or both. We can now interpret the unpredictable effects of mutations of the large Hml i3 loop on sequestration (1, 2): conformational changes of the i3 loop, acting as a molecular switch, determine access to a multi-site binding domain located in the i2 loop and the junctions of the i3 loop. However, one cannot exclude the possibility that the alanine point mutations, particularly in the hinge regions of the i3 loop, also cause conformational changes, rather than disrupt direct binding to target molecules.

The postulated multi-site binding domain of Hml1 responsible for agonist induced sequestration differs fundamentally from those of other classes of membrane receptors where a single contiguous domain was found to mediate internalization (e.g. for low density lipoprotein, transferrin, and epidermal growth factor receptors) (3). Thus, strategies for isolating the cellular factors mediating GPCR cellular trafficking must reflect the need for maintaining the structural integrity of the multi-site binding domain in the native receptor.

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