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Analysis of Domain Responsible for Desensitization of β_1-Adrenergic Receptor

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ABSTRACT—When the wild type β_1-adrenergic receptor (WT-β_1AR) was expressed in Sf9 cells, the β_1AR-stimulated adenylyl cyclase activities were desensitized by prior treatment with isoproterenol. The extent of β_1AR desensitization was not modified, and the onset was not promoted by the overexpression of G protein-coupled receptor kinase 2 (GRK2), GRK5 or GRK6. However, overexpression of the dominant negative mutant of GRK2 appeared to inhibit desensitization of the β_1AR. The change of the potential protein kinase A phosphorylation site located at the intracellular third loop did not affect β_1AR desensitization. Desensitization of the truncated mutant, in which nearly all of the serine and threonine residues from the carboxyl terminus were eliminated, was the same as that of the WT-β_1AR. A deletion mutant that lacked serine and threonine residues of the intracellualr third loop was also desensitized by isoproterenol stimulation. Furthermore, the deletion of serine and threonine residues from both the intracellular third loop and carboxyl terminus did not affect desensitization of the β_1AR. These results suggested that phosphorylation by endogenous GRKs in Sf9 cells contributed to desensitization of the β_1AR and that the regions other than third intracellular loop and carboxyl terminus may be responsible for β_1AR desensitization.

Keywords: β_1-Adrenergic receptor, Sf9 cell, G protein-coupled receptor kinase, Protein kinase A, Desensitization

The β-adrenergic receptors (βARs) belong to members of the superfamily of G protein-coupled receptors and consist of three subtypes (β_1, β_2, β_3) (1). The β_1- and β_2AR-mediated responses are decreased by the continuous presence of agonist (2). This phenomenon is referred to as desensitization. In contast to the β_1- and β_2ARs, the β_3AR is proposed to be resistant to desensitization (3). The mechanism of desensitization of the β_2AR has been characterized at the molecular level as a model receptor (2). Within seconds to minutes of agonist stimulation, the β_2AR is uncoupled from stimulatory heterotrimeric GTP-binding protein (G_s) by phosphorylation of the β_2AR and subsequent binding of β-arrestins to the phosphorylated β_2AR (short-term desensitization). By analyzing the mutated β_2ARs, it was proposed that cAMP dependent protein kinase (PKA) and second messenger-independent G protein-coupled receptor kinases (GRKs) are involved in phosphorylation and desensitization of the β_2AR. PKA is selectively activated by low concentration of agonists and phosphorylates the β_2AR at specific serine residue on the third intracellular loop, as the β_2AR mutant that lacked the consensus PKA site is not desensitized at low concentration of agonists (4, 5). The coupling of the β_2AR phosphorylated by PKA to G_s is impaired without any help of other accessary proteins, as the purified and PKA-phosphorylated β_2AR loses the coupling to G_s in the in vitro reconstitution system (6). At high concentration of agonists, the β_2AR is phosphorylated by both PKA and GRKs (4, 5). It is believed that GRK can phosphorylate an agonist-occupied form of the β_2AR at its carboxyl terminus (2). When these sites are phosphorylated, the β_2AR binds to the β-arrestins, which can cause uncoupling of the phosphorylated β_2AR from G_s. Actually, desensitization is inhibited in the truncated β_2AR at the carboxyl terminus or the mutated β_2AR in which serine and threonine residues at carboxyl terminus were changed to alanine or glycine residues (7).

In contrast to the β_2AR, the mechanism of desensitization of the β_1AR is not clearly understood. The β_1AR possesses one PKA phosphorylation site in the intracellu-

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lar third loop and ten phosphorylatable serine and threonine residues in the carboxyl terminus (8). It was reported that desensitization of the β_1AR involves phosphorylation only by PKA but not by GRKs. In human SK-N-MC cells in which the β_1AR is endogenously expressed, the PKA inhibitor PKI but not the GRK2 inhibitor heparin can block agonist-induced β_1AR desensitization (9). Freedman et al. (10) recently reported that phosphorylation by both PKA and GRKs can involve β_1AR desensitization. They transiently expressed the β_1AR in HEK293 cells and showed that the maximal cAMP production by isoproterenol stimulation is decreased by preincubation with isoproterenol and is further decreased by coexpression of various GRKs. Their data suggested that phosphorylation of the β_1AR by GRKs also plays an important role in the desensitization process.

In the present study, we examined the effects of PKA, GRK2, GRK5 and GRK6 on desensitization of the β_1AR. We constructed the β_1AR mutants that lacked the PKA phosphorylation site and/or potential GRK phosphorylation sites at the carboxyl terminus and expressed them in Sf9 cells. We showed that the carboxyl terminus of the β_1AR was not a major determinant of desensitization in Sf9 cells.

MATERIALS AND METHODS

Materials

[α-^{32}P]ATP (approx. 25 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) and [^{3}H]cAMP (30–50 Ci/mmol), from Moravek Biochemicals, Inc. (Brea, CA, USA). (−)-Isoproterenol·hydrochloride and forskolin were purchased from Sigma (St. Louis, MO, USA); *Pfu* DNA polymerase, from Stratagene (La Jolla, CA, USA); and BaculoGold™ linearized baculovirus DNA, from PharMingen (San Diego, CA, USA). Grace's insect cell culture medium, lactoalbumin hydrolysate and yeastolate solution were from Gibco BRL (Grand Island, NY, USA). A recombinant baculovirus containing GRK2 was a gift from Dr. T. Haga (University of Tokyo, Tokyo) and wild type (WT) *Spodopetra frugipera* (Sf9) cells from Dr. T. Katada (University of Tokyo). The plasmids encoding the human β_1AR (pBC-β_1), rat GRK2 and dominant negative rat GRK2 (K220A-GRK2) were generous gifts from Dr. R.J. Lefkowitz (Duke University, NC, USA). The plasmids encoding human GRK5 and GRK6 were provided by Dr. J.L. Benovic (Thomas Jefferson University, PA, USA).

DNA constructions, preparation of recombinant virus and cell culture

The PCR technique was used for constructing the β_1AR mutants and dominant negative GRK2 modified at the carboxyl terminus (11). Appropriate mutations were confirmed by sequencing by the dideoxy termination method (12). The constructs of the WT- and mutated β_1ARs, GRK5 and GRK6 were subcloned into baculovirus transfer vector pVL1392 (13). To produce the recombinant baculoviruses, Sf9 cells were cotransfected with 2.5 μg of one of the resulting transfer vectors and 0.5 μg of BaculoGold™ linearized baculovirus DNA by the calcium phosphate precipitation method. Sf9 cells were grown as monolayers in Graces insect cell culture medium supplemented with 10% fetal calf serum, lactoalubumin hydrolysate, yeastolate solution and gentamicin (15 μg/ml) at 27℃ (13). The produced recombinant baculoviruses were amplified by infecting a fresh monolayer of Sf9 cells.

Expression and membrane preparation

The WT- and mutated β_1ARs and various GRKs were expressed in Sf9 cells by infection with the produced recombinant baculoviruses. Forty-eight hours after infection, the cells were incubated with 10 μM isoproterenol for the indicated time, mechanically detached, washed four times with ice-cold phosphate-buffered saline, suspended in 5 mM Tris·HCl, pH 7.4, 2 mM EDTA, homogenized and centrifuged at 40,000×g for 10 min. The pellets containing crude membrane fraction were resuspended in 2 mM Hepes, pH 7.4 and used immediately for the adenylyl cyclase assay. Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard (14).

Adenylyl cyclase assay

Adenylyl cyclase activities of membrane preparations were measured according to the method of Salomon et al. (15). The membranes (20–30 μg/tube) were incubated for 30 min at 37℃ with 1 nM–10 μM of isoproterenol, 10 mM NaF or 100 μM forskolin in 50 μl of reaction mixture (0.12 mM ATP, 50 μM GTP, 2.8 mM phosphoenolpyruvate, 0.1 mM cAMP, 1 unit/tube myokinase, 0.2 unit/tube pyruvate kinase, 40 mM Hepes (pH 7.4), 0.8 mM EDTA, 10 mM $MgCl_2$, 1 μCi [α-^{32}P]ATP). The reaction was stopped by the addition of 1 ml of stop solution (0.5 mM ATP, 0.5 mM cAMP, [^{3}H]cAMP (approx. 10,000 cpm)). Produced [α-^{32}P]cAMP was purified through Dowex AG50W-X4 (200–400 mesh) cation exchange resin and aluminum oxide columns. Radioactivities of [α-^{32}P]cAMP and [^{3}H]cAMP was measured with a liquid scintillation counter. Enzyme activities were determined in duplicate. For each experiment, the 100 μM forskolin-stimulated activity was set as 100%, and all other adenylyl cyclase activities were normalized to this value. The basal and maximally stimulated cyclase activities by isoproterenol and EC_{50} values for isoproterenol

were calculated by a computer program, PRISM software (Graphpad software Inc., San Diego, CA, USA), with a nonlinear least-squares regression analysis. Percentage of desensitization was defined as {(isoproterenol-stimulated adenylyl cyclase activity of control membrane) − (isoproterenol-stimulated adenylyl cyclase activity of treated membrane)} / (isoproterenol-stimulated adenylyl cyclase activity of control membrane) × 100 (%).

Radioligand binding studies

To determine expression of the WT- and mutated β_1ARs, the membranes prepared from Sf9 cells were incubated with [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) in 250 μl of 75 mM Tris·HCl, pH 7.5, 12.5 mM $MgCl_2$, 2 mM EDTA. Incubation was carried out for 90 min at 37℃ and terminated by the addition of ice-cold 25 mM Tris·HCl (pH 7.5) containing 1 mM $MgCl_2$, followed by vacuum filtration through Whatman GF/C glass fiber filters. Radioactivity of [^{125}I]-CYP was measured by a gamma counter. Nonspecific binding was determined in the presence of 1 μM (−)-propranolol. The equilibrium dissociation constant and maximal density of binding sites were determined by nonlinear regression analysis using PRISM software.

Data presentation

The data are shown as the mean±S.E.M. from n experiments, as indicated in the Figure legends. However, most of the experiments were done twice. In those cases, the data are shown as the mean values of two experiments. Statistical significance was assessed by the *t*-test.

RESULTS

Effects of GRK2, GRK5 and GRK6 on isoproterenol-induced desensitization of the WT-β_1AR

The WT-β_1AR was expressed in Sf9 cells, incubated in the absence or presence of 10 μM isoproterenol for 2 or

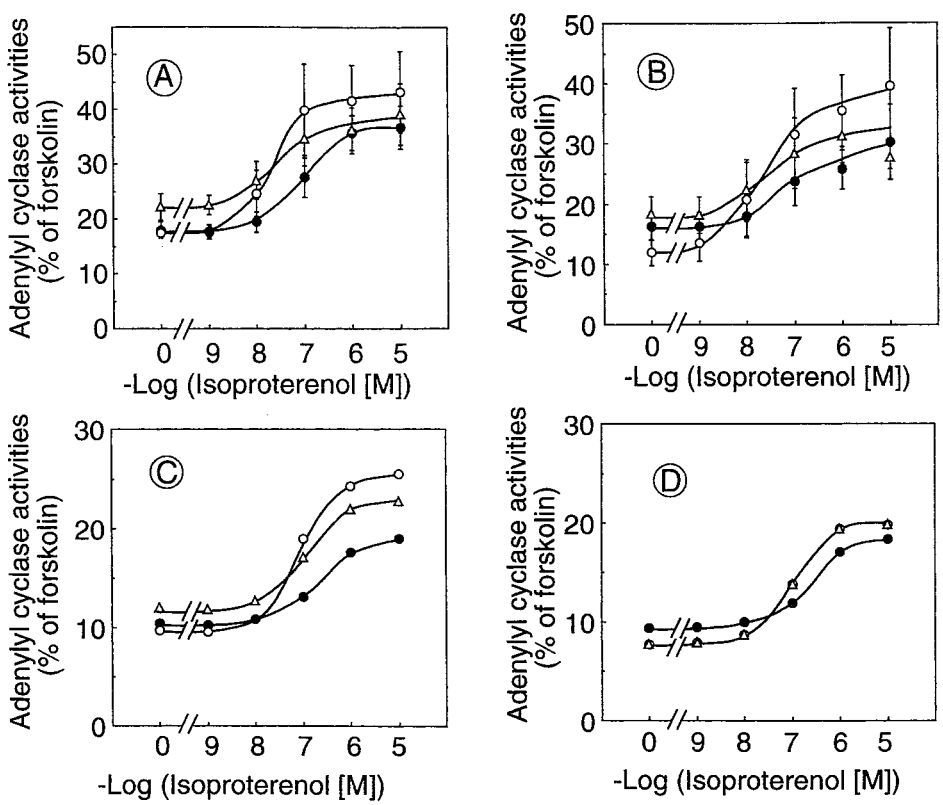


Fig. 1. Effects of GRK2, GRK5 and GRK6 on isoproterenol-induced desensitization of the β_1AR. Sf9 cells expressing the WT-β_1AR (A), the WT-β_1AR and GRK2 (B), the WT-β_1AR and GRK5 (C), or the WT-β_1AR and GRK6 (D) were incubated in the absence (○) or presence of 10 μM isoproterenol for 2 (△) or 30 min (●), membranes were prepared, and then adenylyl cyclase activities were measured as described in Materials and Methods. For each experiment, the 100-μM forskolin-stimulated activity was set as 100%, and all other adenylyl cyclase activities were normalized to this value. The forskolin-stimulated adenylyl cyclase activities were 97.6±30.4 (control, none, n=4); 107.2±32.9 (control, isoproterenol 2 min treatment, n=4); 103.6±32.7 (control, isoproterenol 30 min treatment, n=4); 105.5±21.3 (GRK2, none, n=3); 111.4±20.8 (GRK2, 2 min, n=3); 111.8±22.8 (GRK2, 30 min, n=3); 106.4 (GRK5, none, n=2); 115.4 (GRK5, 2 min, n=2); 115.1 (GRK5, 30 min, n=2); 103.1 (GRK6, none, n=2); 103.8 (GRK6, 2 min, n=2); and 97.2 pmol/mg protein·min (GRK6, 30 min, n=2).

30 min, and membranes were prepared and isoproterenol-stimulated adenyly cyclase activities were then determined. A 2- or 30-min exposure of the cells to isoproterenol induced substantial desensitization of the adenylyl cyclase response (Fig. 1A). Currently, six cDNAs encoding for GRKs (GRK1−GRK6) have been cloned from various species (2). To examine whether GRK2, GRK5 and GRK6 participate in the desensitization of the β_1AR, we expressed the WT-β_1AR alone or with one of these GRKs and determined the effects of overexpression of GRKs on desensitization of the β_1AR. Isoproterenol stimulation increased the adenylyl cyclase activities of Sf9 cells in a concentration-dependent manner. Pretreatment with isoproterenol had two effects on the adenylyl cyclase activities: one was the decreases of the maximally stimulated activities and another was the increases of the basal activities (Fig. 1). The magnitudes of desensitization of control cells with isoproterenol pretreatment for 2 or 30 min were 6.5±5.4% (n=4) or 25.0±6.3% (n=4), respectively (Fig. 1A). Overexpression of GRK2 or GRK5 did not appear to affect desensitization of the isoproterenol-stimulated adenylyl cyclase activities (Fig. 1: B and C). The magnitudes of desensitization in membranes prepared from GRK2- or GRK5-overexpressed cells with isoproterenol pretreatment for 2 min were 13.2±8.0% (GRK2, n=3, P=0.501 vs control) or 10.5% (GRK5, n=2), respectively. The magnitudes of desensitization with isoproterenol pretreatment for 30 min were 18.5±3.5% (GRK2, n=3, P=0.539) and 25.0% (GRK5, n=2). In contrast to GRK2 and GRK5, overexpression of GRK6 seems to diminish desensitization by isoproterenol pretreatment (Fig. 1D). The magnitude of desensitization of GRK6-overexpressed cells were −1.0% (n=2) or 7.2% (n=2) for 2 or 30 min pretreatment with isoproterenol, respectively. The forskolin-stimulated adenylyl cyclase activities were variable between experiments as indicated in the legend of Fig. 1. The variable percentages of desensitization were explained in part by the variable forskolin-stimulated adenylyl cyclase activities.

Effects of dominant negative GRK2 mutant on desensitization of the β_1AR

There is another approach to examine the role of GRK in the desensitization process. Several groups reported that dominant negative GRK2 counteracts the action of endogenous GRK2 and inhibits desensitization. We constructed and expressed a dominant negative GRK2 mutant (C20-K220A-GRK2), in which the lysine residue at position 220 in the catalytic domain was changed to an alanine residue and the last four amino acids from the carboxyl terminus (Ala-Asn-Gly-Leu) were changed to Cys-Val-Leu-Leu to promote membrane translocation by geranylgeranylation. Isoproterenol pretreatment resulted in a time-dependent desensitization in control membranes (Fig. 2A). Overexpression of dominant negative GRK2 appears to impair the effect of isoproterenol pretreatment; i.e., the reduction of decreases of the maximally stimulated adenylyl cyclase activities (Fig. 2B). The magnitudes of desensitization were 10.8% (control, n=2) and 0.0% (C20-K220A-GRK2, n=2) for 2-min pretreatment with isoproterenol and 17.0% (control, n=2) and 8.7% (C20-K220A-GRK2, n=2) for 30-min pretreatment with isoproterenol.

Domains responsible for desensitization of the β_1ARs

To analyze the phosphorylation site(s) responsible for the desensitization, we constructed several β_1AR mutants. The structures of these mutants are shown in Fig. 3. We

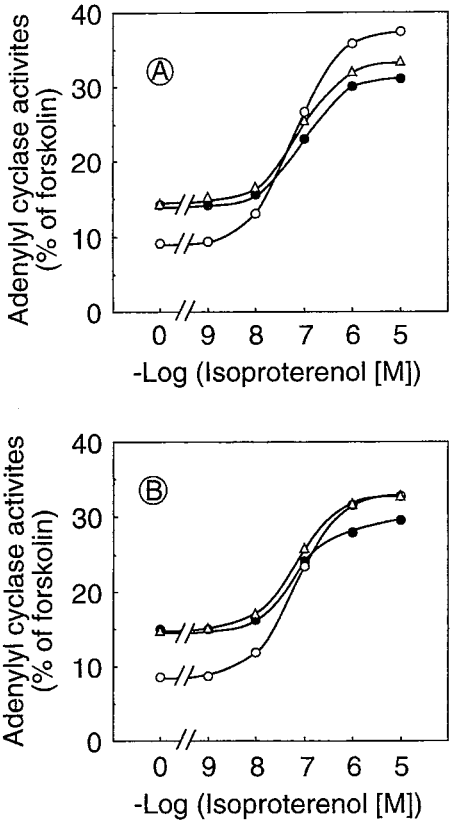


Fig. 2. Effect of dominant negative GRK2 on isoproterenol-induced desensitization of the β_1AR. Sf9 cells expressing the WT-β_1AR (A), the WT-β_1AR and C20-K220A-GRK2 (B) were incubated in the absence (○) or presence of 10 μM isoproterenol for 2 (△) or 30 min (●), membranes were prepared, and then adenylyl cyclase activities were measured as described in Materials and Methods. The adenylyl cyclase activities were normalized as described in Materials and Methods. The 100-μM forskolin-stimulated adenylyl cyclase activities were 97.4 (control, none); 116.7 (control, 2 min); 108.0 (control, 30 min); 91.1 (C20-K220A-GRK2, none); 88.1 (C20-K220A-GRK2, 2 min); and 93.4 pmol/mg protein·min (C20-K220A-GRK2, 30 min). The data are shown as mean values of two separate experiments.

mutated the serine residue at position 312 of the β_1AR (PKAβ_1AR), which is the PKA phosphorylation site located at the intracellular third loop, to determine whether phosphorylation by PKA is involved in β_1AR desensitization. We also constructed the mutant truncated at carboxyl terminus (Ctruncβ_1AR), which is supposed to include a potential GRK phosphorylation site(s). Furthermore, we constructed a double mutant (PKA /Ctruncβ_1AR) that lacked the PKA site in the intracellular third loop and the GRK site(s) at the carboxyl terminus to examine the possibility that PKA and GRK phosphorylation sites cooperatively participate in desensitization. Pretreatment with isoproterenol decreased the isoproterenol-stimulated adenylyl cyclase activities in control membranes (Fig. 4A). The isoproterenol-stimulated adenylyl cyclase activites in the PKA-, Ctrunc- and PKA/Ctrunc mutants were decreased by pretreatment with isoproterenol for 30 min. The magnitudes of desensitization were 29.1±4.2% (control, n=3); 27.3% (PKAβ_1AR, n=2); 21.3% (Ctruncβ_1AR, n=2); and 30.5% (PKA/Ctruncβ_1AR, n=2). Thus, desensitization of the PKAβ_1AR, Ctruncβ_1AR and PKA/Ctruncβ_1AR expressed in Sf9 cells appeared to be same as that of the WT-β_1AR (Fig. 4: A–D), suggesting that other region is important for the β_1AR to be desensitized in Sf9 cells.

We considered the possibility that the GRK phosphorylation site(s) of the β_1AR would be serine and threonine residues in the intracellular third loop, like in G_i-coupled receptors. Thus we expressed the β_1AR mutant that lacked serine and threonine residues of intracellular third loop (3loopDelβ_1AR) in Sf9 cells. We also expressed the double mutant that lacked the phosphorylation site(s) from the intracellular third loop and carboxyl terminus (3loopDel/Ctruncβ_1AR). The third loop deletion mutant showed clear desensitization by isoproterenol pretreatment (Fig. 5A). The combination of deletion of the carboxyl terminus with deletion of the third loop also showed a similar magnitude of desensitization by isoproterenol pretreatment (Fig. 5B). The magnitudes of desensitization were 20.3% (3loopDelβ_1AR, n=2) and 16.3% (3loopDel/Ctruncβ_1AR, n=2), respectively. Prior agonist treatment of the 3loopDelβ_1AR in-

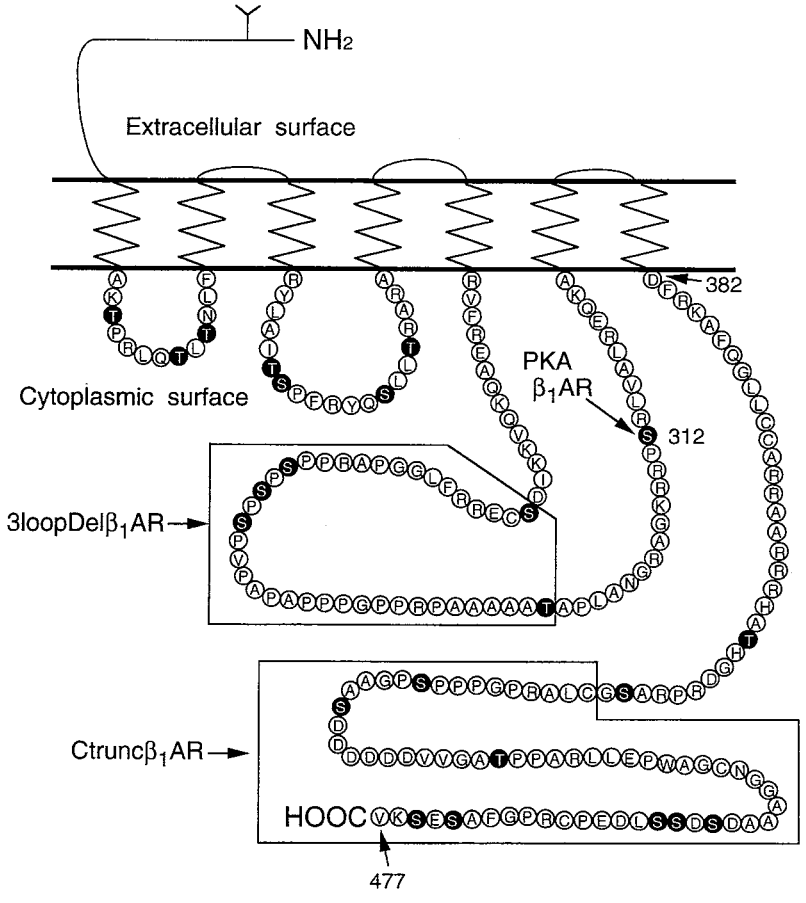


Fig. 3. Structures of wild type and mutant β_1ARs. The structures of the β_1AR mutants used in this study are shown. The construction of these mutants were described in Materials and Methods.

duced desensitization in which the extent was essentially the same as that of the WT-β_1AR (Fig. 5A). The 3loopDel /Ctruncβ_1AR showed essentially the same desensitization as the 3loopDelβ_1AR (Fig. 5B), indicating that the carboxyl terminus of the β_1AR did not mainly contribute to desensitization of the β_1AR.

We determined the maximal binding activity (B_{max}) and dissociation constant (K_d) of the WT- and mutated β_1ARs expressed in Sf9 cells. Table 1 shows that the K_d values of the β_1AR mutants were essentially the same as that of WT-β_1AR and level of expressions were within about twofold in range, suggesting that the various mutations did not affect the binding domains and cellular trafficking of these mutants.

DISCUSSION

It is generally accepted that desensitization of G protein-coupled receptors involves phosphorylation by various protein kinases including PKA, protein kinase C and GRKs (2). The β_1AR is a member of G protein-coupled receptor family. Although the desensitization mechanism of the β_2AR has been characterized at the molecular level, it has not been examined for the β_1AR. In the present report, we studied the effects of PKA and GRKs on β_1AR desensitization at the molecular level by analyzing various mutants and overexpression of GRKs.

At first, we examined whether various GRKs are involved in the desensitization of the β_1AR. We expressed the WT-β_1AR with one of GRK2, GRK5 or GRK6 in Sf9 cells using recombinant baculoviruses and determined the adenylyl cyclase activities after isoproterenol pretreatment. The membrane adenylyl cyclase activities seem to be a better index for desensitization than the determination of cellular cAMP contents, as the β_1AR will be desensitized during not only pretreatment but also second stimulation to examine the effect of pretreatment on cAMP generation. It was reported that the β_2AR ex-

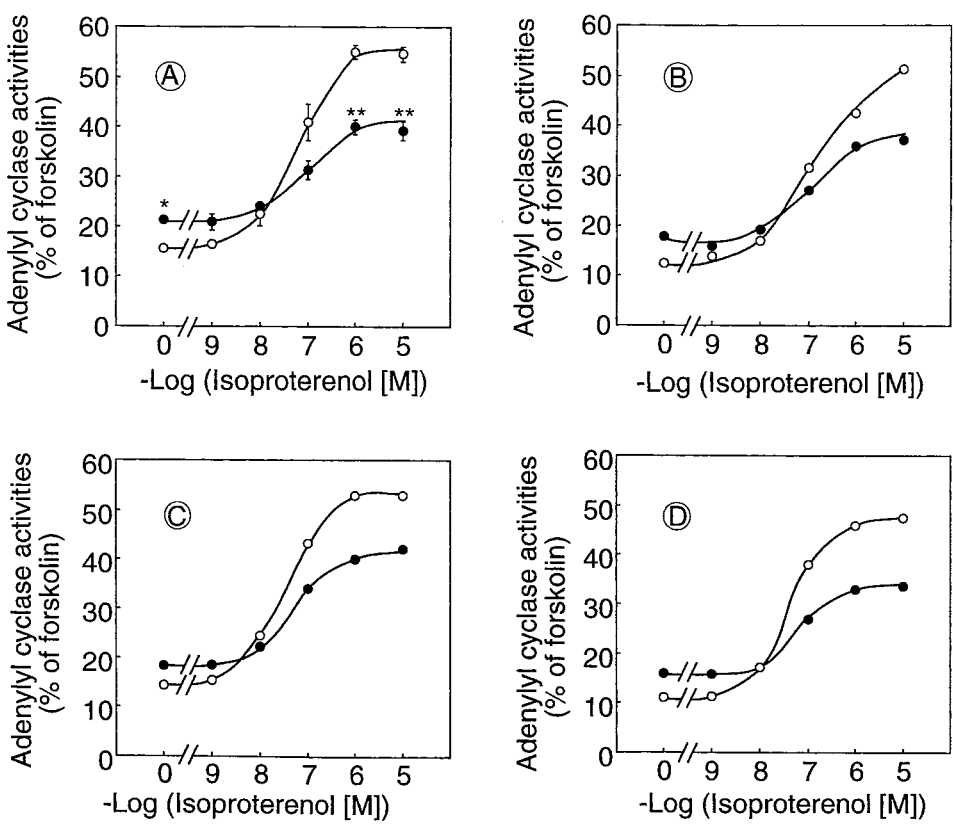


Fig. 4. Effects of mutation of β_1AR on isoproterenol-induced desensitization. Sf9 cells expressing the WT-β_1AR (A), the PKAβ_1AR (B), the Ctruncβ_1AR (C) or the PKA/Ctruncβ_1AR (D) were incubated in the absence (○) or presence (●) of 10 μM isoproterenol for 30 min, membranes were prepared, and then the adenylyl cyclase activities were measured as described in Materials and Methods. The adenylyl cyclase activities were normalized as described in Fig. 1. The 100-μM forskolin-stimulated adenylyl cyclase activities were 140.0±23.5 (WT-β_1AR, none, n=3); 155.0±28.3 (WT-β_1AR, 30 min, n=3); 114.4 (PKAβ_1AR, none, n=2); 111.2 (PKAβ_1AR, 30 min, n=2); 154.9 (Ctruncβ_1AR, none, n=2); 172.3 (Ctruncβ_1AR, 30 min, n=2); 142.1 (Ctrunc/PKAβ_1AR, none, n=2); and 155.9 pmol/mg protein·min (Ctrunc/PKAβ_1AR, 30 min, n=2). **$P<0.01$, *$P<0.05$, significantly different from untreated membranes.

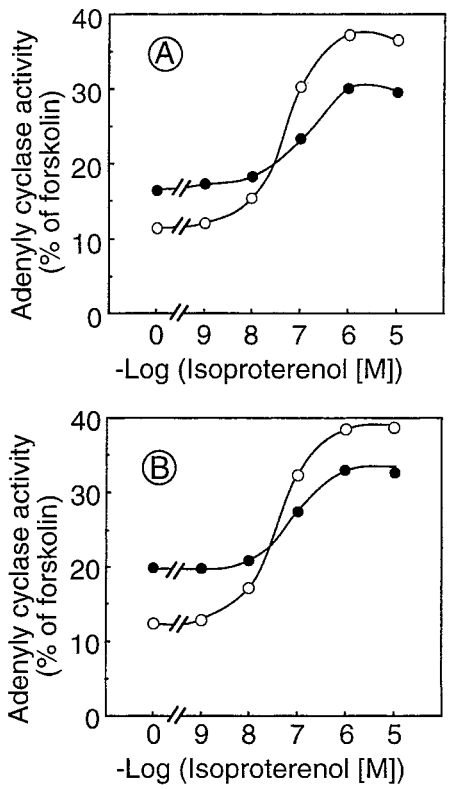


Fig. 5. Effects of deletion of the intracellular third loop on isoproterenol-induced desensitization of β_1AR. Sf9 cells expressing the 3loopDelβ_1AR (A) or the 3loopDel/Ctruncβ_1AR (B) were incubated in the absence (○) or presence (●) of 10 μM isoproterenol for 30 min, membranes were prepared, and then the adenylyl cyclase activities were measured as described in Materials and Methods. The adenylyl cyclase activities were normalized as described in Fig. 1. The 100-μM forskolin-stimulated adenylyl cyclase activities were 151.5 (3loopDelβ_1AR, none); 155.8 (3loopDelβ_1AR, 30 min); 143.2 (3loopDel/Ctruncβ_1AR, none); and 147.1 pmol/mg protein·min (3loopDel/Ctruncβ_1AR, 30 min). The data are shown as mean values of two separate experiments.

pressed in Sf9 cells shows desensitization by isoproterenol pretreatment (16). However, there are no reports in Sf9 cells about analysis of the sites that are important for desensitization. We examined the effect of various GRKs on desensitization by overexpressing with the receptors, although there is a possibility that GRKs bound to the activated receptors and inhibited the coupling to G proteins without contributing to desensitization of the receptors (17). The extent and onset of β_1AR desensitization were not promoted by the overexpression of these GRKs, suggesting that phosphorylation of the β_1AR by GRKs was not involved in desensitization of the β_1AR or that the β_1AR could be already phosphorylated to sufficient stoichiometry by endogenous GRKs or GRK-like protein kinases in Sf9 cells, so that the overexpression of various GRKs could not affect β_1AR desensitization. However, overexpression of GRK6 in Sf9 cells appeared to blunt desensitization of the β_1AR (Fig. 1D). It is assumed that the site (or domain) recognized by GRK is different from the site phosphorylated by the kinase. It suggests that the affinity of GRK6 for the β_1AR is higher than other GRKs, but GRK6 does not phosphorylate the β_1AR efficiently. Then GRK6 bound to the β_1AR can inhibit the phosphorylation by endogenous GRKs in Sf9 cells. This possibility will be resolved by the reconstitution assay with purified proteins. The effects of overexpression of GRKs and introduction of mutations on desensitization were not drastic, suggesting that their effects are of little importance in the respect of physiological context. We expressed the dominant negative mutant of GRK2 (C20-K220A-GRK2) to examine whether the β_1AR could interact with GRK2. It was reported that desensitization of the β_2AR or adenosine A_2 receptor is inhibited by the coexpression of the dominant negative mutant of GRK2 (K220R-GRK2) (18, 19). It was also demonstrated in HEK293 cells that the extent of phosphorylation of the β_1AR is decreased by 50% when the β_1AR is expressed with the dominant negative mutant of GRK2 (10). The extent of β_1AR desensitization appeared to be diminished by overexpression of C20-K220A-GRK2, suggesting that the phosphorylation of the β_1AR by endogenous GRK or GRK-like enzyme in Sf9 cells was involved in desensitization of the β_1AR. It indicates that overexpression of the dominant negative mutant may be a better approach than overexpression of native GRK.

Table 1. Maximal binding activities (B_{max}) and dissociation constant (K_d) values of [^{125}I]-iodocyanopindolol to the wild type and mutant β_1-adrenergic receptors

Receptors	B_{max} (fmol/mg protein)	K_d (pM)
WTβ_1AR	902	22.8
PKAβ_1AR	1080	24.8
Ctruncβ_1AR	476	17.5
PKA/Ctruncβ_1AR	595	21.9
3loopDelβ_1AR	1070	22.1
3loopDel/Ctruncβ_1AR	934	19.7

PKA is assumed to be involved in desensitization of the β_1AR. Zhou and Fishman (9) reported that the PKA inhibitor PKI blocks the isoproterenol-induced desensitization using the permeabilized human SK-N-MC cells. Freedman et al. (10) reported that H-89, one of the PKA inhibitors, blocks the phosphorylation of the β_1AR in HEK293 cells transiently expressed the β_1AR. In the present study, to examine the effect of phosphorylation by PKA on β_1AR desensitization, we constructed the β_1AR mutant (PKAβ_1AR) in which alanine is substituted for the serine at position 312, the residue considered to be the PKA phosphorylation site located in the intracellular

third loop. We showed that the PKAβ_1AR expressed in Sf9 cells was desensitized to essentially the same extent and onset as the WT-β_1AR. One of the reasons why the effect of PKA phosphorylation may be obscured is the high concentration of Mg^{2+} included in the assay mixture, because low concentration of Mg^{2+} in adenyly cyclase assays is important for detecting the effect of PKA on desensitization (20). As desensitization of the β_1AR could be observed under the condition of high Mg^{2+} concentration, it is reasonable to conclude that the phosphorylation by PKA does not play a major role in desensitization of the β_1AR in Sf9 cells. It also suggested that phosphorylation by one of endogenous GRKs or GRK-like enzymes in Sf9 cells contributed to desensitization of the β_1AR.

We constructed the β_1AR mutant truncated at its carboxyl terminus (Ctruncβ_1AR) to determine the GRK phosphorylation site(s) of the β_1AR responsible for desensitization. We observed essentially the same desensitization of the Ctruncβ_1AR mutant as that of the WT-β_1AR. The importance of carboxyl terminus for desensitization was reported in several receptors including the β_2AR (21–27). The replacement of serine and threonine residues of the β_2AR at the carboxyl terminus with alanine or glycine residues or truncation of potential GRK phosphorylation sites of the β_2AR blunted the onset of desensitization and decreased phosphorylation induced by isoproterenol stimulation (4, 7). However, the present study did not support the conclusion that the carboxyl terminus of the β_1AR contained a major phosphorylation site(s) responsible for desensitization. Desensitization of G_i-coupled receptors including α_2AR and m2 muscarinic acetylcholine receptor was mediated by phosphorylation of the intracellular third loop (2). Thus we constructed the third loop deletion mutant of the β_1AR (3loopDelβ_1AR). The 3loopDelβ_1AR showed almost the same extent of desensitization as the WT-β_1AR. To examine the possibility that the carboxyl terminus and intracellular third loop cooperatively contribute to desensitization of the β_1AR, we produced a double-deletion mutant of the third loop and carboxyl terminus (3loopDel/Ctruncβ_1AR). However, the 3loopDel/Ctruncβ_1AR desensitized to essentially the same extent as the 3loopDelβ_1AR and WT-β_1AR. These data suggested that the site(s) of phosphorylation in the β_1AR responsible for desensitization is different from those in the β_2AR and α_2AR. There are two reports that the intracellular loops other than the third intracellular loop and carboxyl terminus play an important role in agonist-induced phosphorylation and desensitization (28, 29). Jockers et al. (28) recently reported that the second intracellular loop of the β_2AR is a new domain responsible for desensitization using the β_2 and β_3AR chimeras. Nakamura et al. (29) reported that the agonist-induced uncoupling of the rat follitropin receptor is facilitated by the agonist-induced phosphorylation of the first and second intracellular loops.

In conclusion, our data suggest that regions other than the intracellular third loop and carboxyl terminus are responsible for desensitization of the β_1AR in Sf9 cells. It remains to be determined whether we can observe similar desensitization of these β_1AR mutants when stably expressed in mammalian cells.

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