The $\alpha_2A/$$\alpha_2C$-adrenergic receptor (AR) undergoes rapid agonist-promoted desensitization due to phosphorylation by G protein-coupled receptor kinases (GRKs) 2 and 3 at serines in the third intracellular loop of the receptor. In contrast, the $\alpha_2A$/AR fails to display such desensitization or phosphorylation, which has been presumed to be due to this receptor lacking GRK phosphorylation sites. However, the $\alpha_2C$/AR has multiple serines and threonines in putative favorable motifs within its third intracellular loop. We considered that the conformation of the third intracellular loop imposed by agonists binding to the transmembrane-spanning domains could be the basis of this subtype-specific property, rather than the presence or absence of phosphoacceptors per se. To address this, $\alpha_2A$/AR third loop chimeric receptors were constructed. In whole cell phosphorylation studies, the $\alpha_2A$ with the $\alpha_2C$ third loop receptor underwent agonist-promoted phosphorylation while the $\alpha_2C$ with the $\alpha_2A$ third loop receptor did not, indicating that the agonist interaction with the parent receptor backbone establishes the phosphorylation phenotype. We postulated then that agonists with diverse structures that distinctly interact with $\alpha_2A$/AR should display different degrees of phosphorylation independent of receptor activation. Indeed, several full and partial agonists were identified, which evoked phosphorylation that was not related to intrinsic activity as established by $[^{35}S]$guanosine 5'-O-(thio)triphosphate binding. Taken together, it appears that phosphorylation of the $\alpha_2A$/AR evoked by agonist is highly sensitive to the conformation of the third intracellular loop induced/stabilized by agonist to such an extent that these properties dictate the extent of phosphorylation of the loop when phosphoacceptors are present, and are the basis for subtype-specific phosphorylation.

Desensitization, defined as a waning of a signal despite the presence of stimulus, is a common biological phenomenon. Many receptors that signal through guanine nucleotide-binding proteins (G proteins) display desensitization in the presence of continuous agonist exposure (1). Such regulation of receptor function is key to the cell being able to integrate the myriad of received signals in order to maintain homeostasis under a variety of physiologic and pathologic conditions. Receptor desensitization can be adaptive or maladaptive within the context of disease (2). Additionally, the process can lead to clinical tachyphylaxis, which limits the therapeutic effectiveness of administered agonists (1).

The most rapid form of desensitization of G protein-coupled receptors is due to phosphorylation, leading to a decrease in coupling of the receptor to its cognate G protein. A family of serine/threonine protein kinases, termed G protein-coupled receptor kinases (GRKs), serves to phosphorylate the agonist-occupied forms of these receptors, and are the kinases responsible for agonist-promoted (homologous) desensitization (3, 4). Within the adrenergic receptor family, the $\beta_2$/adrenergic ($\beta_2$/AR) and $\alpha_2A$-adrenergic ($\alpha_2A$/AR) receptors have been extensively studied in regards to GRK-mediated phosphorylation and desensitization. For the $\beta_2$/AR, serines and threonines in the carboxyl-terminal tail of the receptor have been shown to be phosphorylated by one or more GRKs (5, 6). For the $\alpha_2A$/AR, four serines in the mid-portion of the third intracellular loop within the EESSSS motif, are the residues phosphorylated (7, 8). For both receptors, the critical step to initiation of GRK-mediated phosphorylation is the attainment of the agonist-occupied or stabilized conformation, also termed the “active” conformation. Indeed, in the current model of GRK-mediated phosphorylation, the optimal conformation required for G protein coupling is considered the same as that required for GRK-mediated phosphorylation (3, 4). Partial agonists, then, evoke receptor phosphorylation of the $\beta_2$/AR in direct proportion to their intrinsic activities (9). The $\alpha_2$/ARs consist of three receptor subtypes, denoted $\alpha_2A$, $\alpha_2B$, and $\alpha_2C$. Each couple to the inhibitory G protein $\zeta$, and thus serve to decrease adenylyl cyclase and intracellular cAMP levels in the cell (10). While all three subtypes display similar G coupling, they differ in their propensity to undergo agonist-promoted phosphorylation and desensitization (11). We and others have shown in recombinant expressing cells that while $\alpha_2A$ and $\alpha_2B$ receptors undergo short term agonist-promoted phosphorylation and desensitization, the $\alpha_2C$/receptor does not (12–14). This does not appear to be due to the lack of cellular expression of a particular GRK, as overexpression of GRKs 2, 3, 5, and 6 fail to evoke agonist-promoted phosphorylation of the $\alpha_2C$/AR (14). Comparison of the sequences of the third intracellular loops of the $\alpha_2A$ and $\alpha_2C$/receptors (Fig. 1) shows that both have a number of serines and threonines in similar locations. While the $\alpha_2C$/does not have the $\alpha_2A$/sequence EESSSS, which has been shown in vivo (15) and in

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vi tro (16) to be an excellent substrate for GRK phosphorylation, it does not have DESS within the mid-section of the loop. We have considered that while the residues that might be phosphorylated by GRKs are present in both subtypes, the agonist-promoted intracellular milieu might be different between the two subtypes. This could thus be the basis of the differential phosphorylation of the two subtypes by agonist, rather than the presence or absence of phosphorycceptors per se. To address this, chimeric α2ARs were constructed which were composed of the α2A with substitution of the α2C third intracellular loop, and the α2C with substitution of the α2A third intracellular loop. These two chimera, along with the wild-type α2A and α2C receptors, were expressed in cells and agonist-promoted phosphorylation and desensitization studies undertaken.

EXPERIMENTAL PROCEDURES

Constructs and Mutagenesis—The wild-type human α2AAR and α2C-AR eDNAs were subcloned into the expression vector pCDNA1 modified to contain in-frame the sequence encoding the hemagglutinin epitope tag at the amino terminus. To construct the chimeric receptors, site-directed mutagenesis (17) was carried out on each wild-type template to create the restriction endonuclease sites NheI (transmembrane domain 5) and BamHI (carboxyl terminus of the third intracellular loop) to afford exchange of fragments encompassing the third loop (Fig. 1). The former reactions were also designed so as to maintain α2A or α2C sequence in the transmembrane domains of the chimeras. Thus the chimera consisted of α2AR with the α2AR third intracellular loop, denoted α2AR(3LC), and α2AR with the α2AR third intracellular loop, denoted α2AR(3LC).

Transfection and Cell Culture—Chinese hamster fibroblasts (CHW-1102 cells) were permanently transfected by a calcium phosphate precipitation technique as described (15). Individual clonal lines were 1102 cells) were permanently transfected by a calcium phosphate pre-
spanning segments. The results from competition studies with the agonist epinephrine, however, revealed evidence of retrograde conformational effects of substituting the α2AR third intracellular loop into the α2AAR. As shown in Table I, the $K_i$ for wild-type α2AAR for epinephrine was $347 \pm 46 \text{nM}$. Substitution of the α2CAR loop resulted in a decrease in affinity, with a $K_i$ of $1054 \pm 52 \text{nM}$ for the α2AAR(3LC) receptor. The α2C(3LA) chimera had an epinephrine binding affinity that was not different than wild-type α2CAR (~125 nM). A similar pattern was found with the agonist UK14304, except that only a 2-fold increase in $K_i$ occurred with the α2C(3LC) receptor (data not shown). The functional integrity of the receptors was determined in adenylyl cyclase assays, assessing the inhibition of forskolin-stimulated activity by epinephrine. Each chimeric receptor inhibited adenylyl cyclase by >65%, (Table II, (−) agonist column). Interestingly, the EC$_{50}$ for epinephrine with the α2C(3LC) was lower than that for the other receptors.

The above studies indicated that each of the chimeric receptors bound agonist with high affinity and functionally coupled to the inhibition of adenylyl cyclase. Whole cell receptor phosphorylation studies were then undertaken to test our hypotheses concerning agonist-promoted phosphorylation. Cells expressing each hemagglutinin-tagged receptor were incubated with $[^{32}P]$

phosphate, treated with vehicle or the agonist epinephrine, and receptor-purified by immunoprecipitation using an epitope tag-specific antibody. Results of these studies are shown in Fig. 2. We obtained results similar to those that we previously reported for the two wild-type α2AAR and α2CAR (see Introduction). Thus, the α2AAR underwent agonist-promoted phosphorylation while the α2CAR did not. However, substitution of the α2CAR third intracellular loop into the α2AAR resulted in a receptor that underwent agonist-promoted phosphorylation equivalent to that of wild-type α2AAR. This strongly indicates that the third loop of the α2AR has residues that can be phosphorylated, but that the intracellular milieu of the α2C outside of the third loop and/or the conformational changes in the loop, induced by agonist binding within the pocket established by the transmembrane-spanning domains, do not favor phosphorylation. In either case, one might predict that the α2AAR third loop would not undergo phosphorylation within the context of the α2CAR. This turned out to be so, as in parallel studies the α2C(3LC) receptor failed to undergo agonist-promoted phosphorylation (Fig. 2).

We next considered whether these gain/loss phosphorylation phenotypes were also evident in functional (inhibition of adenylyl cyclase) desensitization studies. Cells in culture were exposed to vehicle or epinephrine, washed, membranes prepared, and agonist-mediated inhibition of forskolin-stimulated adenylyl cyclase activities determined. These results are shown in Table II and Fig. 3. We have previously shown in recombinantly expressing CHW and Chinese hamster ovary cells that α2AAR undergo such desensitization, manifested by a 4–5-fold increase in the EC$_{50}$ with little or no significant change in the maximal extent of adenylyl cyclase inhibition (12). On the other hand, desensitization of α2CAR in Chinese hamster ovary cells is not observed under identical conditions. As shown, these phenotypes are also true in recombinantly expressing CHW cells. Of note, despite the fact that phosphorylation occurs with the α2AAR(3LC) receptor, no agonist-promoted desensitization of this receptor was detected (Fig. 3C). Additionally, substitution of the α2AAR third loop into the α2CAR had no effect on the latter’s lack of functional desensitization (Fig. 3D).

The α2A and α2C receptors have highly similar first and second intracellular loops. The major difference in the short cytoplasmic tail is the lack of a palmitoylated cysteine of α2CAR. We have previously shown that this difference in the tail does not alter agonist-promoted desensitization (20). We thus considered that the conformation established by the transmembrane-spanning backbone of the parent receptor dictates phosphorylation status of the third intracellular loop. This hypothesis was further explored using agonists with substantial differences in structure. Since the extent of GRK2-mediated phosphorylation of the β2AR has been shown to be directly related to the agonist intrinsic activity (9), we first delineated the intrinsic activities of each agonist employed by measuring $[^{35}S]$GTPγS binding. The agents ranged from being full ago-

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**Table II**

**Functional coupling and desensitization of wild-type and chimeric α2ARs.** Cells were exposed to carrier or 10 μM epinephrine for 15 min, washed, membranes prepared, and adenylyl cyclase activities determined as described under “Experimental Procedures.” Results are from four to six independent experiments. *, $p < 0.01$ compared to untreated values.

| Agonist exposure | α2A | α2A(3LC) | α2C | α2C(3LC) |
|------------------|-----|---------|-----|---------|
| EC$_{50}$ (nM)   | (−) | (+)     | (−) | (+)     |
| 2.1 ± 0.3        | 9.7 ± 1.3* | 5.8 ± 0.7 | 9.8 ± 2.4 | 4.4 ± 0.8 |
| EC$_{50}$ (fold change) | 4.63* | 1.68 | 1.03 | 2.14 |
| Inhibition (%)   | 81 ± 3 | 94 ± 3 | 102 ± 5 | 97 ± 5 |

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**Fig. 2.** Agonist-promoted phosphorylation of wild-type and chimeric α2ARs. In A, a representative autoradiogram from a whole cell phosphorylation experiment is shown. In B, mean results from four independent experiments are shown. For the α2C and α2A(3LC) receptors, no receptor band was identified under basal or agonist-exposed conditions; thus, no quantitation is shown.
nists to weak partial agonists, with the lowest intrinsic activity being 0.06. For para-aminoclonidine, SKF43315, BHT920, and oxymetazoline, the extent of agonist-promoted phosphorylation appeared to be related to the intrinsic activity of the agonist (Fig. 4). On the other hand, the $\alpha_2$AR agonist SKF89748, which displays weak partial agonist activity at the $\alpha_2A$AR (intrinsic activity $= 0.06$), evoked phosphorylation that was greater than the phosphorylation evoked by oxymetazoline, which has an intrinsic activity of 0.30. Additionally, UK14304, which was a full agonist, failed to promote phosphorylation to an extent similar to the other full agonist para-aminoclonidine, or the other near full agonists (Fig. 4). Taken together, it appears that phosphorylation of the $\alpha_2$AR evoked by agonist is highly sensitive to the conformation of the third intracellular loop induced/stabilized by agonist to such an extent that these properties dictate the extent of phosphorylation of the loop when phosphoacceptors are present, and are the basis for subtype-specific phosphorylation.

**DISCUSSION**

Although short term agonist-promoted desensitization has been observed with many G protein-coupled receptors, about one-third of those tested appear not to display this regulatory behavior. One might consider, then, that certain cellular events controlled by some receptors are so critical that even under circumstances of prolonged agonist exposure receptor function is maintained.

For the three $\alpha_2$AR subtypes, initial studies revealed similar binding affinities for endogenous catecholamines and similar functional coupling to the inhibition of adenylyl cyclase via $G_i$ coupling. Subsequent studies have revealed important differences between the subtypes in regards to coupling to $G_s$ (10), stimulation of intracellular calcium (21), membrane insertion and trafficking (22), agonist-promoted down-regulation (12), and agonist-promoted phosphorylation and desensitization (12–14). Desensitization of the $\alpha_2$AR was found to be the most robust of the three subtypes, followed by the $\alpha_{2B}$AR (12). The $\alpha_{2C}$AR failed to display functional desensitization (12) or phosphorylation (13) due to agonist exposure in recombinant cells. Site-directed mutagenesis of the human $\alpha_{2A}$AR has shown that agonist-promoted phosphorylation is confined to the third intracellular loop (7) at four serines (15) (residues 296–299, see Fig. 1). Each serine contributes $\approx 25\%$ of the total phosphorylation. However, all four serines must be phosphorylated for agonist-promoted functional desensitization to occur (15). In these studies, the finding that partial phosphorylation did not cause "partial" desensitization is consistent with the notion that the non-visual arrestins have strict requirements for binding to GRK-phosphorylated substrates leading to decreases in coupling to $G_i$. This notion supports the findings of the current work, where we observed phosphorylation of $\alpha_{2A}(3L_C)$, but a
failure of this receptor to display agonist-promoted desensitization. Presumably, the third loop conformation attained is adequate for GRK phosphorylation but not subsequent arrestin binding.

Using the current approach, it might be expected that substitution of the α2A third loop into the α2C receptor would confer phosphorylation, based on the assumption that the primary structure of this loop imparts the relevant features for the process. Similarly, substitution of the α2A third intracellular loop into the α2A would be expected to result in a receptor that failed to undergo agonist-promoted phosphorylation. Instead, we found that the presence or absence of phosphorylation was dependent on the subtype backbone rather than the third intracellular loop. The fact that agonists with different structures promoted markedly divergent degrees of phosphorylation of α2AR (which was unrelated to intrinsic activity) supports the notion that the conformation stabilized by agonist is highly variable and has an important impact on third loop phosphorylation. Interestingly, although we observed these unexpected gain and loss phenotypes, and the differential phosphorylation of α2AR by various agonists, we failed to observe phosphorylation of α2C-AR due to exposure to any agonist tested (data not shown). Thus, although the ligand binding pocket of the α2AR appears to be amenable to modification based on agonist structure, a similar scenario may not exist for α2C-AR. However, since the highly phosphorylatable α2A-AR third intracellular loop fails to undergo phosphorylation in the context of the α2C-AR, the importance of residues outside of the loop is nonetheless clear.

These results support the concept that agonists can stabilize receptor conformation in highly specific ways. This is illustrated in Fig. 5. Here, agonist 1 is shown stabilizing a receptor in a specific R*4 conformation. Such a conformation may allow for effective coupling to G protein, but not for phosphorylation, or some other process. These processes might include other events that are agonist-promoted, such as coupling to a second G protein, internalization, or down-regulation. In this model, other conformations, such as R*2-R*5, might have such properties, and depending on the agonist, specific conformations and thus pathways or properties can be realized. Indeed, we have previously shown that α2AR coupling to Gs (a somewhat inefficient process) is highly dependent on agonist structure (23). For drug development, this is particularly important in that it appears to be possible to design agonists with sufficient intrinsic activities that fail to undergo desensitization. Our findings also suggest that it is overly simplistic to consider agonist-based function to be strictly based on a “universal” active conformation.

In summary, using chimeric-mutagenesis, we have shown that the third intracellular loops of both the α2A-AR and α2C-AR have residues that can be phosphorylated during short term agonist exposure. However, such phosphorylation is present only in the context of the α2A-AR, and is indeed absent when these loops are in the context of the α2C-AR backbone. These results indicate that the subtype ligand binding pockets, which direct third loop conformational changes, have a major influence on subtype-specific phosphorylation of the α2AR.

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α2A/α2C-Adrenergic Receptor Third Loop Chimera Show That Agonist Interaction with Receptor Subtype Backbone Establishes G Protein-coupled Receptor Kinase Phosphorylation

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