Sustained Activation of a G Protein-coupled Receptor via “Anchored” Agonist Binding

MOLECULAR LOCALIZATION OF THE SALMETEROL EXOSITE WITHIN THE β₂-ADRENERGIC RECEPTOR*

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An inherent therapeutic limitation of many G protein-coupled receptor agonists is a short duration of action due to rapid dissociation from receptors. Salmeterol is a modified β-adrenergic receptor (βAR) agonist that has a long duration of action at the β₂AR (but not the β₁AR) both in vitro and in vivo and that is persistent despite extensive washout of the agonist. It has been proposed that salmeterol binds not only to the active site of the β₂AR (localized to receptor transmembrane spanning domains (TMDs) 3 and 5) but also to another site (termed the “exosite”) that anchors it to the receptor and provides for repetitive active-site binding events. To identify the location of this exosite, we used site-directed mutagenesis to replace β₂AR amino acids 149–173 (within TMD4) with β₁AR sequence. The resulting constructs were then expressed in COS-7 cells for radioligand binding studies. Using this approach, when this domain was replaced with the analogous β₁AR sequence, the ability of salmeterol to persist at the receptor under washout conditions was reduced by 67%. The results from more selective mutants (S-149-166, S-164-173, and S-149-158)) indicated that a limited 10-amino acid region (β₂AR residues 149–158), localized at the interface of the cytoplasm and the transmembrane domain, contains a critical determinant for exosite binding. Whereas CHW cells stably expressing wild-type β₂AR displayed persistent salmeterol-promoted cAMP accumulation despite agonist washout, substitution of β₂AR residues 149–158 with β₁AR sequence resulted in a 50% attenuation of salmeterol-promoted cAMP accumulation under identical washout conditions. A reverse chimera was also studied, which consisted of a substitution of β₂AR residues 152–156 into the β₁AR. This substitution was found to confer exosite binding to the β₁AR. None of these mutations decreased the affinity of salmeterol for the receptor at the active site as assessed in competition binding studies. Anchored binding to this motif thus represents a novel mechanism by which agonists like salmeterol can repetitively activate receptors. Conceivably, with other G protein-coupled receptors that have similar motifs, anchored ligands can be designed to provide for long durations of action by this mechanism.

The active sites of the β₂-adrenergic receptor (β₂AR)1 for binding catecholamine-like agonists lie within the transmembrane spanning domains (TMDs) of the receptor and include key interactions at Ser-204 and Ser-207 of TMD5 (which form hydrogen bonds with hydroxyl groups on the catecholamine ring) and Asp-113 of TMD3 (which interacts with the amine head group) (1–3). Salmeterol is an analogue of the saligenin ethanolamine salbutamol (albuterol), with an aralkyloxyalkyl substitution at the amine group (Fig. 1a). In addition to increased lipophilicity, salmeterol exhibits binding and functional characteristics that suggest it binds not only to the active site but also to another site providing for a long duration of action (4–6). In tissue organ bath and cell culture systems, a single exposure to salmeterol results in persistent activation of β₂AR despite removal of the drug and extensive washing. Furthermore, although this persistent activation may be readily reversed by βAR antagonists, subsequent washout of the antagonist results in reassertion of the agonist activity (4, 7, 8). Kinetic analyses of the interaction between salmeterol and artificial lipid membranes suggest that this prolongation of action is not due solely to its increased lipophilicity; rather, the data implicate an additional membrane-bound factor(s) (such as the receptor) to explain the persistence of the ligand-receptor interaction (9). Furthermore, such a prolonged duration of action is not observed in tissue and cell preparations expressing the β₁AR, even though salmeterol does bind to this receptor (albeit with a lower affinity than to the β₂AR) (8). In addition, a recent study examining the functional activation of β₂AR expressed in L cells has also suggested that salmeterol is able to access the receptor despite concurrent blockade, apparently by forming a high affinity interaction with the receptor at a second, undefined locus (6).

Two mechanisms of action of salmeterol have been considered to account for these observations, 1) the drug, which is highly lipophilic, interacts with the lipid bilayers of the cell membrane in the vicinity of the receptor thereby providing a local depot, or 2) it specifically binds to the β₂ receptor itself at an anchoring region that allows for persistent and/or repetitive stimulation via the active site. This latter interaction, commonly referred to as “exosite binding,” would be a novel mechanism for G protein-coupled receptor agonism, and the molecular domains responsible for this potential interaction were investigated. To distinguish between the two aforementioned mechanisms, we developed a recombinant cell model by which

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1 The abbreviations used are: β₂AR, β₂-adrenergic receptor; β₁AR, β₁-adrenergic receptor; ICP, 125I-labeled cyanopindolol; TMD, transmembrane spanning domain; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium.
the interaction of salmeterol with the β₂AR could be measured. We then used this model system, together with site-directed mutagenesis of the receptor, to explore the molecular basis of this interaction. We demonstrate herein that salmeterol indeed binds to the β₂AR in a receptor-specific, “exosite”-type manner; furthermore, the molecular determinants of this novel binding mechanism are localized to a small domain within the transmembrane-spanning domain of the receptor, which is distinct from those regions classically associated with the receptor active site. Thus, the “anchored” binding of salmeterol to the β₂AR represents a novel ligand interaction of an agonist with a G protein-coupled receptor.

EXPERIMENTAL PROCEDURES

Constructions—Plasmids encoding the cDNAs for the wild-type β₂AR and β₁AR were used as described previously (10). Mutations of the wild-type β₂AR in which various sequences were replaced by the analogous region of the β₁AR were constructed by site-directed mutagenesis similar to that previously reported (11); briefly, the Kpn I-Pst I fragment of the β₂AR was subcloned into M13 phage, and oligonucleotide-directed mutagenesis was performed by the method of Kunkel (12), after which the mutated fragment was reintroduced into the wild-type β₂AR-expressing plasmid β₂PC12BI. Mutations of the β₁AR were similarly constructed, except that the entire coding region was subcloned into M13 phage; following mutagenesis, the Bst XI-Ase I fragment was subcloned into the closely related vector containing the otherwise wild-type sequence. All mutations were confirmed by dideoxy sequencing. The predicted protein sequences of the β₂AR wild-type and mutated receptors are depicted in Fig. 1. In each case, numbering of the mutation refers to the regions of the wild-type receptor that were replaced by analogous sequence. Specifically, the largest mutation (mutant β₂AR TMD4 residues 149–173) consisted of replacement of the entire β₂AR TMD4 (residues 149–173) with the analogous β₁AR sequence. Similarly, smaller mutations included substitution of the β₂AR TMD4 amino acids in closest proximity to the cytoplasmic face of the receptor (mutant β₂AR S148–158), residues of the lower two-thirds of the β₂AR TMD4 (mutant β₂AR S148–166), and residues closest to the extracellular face of the receptor (mutant β₂AR S164–173), with the analogous β₁AR sequences.

Transfections and Cell Culture—Plasmid DNA containing each receptor construct were transiently expressed in subconfluent COS-7 cells by either electroporation or by the DEAE/dextran technique (13). 1–10 μg of plasmid DNA was used for either transfection procedure and was adjusted to yield equivalent receptor expression levels, as determined in radioligand binding studies described below. Cells were maintained in DMEM and fetal calf serum (10%) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO₂ at 37 °C. Cells were assayed when confluent, typically 48 h following transfection. For functional studies, plasmids were stably transfected in CHW-1102 cells by calcium phosphate precipitation as described previously (10); 30 μg of plasmid DNA were cotransfected with 3 μg of pSV2neo, and recombinant cells were selected in 300 μg/ml G418. Cell lines resistant to G418 were maintained in DMEM with serum, antibiotics, and 80 μg/ml G418, and βAR expression was determined by radioligand binding as described below. Clonal lines expressing equivalent amounts of receptor (~600 fmol/mg protein) were selected for study.

Radioligand Binding Assays—Radioligand binding studies were performed in COS-7 membranes in a manner similar to that described previously (10, 11). Briefly, confluent COS-7 cultures were scraped in 5 mM Tris (pH 7.4), 2 mM EDTA at 4 °C, and crude membranes collected and recentrifuged, and supernatant was removed for cAMP determination. After such washout, but 38 μM salmeterol. After 10 min, the cells were washed extensively at room temperature with calcium-free phosphate-buffered saline (PBS) at a constant flow rate of 20 ml/min for the indicated times using a multichannel peristaltic pump. (In preliminary studies, analysis of washout after such a wash revealed no detectable agonist by high performance liquid chromatography.) Cells were then lysed, membranes prepared, and the apparent receptor density (i.e. the number of receptors not occupied by drug) was determined by radioligand binding as described above. Because of day-to-day variability in the transient receptor expression levels (range, 400–6000 fmol/mg protein), apparent receptor density was normalized as the percentage of 125I-cyanopindolol binding sites observed in similarly treated membranes preincubated with vehicle alone. For wild-type β₂AR, 38.2 ± 2.3% of the receptor remained occupied by salmeterol after this washout procedure, indicative of persistent binding to the wild-type receptor. Subsequent data are presented as the loss of this exosite binding activity for the various mutant receptors.

cAMP Accumulation Studies—Functional studies were performed using confluent 35-mm tissue culture dishes containing stably-transfected CHW-1102 cells expressing either wild-type or mutated β₂AR at equivalent expression levels as described above. Media from each dish were aspirated, cells washed twice with PBS, and 1 ml of serum-free DMEM containing either the indicated concentration of agonist plus vehicle or vehicle alone was added. Dishes were incubated for 10 min at 37 °C, after which the media were removed and saved for measurement of cAMP. (In preliminary studies, salmeterol-promoted accumulation of cAMP in CHW cells expressing either wild-type or mutated β₂AR was linear and equivalent up to at least 30 min.) The cells were washed by perfusion with PBS for 2.5 min at room temperature in the same manner as described for the binding studies above. At the end of this perfusion, the PBS was aspirated, 1 ml of serum-free DMEM (without agonist) was added, the cells were returned to the 37 °C incubator for 10 min, and the supernatant was then removed for cAMP determination. This process of washing, incubation, and cAMP determination was repeated until the total aggregate perfusion time reached 15 min. Total cAMP content in the saved DMEM fractions was determined by an acetylated radioimmunoassay method as described previously (11, 16). The cAMP content of each fraction, determined as pmol/ml media, was expressed as the percentage of cAMP measured in the initial fraction obtained prior to any washing.

Data Analyses—Comparisons between means were by paired or unpaired t tests, as appropriate. p values < 0.05 were considered significant. Values ± S.E. of n tests were shown unless otherwise indicated. Radioligand binding data were analyzed by nonlinear regression of raw counts, using software from GraphPad (San Diego, CA). Kᵣ values were determined from EC₅₀ values by the method of Cheng and Prusoff (17).

Materials—DMEM and fetal calf serum were from JRH Biosciences. G418 was from Life Technologies, Inc. ICYP was from DuPont NEN. 125I-cAMP-tyrosine methyl ester, used in the cAMP radioimmunoassays, was from Hazelton-Washington. Oligonucleotides were from the University of Cincinnati DNA Core Facility. Restriction endonucleases were from New England BioLabs. Salmeterol and formoterol were a gift from Glaxo-Wellcome. All other reagents were from Sigma.

RESULTS

In order to develop a useful model of salmeterol/β₂AR binding, initial experiments were carried out using the β₂AR and the closely related β₁AR subtype expressed in the same parental cell. Human β₂AR (18) and β₁AR (19) were recombinantly expressed in COS-7 cells and exposed to 100 μM isoproterenol (a short acting agonist), 10 μM formoterol (a moderately long-acting agonist thought to act via a membrane depot effect), or 1 μM salmeterol. After 10 min, the cells were washed extensively, and the number of receptors occupied by drug was assessed by means of radioligand binding. For β₂AR, <2% of the receptors remained occupied by isoproterenol or formoterol after this washout, but 2.3% (± 0.6%) of β₂AR receptors (i.e. 242 fmol/mg protein, n = 11) remained occupied by salmeterol. For the β₁AR, <2% of the receptors remained occupied after treatment with any of the three agonists, even at concentra-
tions up to 10 times their respective $K_i$ (as determined in radioligand binding studies; see Table I and discussion below) and subsequent washout. Given that these two receptors were expressed in an identical membrane environment, these results suggest that salmeterol’s persistence at $\beta_2AR$ results from its binding specifically to a domain within the $\beta_2AR$ receptor itself, rather than simply within the membrane bilayers.

To identify a specific domain within the $\beta_2AR$ that is required for persistent binding, we constructed chimeric $\beta_2AR$ having $\beta_1AR$ sequences substituted into TMD4 (Fig. 1b). This segment was chosen for mutagenesis because it displays the greatest difference in primary amino acid sequence between $\beta_1AR$ and $\beta_2AR$ TMDs (~55% identity), and because earlier mutagenesis studies by our group (20) and others (21) have suggested that this domain may be important in agonist side chain interactions with $\beta_2AR$. Of note, the TMD3 and TMD5 segments that have the key determinants of active site binding are virtually identical (19) between the two receptors. Radioligand binding data from competition studies for these receptors are shown in Fig. 2 and are summarized in Table I. As indicated in the table, substitution of the entire $\beta_1AR$ TMD4 into the $\beta_2AR$ (mutant $\beta_2S$-(149–173)) had no significant effect on the affinity of the radioligand ICYP for the receptor (dissociation constant $K_i = 41.7 \pm 2.3$ versus $38.0 \pm 5.6$ pM for wild type, $n = 3$). In addition, in radioligand binding competition studies this mutant displayed no significant differences in affinity for isoproterenol (inhibition constant $K_i = 67.0 \pm 4.0$ versus $105 \pm 17.0$ nM) or epinephrine (327 ± 52.4 versus 477 ± 69.9 nM) compared with the wild-type $\beta_2AR$. Importantly, the affinity of the $\beta_2S$-(149–173) chimera (as well as each of the other $\beta_2AR$ chimeras studied) for salmeterol was no different than that observed for the wild-type $\beta_2AR$ ($K_i = 0.63 \pm 0.09$ versus $1.29 \pm 0.51$ nM, respectively; $n = 6–8$, $p = 0.30$). No major alterations in affinities were detected with any of the other receptors, and the indicated agonists, although small, statistically significant differences were noted in some cases (Table I). It should be noted that although one report (21) using different chimeras suggested that $\beta_2AR$ versus $\beta_1AR$ agonist specificity is predominantly dictated by TMD4, this was not observed in the current study (Fig. 2). Thus, neither the $\beta_2AR$ rank order of agonist affinities (isoproterenol $>$ epinephrine $>$ norepinephrine) was changed by these mutations nor was the affinity of the $\beta_2AR$-specific agonist albuterol altered. Taken together, these results suggest that the overall integrity of the active binding site for agonists remained intact for each mutant receptor.

As described above, wild-type $\beta_2AR$ display persistent occupation of receptors by salmeterol after extensive washing of the cells. The mutant receptors were thus studied in a similar fashion, with the extent of persistent binding of salmeterol to wild-type $\beta_2AR$ (termed exosite binding) serving as the control. The results of these studies are shown in Fig. 3. Substitution of
Inhibition constants \( K_i \) and dissociation constants \( K_d \) were determined in ICYP radioligand binding assays in the presence of GTP as described under "Experimental Procedures." Data shown are mean \pm S.E. for three to eight experiments, each performed in duplicate. In general, effects on agonist affinities were small, relative to wild type \( \beta_2 \text{AR} \). No differences in salmeterol affinity were observed for any of the \( \beta_2 \text{AR} \) chimeras studied. \( \beta_2 \text{AR} \) data are presented for comparative purposes only. ISO, isoproterenol; EPI, epinephrine; NOREPI, norepinephrine; ND, not determined.

| Construct | ISO | EPI | NOREPI | Albuterol | Formoterol | Salmeterol | ICYP |
|-----------|-----|-----|--------|-----------|------------|------------|------|
| \( \beta_2 \text{AR} \) | 105 ± 17.0 | 477 ± 69.9 | 16.5 ± 4.1 | 2.15 ± 0.51 | 12.7 ± 0.57 | 1.29 ± 0.51 | 38.0 ± 5.6 |
| \( \beta_2 \text{S(149–173)} \) | 67.0 ± 4.0 | 327 ± 52.4 | 5.5 ± 0.1* | 1.10 ± 0.05 | 11.5 ± 1.2 | 0.63 ± 0.09 | 41.7 ± 2.3 |
| \( \beta_2 \text{S(149–166)} \) | 222 ± 45.4* | 484 ± 72.6 | 10.5 ± 2.4* | 1.06 ± 0.11* | 14.4 ± 1.2 | 1.35 ± 0.18 | ND |
| \( \beta_2 \text{S(164–173)} \) | 228 ± 27.2* | 350 ± 68.0 | 15.1 ± 4.7 | 0.91 ± 0.08 | 15.4 ± 0.5* | 0.36 ± 0.06 | ND |
| \( \beta_2 \text{S(149–158)} \) | 284 ± 30.4* | 1570 ± 144* | 22.9 ± 3.8 | 2.04 ± 0.72 | 21.9 ± 1.3* | 1.85 ± 0.59 | 43.9 ± 3.9 |
| \( \beta_2 \text{AR} \) | 81.5 ± 9.2 | 1680 ± 688 | 0.54 ± 0.03 | 39.4 ± 7.2 | 424 ± 93.7 | 826 ± 85.4 | ND |

* \( p < 0.05 \) versus \( \beta_2 \text{AR} \).

### Fig. 2
ICYP radioligand competition data for COS-7 cells expressing either wild-type \( \beta_2 \text{AR} \) or \( \beta_2 \text{S(149–173)} \). COS-7 cells were transiently transfected with either wild-type \( \beta_2 \text{AR} \) (solid symbols) or \( \beta_2 \text{S(149–173)} \) (open symbols) constructs, membranes prepared, and competition binding assays performed in the presence of GTP and the indicated concentrations of the agonists isoproterenol (\( \square \)), epinephrine (\( \bigcirc \)), and norepinephrine (\( \blacksquare \)) as described under "Experimental Procedures." In each case, the data were fit to a single-site binding model using software from GraphPad and inhibition constants \( K_i \) determined from EC\( _{50} \) data by the method of Cheng and Prusoff (17). For each of the agonists studied, little or no difference in agonist affinity was observed between the wild-type and mutated receptor; in addition, no effect on the typical \( \beta_2 \text{AR} \) rank-order profile (isoproterenol > epinephrine > norepinephrine) was observed. Shown are mean data \pm S.E. for four or five experiments; see also Table I.

### Fig. 3
Exosite binding of salmeterol to wild-type and mutant \( \beta_2 \text{AR} \). COS-7 cells expressing each of the receptors were preincubated at 37 °C for 10 min with either 1 \( \mu \text{M} \) salmeterol or vehicle, the media removed, and the cells washed continuously at room temperature with PBS at a constant flow rate of 20 ml/min for 30 min. Cells were then lysed, membranes prepared, and the apparent receptor density (i.e., the number of \( \beta_2 \text{AR} \) not occupied by drug) was determined as described under "Experimental Procedures." With \( \beta_2 \text{AR} \), 38.2 ± 2.3% of the receptor remained occupied by salmeterol after this washout procedure, indicative of persistent binding to the wild-type receptor. Data are presented as the percent loss of this amount of exosite binding for the various mutant receptors. Treatment with isoproterenol and formoterol revealed no persistent binding in the wild-type \( \beta_2 \text{AR} \) or the mutant receptors (data not shown).

\( \beta_2 \text{AR} \) or \( \beta_2 \text{S(149–158)} \) were established, and the ability of isoproterenol or salmeterol to activate each receptor under washout conditions was examined. Receptor expression was equivalent for the two lines studied (635 ± 61 fmoi/mg protein for \( \beta_2 \text{AR} \) versus 664 ± 48 fmoi/mg protein for \( \beta_2 \text{S(149–158)} \), \( n = 5 \)). Fig. 4 presents the cAMP content determined in the supernatants of CHW cells expressing each receptor before washout (perfusion time = 0) and following each subsequent 2.5-min perfusion washout (for simplicity, these perfusion times were summed and expressed as an aggregated time for each data point). As shown in the figure, salmeterol-promoted cAMP accumulation in CHW cells expressing wild-type \( \beta_2 \text{AR} \) remained at or near initial values despite repeated washings, indicating persistent activation of the receptor by retained agonist. In contrast to the results obtained with the wild-type \( \beta_2 \text{AR} \), but consistent with the radioligand binding studies described above, salmeterol-promoted cAMP levels in cells expressing \( \beta_2 \text{S(149–158)} \) demonstrated a gradual decline. Thus, at 10 min aggregate perfusion (washout) time, cAMP levels for the wild-type \( \beta_2 \text{AR} \) were still 91.9 ± 2.7% of the control (prewashout) value versus 40.5 ± 7.5% for the \( \beta_2 \text{S(149–158)} \) receptor, a reduction of 56% (\( n = 5 \), \( p = 0.0013 \)). Persistent receptor activation was not observed following exposure of cells...
expressing either receptor to isoproterenol at high concentration (1 μM). After 10 min aggregate washout time, the cAMP accumulation in cells expressing β2AR was 20.6 ± 3.0% of the initial value; for cells expressing β2S-(149–158), the corresponding value was 15.2 ± 8.2% (n = 5, p = 0.55).

To examine further the role of the small β2AR domain to function as a salmeterol exosite binding domain, we substituted the Val-Ile-Ile-Leu-Met β2AR sequence (residues 152–156) into TMD4 of the wild-type β2AR. The ability of salmeterol to persist at this reverse chimera, termed β2S-(177–181), despite washout conditions was then assessed in a similar manner as described for the β2AR constructs above. As shown in Fig. 5, under the reduced washout stringency conditions described in the figure legend, wild-type β2AR expressed in COS-7 cells did not exhibit any retained binding of salmeterol (−0.6 ± 1.8% change in apparent receptor density versus untreated control, n = 5). In contrast, COS-7 cells expressing β2S-(177–181) demonstrated a 13.6 ± 2.2% (n = 5) loss of apparent receptor density following treatment with salmeterol, indicating retained receptor occupancy by drug. This difference, which represents a gain in exosite binding, was highly significant (p = 0.001). These results were not due to an increase in salmeterol affinity for the mutant versus wild-type β2AR; in fact, the affinity of salmeterol for the β2S-(177–181) chimera was actually reduced slightly relative to the wild-type β2AR (Kᵣ = 1130 ± 77 (n = 5) versus 826 ± 85 (n = 8) nM, respectively; p = 0.03).

**DISCUSSION**

As introduced earlier, salmeterol is a modified β2AR agonist that exhibits markedly prolonged duration of activity (>15 h) in multiple physiologic preparations. Although salmeterol is highly lipophilic (log octanol:H₂O partition coefficient = 3.88 (4)), the activity of salmeterol in such preparations is not adequately explained by increased lipophilicity alone. For example, although activation of β2AR by salmeterol is blocked by typical βAR antagonists (indicating that the drug acts via the receptor), this activity recurs rapidly following washout of the antagonist. Kinetic studies have demonstrated that the drug dissociates from model lipid membranes much faster (t₁/₂ ≈ 25 min) than is observed in vivo, suggesting that other components of the native cell membrane architecture, including perhaps the receptor itself, are involved in prolonging the effective duration of action of the drug (9).

Based on studies such as these, a unique model of salmeterol binding to the β2AR has been proposed. According to this model, salmeterol binds not only to the receptor active site but in addition to a second locus within the receptor. This second locus, termed the exosite, allows the drug to persist at the receptor even in the presence of concurrent antagonist occupancy at the active site. By anchoring the agonist to the receptor, the concentration of agonist available for active site binding might be maintained, rather than decline, due to degradation, redistribution, and/or other elimination pathways. Since the equilibrium between activated (agonist-bound) and inactivated receptor depends, in part, on the concentration of agonist available to the receptor, the net result of anchored ligand binding would be an increase in the probability of receptor activation and thus a prolongation of effective agonist duration of action.

The goal of the current study was to identify the molecular basis of such an exosite. We hypothesized that if such a domain existed, alteration of these amino acids would attenuate the ability of salmeterol to persist at the receptor. In order to demonstrate the specificity of such a domain, we chose to substitute candidate regions of the β2AR with corresponding regions of the β1AR. In doing so, we made use of the observation that although salmeterol binds to the β1AR, salmeterol does not exhibit exosite binding to this receptor despite the fact that the β1AR and β2S-(177–181) display a moderate degree of homology within the putative ligand binding domains. We selected the fourth transmembrane spanning domain (TMD4) for study for several reasons. First, the greatest degree of dissimilarity among the TMDs of these receptors is observed in TMD4; if exosite binding is indeed unique to the β2AR, one might expect it to occur in a region more dissimilar to the β1AR. Second, using chimeric β1AR/β2AR constructs Frielle et al. (21) demonstrated that while multiple domains appeared to be involved in determining the subtype specificity of agonist binding, the ma...
FIG. 6. Predicted interaction of salmeterol with the $\beta_2$-adrenergic receptor. Shown are the seven TMDs of the receptor, modeled by analogy to bacteriorhodopsin (25), as viewed from within the cell membrane. Key active site hydrostatic interactions at Asp-113 and Ser-204/Ser-207 of the third and fifth transmembrane spanning domains, respectively, are indicated by dashed red lines. The portions of the fourth TMD found to have critical residues for the salmeterol exosite (residues 149–158) are indicated. The figure was generated by modeling the $\beta_2$-adrenergic receptor using the WHATIF program (26, 27), then using the docking and molecular visualization routines in the PCImDad (Molecular Applications Group, Palo Alto, CA) software package.
ounds is not changed by the altered position of the oxygen (4), this further supports the concept that the mechanism of salmeterol's persistent action is not simply one of a nonspecific lipophilic integration of the drug within the membrane but a specific interaction with a distinct exosite domain of the β2AR. This interaction may nonetheless be related to the lipophilic nature of the salmeterol side chain since the residues identified as the anchor in the current study comprise the most hydrophobic region of TMD4.

In summary, the present work confirms the molecular basis for binding of salmeterol to a region of the β2AR distinct from the active binding site. Elimination of this exosite domain results in loss of the ability of salmeterol to persist at the receptor, without affecting the overall affinity for the active site as determined by radioligand binding analyses. Taken together, this provides a molecular mechanism to explain, in part, salmeterol's prolonged physiologic duration of bronchodilation, as well as the ability of salmeterol to persevere at the receptor despite reversible active site blockade by antagonists. This type of binding may have significant implications for other G protein-coupled receptors. As has been demonstrated in clinical trials, inhaled salmeterol provides for a long duration of bronchodilation in the treatment of asthma, resulting in substantially improved control of the disease over that provided by traditional short-acting agents (23). To date, the mechanism of salmeterol's long duration of action had not been delineated and indeed the existence of an exosite-type mechanism intensely debated (24). With the results of the current work, which identifies a specific domain within the β2AR that provides for exosite binding, the possibility exists that ligands for other G protein-coupled receptors could be designed based on this mechanism to provide for repetitive activation, thereby enhancing the therapeutic utility of drugs acting via these receptors.

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