

# Structural basis for receptor subtype-specific regulation revealed by a chimeric $\beta_3/\beta_2$ -adrenergic receptor

(desensitization/phosphorylation/down-regulation/adenylyl cyclase)

STEPHEN B. LIGGETT\*, NEIL J. FREEDMAN†, DEBRA A. SCHWINN‡, AND ROBERT J. LEFKOWITZ†§¶

\*Departments of Medicine (Pulmonary), Molecular Genetics and Pharmacology, University of Cincinnati Medical Center, Cincinnati, OH 45267; and Departments of †Medicine (Cardiology), ‡Biochemistry, and ‡Anesthesiology and Pharmacology, Duke University Medical Center and the Howard Hughes Medical Institute, Durham, NC 27710

Contributed by Robert J. Lefkowitz, December 18, 1992

**ABSTRACT** The physiological significance of multiple G-protein-coupled receptor subtypes, such as the  $\beta$ -adrenergic receptors ( $\beta$ ARs), remains obscure, since in many cases several subtypes activate the same effector and utilize the same physiological agonists. We inspected the deduced amino acid sequences of the  $\beta$ AR subtypes for variations in the determinants for agonist regulation as a potential basis for subtype differentiation. Whereas the  $\beta_2$ AR has a C terminus containing 11 serine and threonine residues representing potential sites for  $\beta$ AR kinase phosphorylation, which mediates rapid agonist-promoted desensitization, only 3 serines are present in the comparable region of the  $\beta_3$ AR, and they are in a nonfavorable context. The  $\beta_3$ AR also lacks sequence homology in regions which are important for agonist-mediated sequestration and down-regulation of the  $\beta_2$ AR, although such determinants are less well defined. We therefore tested the idea that the agonist-induced regulatory properties of the two receptors might differ by expressing both subtypes in CHW cells and exposing them to the agonist isoproterenol. The  $\beta_3$ AR did not display short-term agonist-promoted functional desensitization or sequestration, or long-term down-regulation. To assign a structural basis for these subtype-specific differences in agonist regulation, we constructed a chimeric  $\beta_3/\beta_2$ AR which comprised the  $\beta_3$ AR up to proline-365 of the cytoplasmic tail and the C terminus of the  $\beta_2$ AR. When cells expressing this chimeric  $\beta_3/\beta_2$ AR were exposed to isoproterenol, functional desensitization was observed. Whole-cell phosphorylation studies showed that the  $\beta_2$ AR displayed agonist-dependent phosphorylation, but no such phosphorylation could be demonstrated with the  $\beta_3$ AR, even when  $\beta$ AR kinase was overexpressed. In contrast, the chimeric  $\beta_3/\beta_2$ AR did display agonist-dependent phosphorylation, consistent with its functional desensitization. In addition to conferring functional desensitization and phosphorylation to the  $\beta_3$ AR, the C-terminal tail of the  $\beta_2$ AR also conferred agonist-promoted sequestration and long-term receptor down-regulation.

Three distinct subtypes of  $\beta$ -adrenergic receptors ( $\beta$ ARs) have been identified by classic pharmacologic means and molecular cloning (1, 2).  $\beta_1$ -,  $\beta_2$ -, and  $\beta_3$ AR all couple to  $G_s$ , and in the presence of agonist they transduce a signal that results in increased intracellular cAMP. Each of these receptors also binds the endogenous agonists epinephrine and norepinephrine, albeit with some differences in their relative potencies. The relevance of  $\beta$ AR subtypes is thus obscure, since all couple to the same effector and all utilize the same endogenous agonists. Previous work by several groups has focused on identifying regions of the  $\beta_1$ - and  $\beta_2$ AR which provide for antagonist or agonist subtype-specific binding (3,

4). In contrast, little is known about how  $\beta$ ARs may be distinguished in properties “downstream” of ligand binding.

We considered that perhaps one reason for the existence of  $\beta$ AR (and other receptor) subtypes might reside in the manner in which they are regulated. One form of G-protein-coupled receptor regulation with important physiologic and therapeutic relevance is that resulting from continuous exposure of receptors to agonists. This phenomenon, termed desensitization, is an adaptive process found in a number of diverse signal transduction systems (1, 2). It serves to limit the cellular response to a continuously present stimulus. Rapid (within seconds) agonist-promoted desensitization of the  $\beta_2$ AR appears to be primarily due to phosphorylation of the receptor by several protein kinases, including the  $\beta$ AR kinase ( $\beta$ ARK) and the cAMP-dependent kinase, protein kinase A (PKA) (5–7). The sites of  $\beta$ ARK phosphorylation appear to reside in serines and threonines in the C-terminal portion of the  $\beta_2$ AR (5, 6). In addition, the third intracellular loop and proximal cytoplasmic tail of the  $\beta_2$ AR contain consensus sequences for PKA-mediated phosphorylation (5), with the third intracellular loop site appearing to be the more critical for cAMP dependent desensitization (8). Examination of the molecular structure of the three  $\beta$ AR subtypes (9–11) reveals a striking difference between the  $\beta_2$ - and  $\beta_3$ AR. In contrast to the 11 serines and threonines in the distal portion of the cytoplasmic tail of the  $\beta_2$ AR, there are only 3 serines in the same region of the  $\beta_3$ AR. In addition to this apparent paucity of potential  $\beta$ ARK phosphorylation sites, the  $\beta_3$ AR lacks consensus sequences for PKA-mediated phosphorylation.

Receptor sequestration and down-regulation are two other processes, independent of receptor phosphorylation, that also occur during agonist-promoted desensitization of the  $\beta_2$ AR. Sequestration is a rapid (seconds to minutes) movement (internalization) of receptors from the cell surface to an intracellular compartment. As discussed below, the  $\beta_3$ AR lacks an apparently critical 10-amino acid sequence motif in the cytoplasmic tail which has been implicated as playing a role in  $\beta_2$ AR sequestration (12). Finally, after long-term (hours) agonist exposure, the net cellular expression of  $\beta_2$ AR becomes decreased (regardless of localization), a process termed down-regulation. Two (but not necessarily all) of the requirements for full agonist-promoted down-regulation of the  $\beta_2$ AR are PKA phosphorylation sites (13) and tyrosine residues in the cytoplasmic tail (14). Again, the  $\beta_3$ AR lacks both of these determinants.

Thus the  $\beta_3$ AR appears to lack a number of the molecular features for agonist-promoted regulation which have thus far been established with the  $\beta_2$ AR. This raises the interesting

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations:  $\beta$ AR,  $\beta$ -adrenergic receptor;  $\beta$ ARK,  $\beta$ AR kinase; PKA, cAMP-dependent protein kinase (protein kinase A);  $^{125}$ I-CYP,  $^{125}$ I-labeled cyanopindolol.

¶To whom reprint requests should be addressed at: Box 3821, Duke University Medical Center, Durham, NC 27710.

question as to whether different subtypes of adrenergic receptors may have evolved to meet differing needs for agonist regulation. To investigate this, we expressed  $\beta_2$ AR,  $\beta_3$ AR, and a chimeric  $\beta_3/\beta_2$ AR in CHW and COS-7 cells and examined the various components of such regulation by agonists.

## METHODS

**Constructs.** The expression vector pBC $\beta_3$  was constructed by ligating *Sma* I/*Acc* I and *Acc* I/*Bam*HI digests of the human  $\beta_3$ AR cDNA into *Sma* I/*Bam*HI sites of the mammalian expression vector pBC12BI (15). The chimeric  $\beta_3/\beta_2$ AR construct (p $\beta_3/\beta_2$ ) was made by ligating an *Acc* III/*Kpn* I pBC $\beta_3$  fragment with a *Stu* I/*Kpn* I pBC $\beta_2$  (3) fragment. This construct contained 6 bp of 5'  $\beta_3$ AR untranslated sequence, the coding block of the  $\beta_3$ AR up to that which encodes Pro-365, and the coding sequence which corresponds to the last 65 amino acids of the  $\beta_2$ AR (see Fig. 1). To determine the capacity of the three receptors to undergo agonist-promoted phosphorylation, additional constructs based on pBC $\beta_3$ , pBC $\beta_2$ , and pBC $\beta_3/\beta_2$  were made, each having a sequence inserted at the N terminus which encodes a 9-amino acid epitope (YPYDVPDYA) which is recognized by the monoclonal antibody 12CA5 (16). By using this approach each receptor was tagged with the epitope and could be immunoprecipitated with the same antibody. All mutated sequences were confirmed by dideoxy sequencing. The construct consisting of the cDNA encoding bovine  $\beta$ ARK1 (pBC $\beta$ ARK) is as described (17).

**Cell Culture and Transfections.** CHW cells (Chinese hamster fibroblasts) were cotransfected with pBC $\beta_3$ , pBC $\beta_2$ , or pBC $\beta_3/\beta_2$  and pSV2Neo in a manner similar to that described (5, 6), using precipitation with calcium phosphate. For studies of desensitization, sequestration, and down-regulation, receptor expression of each subtype in CHW cells was 200–300 fmol/mg. For phosphorylation studies, COS-7 cells were transiently transfected by the DEAE-dextran method with epitope-tagged pBC $\beta_2$ , pBC $\beta_3$ , or pBC $\beta_3/\beta_2$  and, in some cases, pBC $\beta$ ARK.

**Desensitization and Adenylyl Cyclase Assays.** Cells at 90% confluence were incubated for 2 hr with fresh medium without calf serum, then exposed to medium alone (control) or 100  $\mu$ M isoproterenol for 30 min. The flasks were then placed on ice; the cells were washed four times with 20 ml of cold phosphate-buffered saline (PBS), detached, and lysed by scraping in 5 mM Tris-HCl, pH 7.4/2 mM EDTA buffer; and membranes were prepared as described (6). Adenylyl cyclase assays were performed by the method of Solomon as modified (6).

**Sequestration, Down-Regulation, and Ligand Binding.** For these studies cells were exposed to 100  $\mu$ M isoproterenol for the times indicated at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. To delineate the sequestered pool of receptors, whole-cell binding studies were performed in a manner similar to that previously described (5). For the determination of agonist-promoted down-regulation of  $\beta$ AR,  $^{125}$ I-labeled cyanopindolol ( $^{125}$ I-CYP) binding was performed with washed membranes as described (18). Specific binding was defined as the difference between binding of 1.2 nM  $^{125}$ I-CYP in the absence or presence of 10  $\mu$ M CGP12177 (which defines nonspecific binding), and it was normalized to protein.

**Immunoprecipitation and Phosphorylation of  $\beta$ ARs.** Agonist-promoted whole-cell phosphorylation studies were carried out in a manner similar to that we have described for the  $\alpha_2$ -adrenergic receptor (19), except that epitope-tagged receptors were transiently expressed in COS-7 cells and immunoprecipitation was carried out with the monoclonal antibody 12CA5 (ref. 16; Berkeley Antibody, Richmond, CA). Transient expression of the  $\beta$ ARs in COS-7 cells rather than stable expression in CHW cells was utilized to obtain higher expression levels suitable for whole-cell phosphorylation studies. In preliminary studies, epitope-tagged receptors in COS-7 cells were metabolically labeled with [ $^{35}$ S]methionine and immunoprecipitated with the 12CA5 antibody by the methods described above. Autoradiograms from such experiments revealed bands representing the  $\beta_2$ AR, the  $\beta_3$ AR, and the chimeric  $\beta_3/\beta_2$ AR at the appropriate molecular weights (data not shown).

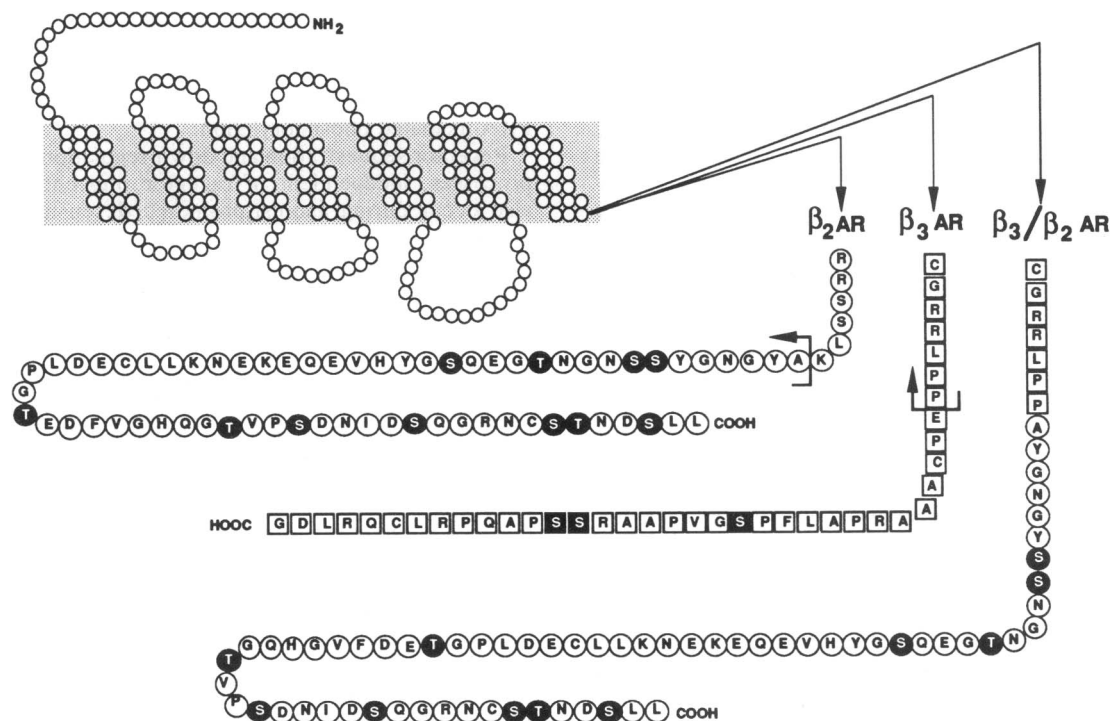


FIG. 1. Primary amino acid sequences of the C-terminal regions of the human  $\beta_2$ AR,  $\beta_3$ AR, and the chimeric  $\beta_3/\beta_2$ AR. Darkened residues indicate potential phosphorylation sites for  $\beta$ ARK.

**RESULTS**

On the basis of the marked differences between the amino acid sequences of the  $\beta_2$ AR and the  $\beta_3$ AR in regions thought to be important for agonist-promoted regulation (Fig. 1), we expressed these two receptors as well as a chimeric  $\beta_3/\beta_2$ AR in CHW cells and examined the mechanisms of receptor regulation after exposure to the full agonist isoproterenol. When expressed in CHW cells, all three of these receptors were functionally capable of coupling to  $G_s$  and stimulating adenylyl cyclase activity. The rank order of potency for agonists for stimulating adenylyl cyclase was isoproterenol > BRL 37344 > epinephrine > norepinephrine as expected for the  $\beta_2$ AR, and was isoproterenol = BRL 37344 > norepinephrine > epinephrine for the  $\beta_3$ AR as recently described (20). The chimeric  $\beta_3/\beta_2$ AR exhibited the same rank order of potency for agonists for stimulating adenylyl cyclase as did the wild-type  $\beta_3$ AR (data not shown). In membranes from these cells, basal and maximal isoproterenol-stimulated adenylyl cyclase activities, respectively, were as follows, in pmol/min per mg of protein:  $\beta_2$ AR,  $4.7 \pm 1.0$  and  $16.3 \pm 2.5$ ;  $\beta_3$ AR,  $5.9 \pm 1.9$  and  $12.3 \pm 3.3$ ; and chimeric  $\beta_3/\beta_2$ AR,  $3.2 \pm 1.0$  and  $18.5 \pm 3.7$  ( $n = 4$ , mean  $\pm$  SEM).

Fig. 2 shows agonist-stimulated adenylyl cyclase activities in membranes prepared from cells expressing the  $\beta_2$ AR

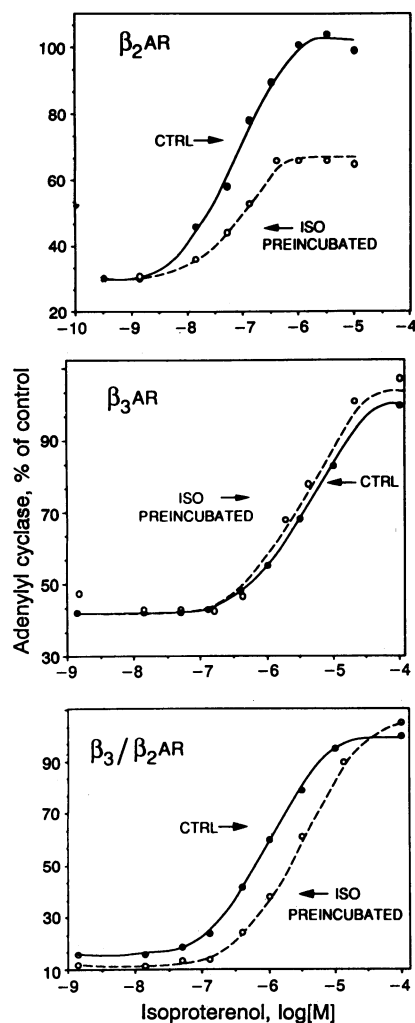


FIG. 2. Effects of short-term agonist exposure on  $\beta$ AR subtype function. Cells expressing  $\beta_2$ AR,  $\beta_3$ AR, or the chimeric  $\beta_3/\beta_2$ AR were exposed to  $100 \mu\text{M}$  isoproterenol (ISO) or medium alone (CTRL) for 30 min and washed, and membrane adenylyl cyclase activities were determined. Data shown are the means of four experiments.

preincubated with medium alone or medium with  $100 \mu\text{M}$  isoproterenol. The  $\beta_2$ AR showed a marked desensitization after a 30-min exposure to agonist. This was manifested by both an  $\approx 3$ -fold increase in the  $\text{EC}_{50}$  for isoproterenol ( $15.1 \pm 3.3$  to  $53.3 \pm 5 \text{ nM}$ ,  $P < 0.01$ ) and an  $\approx 25\%$  decrease in the maximal stimulation. In marked contrast, no desensitization of the  $\beta_3$ AR was found under the same conditions. As shown in Fig. 2, no significant changes in the  $\text{EC}_{50}$  for isoproterenol ( $4.3 \pm 0.6$  to  $2.2 \pm 0.6 \mu\text{M}$ ,  $P = \text{not significant}$ ) or in the maximal adenylyl cyclase activity were noted after cells expressing the  $\beta_3$ AR were exposed to agonist under the same conditions that provided for desensitization of the  $\beta_2$ AR.

To assign molecular determinants to such a difference in agonist-promoted desensitization patterns, we constructed a chimeric  $\beta_3/\beta_2$ AR that has the  $\beta_2$ AR cytoplasmic tail substituted into the  $\beta_3$ AR at the position indicated in Fig. 1. Thus, the putative phosphorylation sites for  $\beta$ ARK, the kinase involved in the homologous form of  $\beta_2$ AR desensitization, were present in this chimera. Note that the potential sites of phosphorylation by PKA, the mediator of the heterologous form of desensitization of the  $\beta_2$ AR, were not added to the chimeric  $\beta_3/\beta_2$ AR. Fig. 2 shows the results of adenylyl cyclase studies with membranes from cells expressing the chimeric  $\beta_3/\beta_2$ AR exposed to  $100 \mu\text{M}$  isoproterenol for 30 min. In contrast to what was found with the  $\beta_3$ AR, the chimeric  $\beta_3/\beta_2$ AR did display agonist-dependent desensitization, manifested by an increase in the  $\text{EC}_{50}$  for isoproterenol (from  $0.9 \pm 0.3$  to  $3.4 \pm 0.3 \mu\text{M}$ ,  $P < 0.01$ ). This rightward shift in the dose-response curve represents an  $\approx 40\%$  desensitization when one examines the adenylyl cyclase activities at a submaximal concentration ( $1 \mu\text{M}$ ) of isoproterenol (Fig. 2). There was, however, no decrease in the maximal level of stimulation. That the desensitization pattern found with this chimeric receptor is not identical to that of the  $\beta_2$ AR is not altogether surprising, since the chimera does not have the PKA sites found in the  $\beta_2$ AR.

To determine if receptor phosphorylation might in fact be the mechanism by which the  $\beta_2$ AR C-terminal tail conferred desensitization to the  $\beta_3$ AR, each receptor was expressed in COS-7 cells and agonist-promoted receptor phosphorylation was determined. The results of these studies are depicted in Fig. 3. When cells were transfected with vector alone (i.e., pBC12BI) no phosphorylation signal in the molecular weight region of interest was noted. As expected, the  $\beta_2$ AR displayed agonist-dependent phosphorylation, a result that has been previously found with a number of different cell types and conditions (1, 2). However, the  $\beta_3$ AR showed no agonist-dependent phosphorylation, consistent with its lack of agonist-promoted desensitization in the functional assays. Even under conditions where  $\beta$ ARK was overexpressed, no agonist-dependent phosphorylation of the  $\beta_3$ AR could be dem-

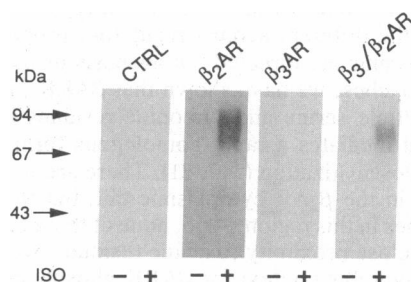


FIG. 3. Agonist-promoted phosphorylation of  $\beta$ AR subtypes. Epitope-tagged  $\beta_2$ AR,  $\beta_3$ AR, and the chimeric  $\beta_3/\beta_2$ AR were transiently expressed in COS-7 cells and whole-cell phosphorylation of receptors in response to agonist exposure was assessed. Shown are the results from a single experiment, representative of four performed. ISO, isoproterenol; CTRL, control transfected with vector alone.

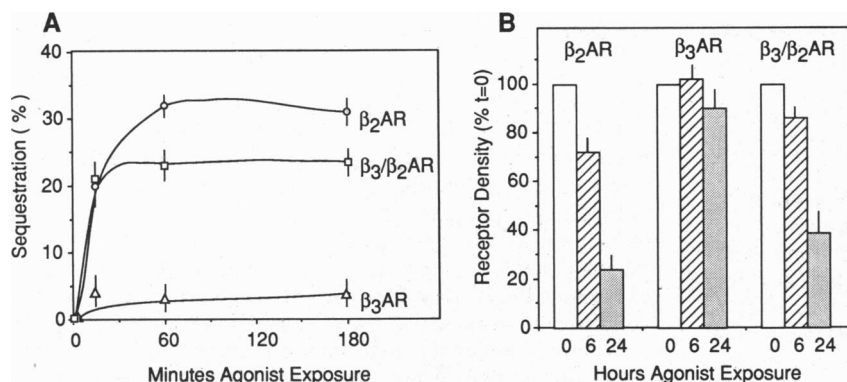


FIG. 4. Agonist-promoted sequestration and down-regulation of  $\beta$ AR subtypes. CHW cells expressing  $\beta_2$ AR,  $\beta_3$ AR, or  $\beta_3/\beta_2$ AR were exposed to 100  $\mu$ M isoproterenol for the indicated times.  $^{125}$ I-CYP binding was measured in whole cells to delineate the degree of sequestered receptors induced by short-term exposures (A) or in membranes to determine the degree of net receptor loss (down-regulation) after long-term agonist exposure (B). Data are from four experiments.

onstrated (data not shown). On the other hand, the chimeric  $\beta_3/\beta_2$ AR clearly showed an agonist-promoted phosphorylation (Fig. 3). This observation is consistent with the idea that it is the phosphorylation sites in the  $\beta_2$ AR C-terminal tail that provide for desensitization in the chimeric  $\beta_3/\beta_2$ AR.

We next examined the potential for each receptor to undergo agonist-promoted sequestration and down-regulation. As shown in Fig. 4A, agonist-promoted sequestration of the  $\beta_2$ AR is evident within minutes of exposure, and is maximal by  $\approx 1$  hr. In contrast, no significant agonist-promoted sequestration of the  $\beta_3$ AR was detected over the same time course. However, the chimeric  $\beta_3/\beta_2$ AR, which contains a region of the  $\beta_2$ AR in the cytoplasmic tail which appears to be important for sequestration, indeed does display agonist-promoted sequestration, with the same time course and to nearly the same maximal extent as the  $\beta_2$ AR (Fig. 4A).

Agonist exposure over more prolonged periods induces a marked down-regulation of  $\beta_2$ AR expression. Given that the  $\beta_3$ AR lacks at least two determinants for this process, we assessed the potential of the  $\beta_3$ AR and the  $\beta_3/\beta_2$ AR chimera to undergo down-regulation after long-term agonist exposure. As shown in Fig. 4B, the  $\beta_2$ AR displays a time-dependent decrease in receptor expression during long-term agonist exposure, with only 24%  $\pm$  5% of receptors remaining after 24 hr of exposure. On the other hand, the  $\beta_3$ AR displayed virtually no such down-regulation. As was the case for the short-term processes, the cytoplasmic tail of the  $\beta_2$ AR was found to confer agonist-promoted down-regulation to the  $\beta_3$ AR. With the chimeric  $\beta_3/\beta_2$ AR, 39%  $\pm$  11% of receptors remained after 24 hr of agonist exposure, although after 6 hr of exposure there was minimal down-regulation noted (Fig. 4B).

## DISCUSSION

On the basis of a number of differences between the primary amino acid sequences of the  $\beta_2$ AR and the  $\beta_3$ AR, we hypothesized that one feature of  $\beta$ AR subtype differentiation may lie in the different agonist regulatory properties of the subtypes. Using site-directed mutagenesis of the  $\beta_2$ AR and other approaches, we have shown that  $\beta$ ARK phosphorylation of multiple serine and threonine residues in the cytoplasmic tail mediates a rapid homologous form of agonist-promoted desensitization (5–7, 21). There are 11 serines and threonines in the  $\beta_2$ AR cytoplasmic tail, but the  $\beta_3$ AR has only 3 serines in this region. Also, none of these serines of the  $\beta_3$ AR is in close proximity to acidic residues, which provide the most favorable context for  $\beta$ ARK phosphorylation (22). Indeed, there are 10 Asp or Glu residues in the human  $\beta_2$ AR C-terminal region, compared with 2 such residues in the same region of the human  $\beta_3$ AR. In addition to the apparent absence of potential  $\beta$ ARK phosphorylation sites, the PKA consensus sequence Arg-Arg-Xaa-Ser (RRXS in single-letter code), which is found twice in the  $\beta_2$ AR, is not present in any intracellular region of the  $\beta_3$ AR. Thus the  $\beta_3$ AR appears to

lack the major determinants for rapid agonist-promoted desensitization.

Another process that occurs during short-term agonist exposure to  $\beta_2$ AR is a movement (within seconds to minutes) of receptors away from the cell surface. The role of sequestration in agonist-promoted regulation of the  $\beta_2$ AR is controversial (23). Sequestration of  $\beta_2$ AR may be responsible for a small component of agonist-promoted desensitization (6, 21, 23) or for resensitization after desensitization (24), or it may participate in down-regulation (23). The molecular determinants for sequestration of the  $\beta_2$ AR are also unclear. A short sequence (SSNGNTGEQS), located within the midregion of the cytoplasmic tail of the  $\beta_2$ AR, has been identified as being potentially important for agonist-promoted sequestration (12), but it is likely that other determinants participate as well. The  $\beta_3$ AR does not have any region in the cytoplasmic tail with homology to this  $\beta_2$ AR sequence motif.

After long-term agonist exposure, marked down-regulation of receptor expression is observed. Several mechanisms have been shown to be important for agonist-promoted  $\beta_2$ AR down-regulation, including those operating at the levels of transcription, mRNA stability, and protein degradation (reviewed in refs. 1 and 2). At the receptor level, Valiquette *et al.* (14) have shown that tyrosine residues at amino acid positions 350 and 354 in the cytoplasmic tail of the  $\beta_2$ AR appear to play roles in conferring agonist-promoted down-regulation. When these were replaced by alanines, the mutated  $\beta_2$ AR underwent  $\approx 50\%$  less down-regulation as compared with the wild type (14). The third intracellular loop PKA consensus sequence, located in the  $\beta_2$ AR, also appears to be required for full agonist-promoted down-regulation (13, 25). Alteration of this site depressed agonist-promoted down-regulation by  $\approx 50\%$  (13). With respect to these determinants of long-term agonist-promoted regulation, the  $\beta_3$ AR has no tyrosine residues in the cytoplasmic tail, and as stated earlier, no PKA consensus sequences.

Our results suggest that one level of significance of  $\beta$ AR subtypes reside in their potential for differing patterns of agonist-promoted regulation. In terms of short-term regulation, we have shown that the  $\beta_3$ AR is relatively refractory to agonist-promoted desensitization, a result consistent with its paucity of potential phosphorylation sites. Whole-cell phosphorylation studies failed to show any agonist-dependent phosphorylation of the  $\beta_3$ AR during agonist exposure, under the same conditions where the  $\beta_2$ AR undergoes a distinct agonist-dependent phosphorylation (Fig. 3). It should be noted that no agonist-dependent  $\beta_3$ AR phosphorylation could be shown even under conditions of overexpression of  $\beta$ ARK. Our findings of absent agonist-promoted desensitization and phosphorylation of recombinant human  $\beta_3$ AR are consistent with recent data from Granneman (26) which show a lack of desensitization of  $\beta_3$ AR-mediated lipolysis in rat white adipose tissue. Moreover, we were able to confer such desensitization to the  $\beta_3$ AR by substituting the serine- and threonine-rich C-terminal tail of the  $\beta_2$ AR. Consistent with the idea

that this domain conferred desensitization by providing phosphorylation sites is our observation of agonist-dependent phosphorylation of the chimeric  $\beta_3/\beta_2$ AR.

Two other  $\beta_2$ AR agonist-promoted regulatory events, which are independent of receptor phosphorylation, are also absent from the  $\beta_3$ AR but could be conferred by substitution of the  $\beta_2$ AR cytoplasmic tail. Agonist-promoted sequestration did not occur with the  $\beta_3$ AR, yet was nearly fully restored in the  $\beta_3/\beta_2$ AR chimera. Similarly, long-term agonist exposure failed to significantly reduce  $\beta_3$ AR cellular expression, while the  $\beta_2$ AR underwent  $\approx 75\%$  down-regulation under the same conditions. Substitution of the cytoplasmic tail of the  $\beta_2$ AR conferred down-regulation to the  $\beta_3$ AR, as demonstrated with the  $\beta_3/\beta_2$ AR chimera. The mechanisms and molecular determinants for agonist-promoted sequestration and down-regulation of the  $\beta_2$ AR are less well defined as compared with those of short-term desensitization, which has been shown to be mediated primarily by receptor phosphorylation. Thus the manner in which the C-terminal tail of the  $\beta_2$ AR conferred sequestration and down-regulation to the  $\beta_3$ AR is not clear at present.

In terms of down-regulation, tyrosine residues in the cytoplasmic tail, as discussed above, may represent one determinant of the process. However, it is not surprising that the substitution of the  $\beta_2$ AR tail alone does not confer the capacity for full long-term down-regulation since, at a minimum, the third intracellular loop PKA sites have also been shown to be important in this process (13). It should be noted that in these studies we are utilizing recombinant receptors whose expression in CHW cells is driven by elements within our expression vector. It is thus not possible to address other factors, such as those that may affect transcription via elements within the natural promoters of these receptors, in the current study of long-term agonist regulation. Studies by several groups using naturally expressed  $\beta_3$ AR have addressed this, with various results (18, 27). Nevertheless, the striking lack of down-regulation of the  $\beta_3$ AR as compared with the  $\beta_2$ AR, under identical conditions utilizing the same expression vector, points to receptor subtype-specific regulation.

We have previously identified, by site-directed mutagenesis, some of the molecular determinants of agonist-mediated regulation of the  $\beta_2$ AR (5–7, 12–14, 24, 25). Here we have taken advantage of the opportunity offered by the  $\beta_3$ AR to test the generality of the importance of these C-terminal regions in imparting agonist-promoted regulation, by examining the properties of a native  $\beta$ AR which lacks several of these elements. The lack of homologous motifs in the  $\beta_3$ AR, the lack of short- and long-term agonist-promoted regulation of the  $\beta_3$ AR, and the demonstration of such regulation in the chimeric  $\beta_3/\beta_2$ AR are all consistent with our proposed molecular basis for subtype-specific  $\beta$ AR desensitization. The fact that the chimeric  $\beta_3/\beta_2$ AR containing the C-terminal tail of the  $\beta_2$ AR demonstrated all of the regulatory phenomena of the  $\beta_2$ AR, albeit not completely, further confirms the importance of the receptors' C-terminal domains in directing agonist-promoted regulation.

That receptor subtypes may importantly differ in the ways in which they are regulated by agonists may be a feature common to numerous other receptor subtypes. Indeed, few differences in coupling to G proteins or in binding to epinephrine and norepinephrine have been noted to date among the various  $\alpha_1$ - or  $\alpha_2$ -adrenergic receptor subtypes. Thus, perhaps the specific tissue distributions of adrenergic receptor subtypes are based in part on varying requirements for agonist-mediated regulation. The  $\beta_3$ AR is presumably localized primarily to adipose tissue in humans and provides for catecholamine-mediated lipolysis. Thus, perhaps the lack of desensitization of this receptor during continuous agonist

exposure may fulfill the need for a sustained output of energy substrates during prolonged increased sympathetic activity.

We thank Brian Kobilka and Sam Mostafapour for epitope tagging the  $\beta_3$ AR and the chimera. This work was supported in part by National Institutes of Health Grants HL45967 (S.B.L.), HL02490 (D.A.S.), and HL16037 (R.J.L.).

- Liggett, S. B. & Raymond, J. R. (1993) in *Catecholamines*, ed. Bouloux, P. M. (Saunders, London), in press.
- Liggett, S. B. & Lefkowitz, R. J. (1993) in *Regulation of Cellular Signal Transduction Pathways by Desensitization and Amplification*, eds. Sibley, D. D. & Houslay, M. (Wiley, New York), in press.
- Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240**, 1310–1316.
- Frielle, T., Daniel, K. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9494–9498.
- Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G. & Lefkowitz, R. J. (1988) *Nature (London)* **333**, 370–373.
- Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G. & Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264**, 12657–12665.
- Liggett, S. B., Bouvier, M., Hausdorff, W. P., O'Dowd, B., Caron, M. G. & Lefkowitz, R. J. (1989) *Mol. Pharmacol.* **36**, 641–646.
- Clark, R. B., Friedman, J., Dixon, R. A. & Strader, C. D. (1989) *Mol. Pharmacol.* **36**, 343–348.
- Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Krancke, U., Caron, M. G. & Lefkowitz, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 46–50.
- Frielle, T., Collins, S., Daniel, K. W., Caron, M. G., Lefkowitz, R. J. & Kobilka, B. K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7920–7924.
- Emorine, L. J., Marullo, S., Briand-Sutren, M., Patey, G., Tate, K., Delavier-Klutchko, C. & Strosberg, A. D. (1989) *Science* **245**, 1118–1121.
- Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G. & Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2979–2983.
- Bouvier, M., Collins, S., O'Dowd, B. F., Campbell, P. T., De Blasi, A., Kobilka, B. K., MacGregor, C., Irons, G. P., Caron, M. G. & Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264**, 16786–16792.
- Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5089–5093.
- Cullen, B. (1987) *Methods Enzymol.* **152**, 684–704.
- Nimar, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M. & Lerner, R. A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4949–4953.
- Benovic, J. L., De Blasi, A., Stone, W. C., Caron, M. G. & Lefkowitz, R. J. (1989) *Science* **246**, 235–240.
- Thomas, R. F., Holt, B. D., Schwinn, D. A. & Liggett, S. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4490–4494.
- Liggett, S. B., Ostrowski, J., Chesnute, L. C., Kurose, H., Raymond, J. R., Caron, M. G. & Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 4740–4746.
- Liggett, S. B. (1992) *Mol. Pharmacol.* **42**, 634–637.
- Lohse, M. J., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1990) *J. Biol. Chem.* **265**, 3202–3209.
- Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J. & Benovic, J. L. (1991) *Biochemistry* **30**, 5118–5125.
- Perkins, J. P., Hausdorff, W. P. & Lefkowitz, R. J. (1991) *Mechanisms of Ligand-Induced Desensitization of  $\beta$ -Adrenergic Receptors*, ed. Perkins, J. P. (Humana, Clifton, NJ), pp. 125–180.
- Yu, S. S., Lefkowitz, R. J. & Hausdorff, W. P. (1993) *J. Biol. Chem.* **268**, 337–341.
- Campbell, P. T., Hnatowich, M., O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J. & Hausdorff, W. P. (1990) *Mol. Pharmacol.* **39**, 192–198.
- Granneman, J. G. (1992) *J. Pharmacol. Exp. Ther.* **261**, 638–642.
- Granneman, J. G. & Lahners, K. N. (1992) *Endocrinology* **130**, 109–114.