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ACCELERATED COMMUNICATION

Identification of a Specific Site Required for Rapid Heterologous Desensitization of the β-Adrenergic Receptor by cAMP-Dependent Protein Kinase

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SUMMARY

The molecular basis for heterologous desensitization of the β-adrenergic receptor (βAR) was investigated by site-directed mutagenesis of the βAR protein. Rapid heterologous desensitization of agonist-stimulated adenylyl cyclase activity was observed when L cells expressing the wild-type βAR were incubated with 50 nM epinephrine. This desensitization response could be mimicked in a cell-free system by incubation with cAMP-dependent protein kinase (cA·PK). Deletion of amino acid residues 259–262 from the βAR, removing one of the two consensus sequences in the receptor for phosphorylation by cA·PK, abolished the ability of the receptor to undergo rapid heterologous desensitization. In contrast, deletion of the other cA·PK consensus sequence (residues 343–348) or truncation of the Ser/Thr-rich C-terminal tail of the βAR (deletion of residues 354–418) did not affect this heterologous desensitization process. These results suggest that the action of cA·PK on amino acid residue(s) contained within the sequence 259–262 of the βAR is required for rapid heterologous desensitization of the receptor in response to agonists.

The βAR is a member of a family of receptor proteins that mediate their actions through activation of guanine nucleotide-binding regulatory proteins. Binding of adrenergic agonists to the receptor causes activation of the guanine nucleotide-binding protein G_s, resulting in the stimulation of adenylyl cyclase and the intracellular accumulation of cAMP (1). Continuous exposure of cells to agonists leads to desensitization of the responsiveness to further agonist stimulation. Several distinct but interrelated mechanisms are involved in the overall cellular desensitization response (2–4). At the level of the βAR, two categories of desensitization have been described. Heterologous desensitization in response to β-adrenergic agonists denotes an attenuation of the activity of the βAR as well as of other receptors coupled to the G_s/adenylyl cyclase system. In contrast, homologous desensitization is specific for the βAR, and the activity of other receptors is not affected. Homologous desensitization of the βAR is characterized by a rapid uncoupling of the receptor from G_s and sequestration of the receptor away from the cell surface, followed by a slower down-regulation of total cell-associated receptor (3, 4). This homologous desensitization response correlates temporally with receptor phosphorylation, which has been attributed to a recently described βAR-specific kinase (5). Heterologous desensitization has also been associated with receptor phosphorylation, postulated to be mediated by either cA·PK or protein kinase C (2, 6). One type of heterologous desensitization, which has been extensively characterized in our laboratory, is a rapid attenuation of adenylyl cyclase responsiveness that is observed in response to low physiological concentrations of agonists (10–50 nM epinephrine or PGE_1) (7–9). This rapid heterologous desensitization is characterized by a 2–3-fold increase in the K_{act} for agonist-stimulated adenylyl cyclase activity, with no accompanying decrease in the V_{max}, such that the extent of desensitization is maximal (45–55%) when measured at concentrations near the K_{act}. Two lines of evidence suggest that this form of heterologous desensitization is mediated by cA·PK. First, this process does not occur in either the kin^- or the cyc^- mutants of S49 cells, which lack either cA·PK or the capacity for agonists to activate the kinase, respectively (7, 8). Secondly, this heterologous desensitization response can be mimicked in a cell-free system by addition of the catalytic subunit of $cA·PK_c$ (9).

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ABBREVIATIONS: βAR, β-adrenergic receptor; cA·PK, cAMP-dependent protein kinase; PGE_1, prostaglandin E_1; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $cA·PK_c$, catalytic subunit of cAMP-dependent protein kinase.

The recent cloning and expression of the gene encoding the mammalian β_2AR (10) has allowed the application of genetic techniques to the elucidation of the molecular mechanisms of these desensitization processes. Homologous desensitization of the βAR expressed in mouse L cells (11, 12), Chinese hamster ovary cells (13), and *Xenopus* oocytes (14) has been characterized, with the processes in each of these systems being similar to those observed in native cell lines. Truncation of the Ser-rich C-terminal tail of the receptor does not alter the level of agonist-promoted receptor sequestration or of desensitization of βAR-stimulated adenylyl cyclase activity in response to high concentrations of hormone (11, 14). More recent studies indicate that removal of these Ser residues, either by truncation of the C-terminus or by molecular replacement of Ser residues with Gly or Ala, decreases the initial rate of homologous desensitization but does not change the ultimate extent of the process (12, 13).

The hamster β_2AR contains two consensus sequences for phosphorylation by cA·PK [defined as Lys/Arg-X-(X)-Ser-X] (15). One of the sites (Arg^{259}-Arg-Ser-Ser-Lys^{263}) is located on the third intracellular loop of the receptor, whereas the other site (Arg^{343}-Arg-Ser-Ser-Ser-Lys^{348}) is located in the C-terminal domain (10). Deletion mutagenesis experiments demonstrated that these sites are not required for homologous desensitization to occur (11). In the present report, we have used deletion mutagenesis to determine whether these putative cA·PK sites are involved in cA·PK-mediated heterologous desensitization of the βAR in mouse L cells. The cA·PK consensus site in the third intracellular loop of the receptor is essential for rapid cA·PK-mediated heterologous desensitization. In contrast, the site near the C-terminus is not required for heterologous desensitization of the receptor.

Materials and Methods

Mutagenesis and cell culture. The cloning and oligonucleotide-directed mutagenesis have been described previously (10). Mutants D(259–262)βAR, D(343–348)βAR, D(259–262)D(343–348)βAR, and T(354)βAR, expressed in L cells, were published as D(k_1)βAR, D(k_2)βAR, D(k_{12})βAR, and T(354)βAR, respectively in Ref. 11. Cells were cultured at 37° in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and Geneticin at 0.4 mg/ml.

Cell incubations and membrane preparations. The cells were treated at 37° for 5–7 min with either 50 nM epinephrine in 1 mM thiourea and 0.1 mM ascorbate or 1 μM PGE_1 in 0.02% ethanol. Controls were treated with either ascorbate/thiourea or ethanol. The cells were then washed twice with cold HME buffer (20 mM HEPES, pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 2 mM tetrasodium pyrophosphate, 10 μg/ml trypsin inhibitor, and 0.1 mg/ml bovine serum albumin), scraped in HME plus 10 μg/ml leupeptin, and homogenized with three strokes of a type B Dounce homogenizer. The lysates were layered over gradients of 23 and 43% sucrose in HE buffer (20 mM HEPES, pH 8, 1 mM EDTA), and membranes were isolated by centrifugation at 25,000 rpm in a Beckman SW27 rotor for 45 min. The band at the sucrose interface was removed and, before being assayed, was washed once in 19% sucrose in HE with a 15-min centrifugation at 140,000 × *g*. The pellets were suspended in HE for adenylyl cyclase assays. Adenylyl cyclase was assayed by modification (8) of the method of Salomon *et al.* (16).

Cell-free desensitization. cA·PK_c was prepared from bovine heart as previously described (9) and was dialyzed against 40 mM HEPES, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol. For the cell-free desensitization experiments, washed membranes were combined with cA·PK_c or its buffer and hormone additions. The samples were preincubated for 5 min at 30° with 50 mM HEPES pH 7.7, 1 mM EDTA, 1.47 mM $MgCl_2$, 8 mM creatine phosphate, 16 units/ml creatine kinase, 0.2 mM ATP, 10 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, 1 mM benzamidine, 10 μg/ml leupeptin, 0.1 mg/ml bovine serum albumin. To assess adenylyl cyclase activity, approximately 2 μCi of [α-^{32}P]ATP were added, and the incubation was continued for 10 min.

Results

Epinephrine-induced desensitization of adenylyl cyclase in intact cells. To determine whether L cells expressing the wild-type hamster βAR undergo rapid heterologous desensitization upon exposure to low concentrations of agonists, L cells were incubated with either 50 nM epinephrine or 1 μM PGE_1 for 5–7 min, membranes were isolated from the cells, and adenylyl cyclase activity was measured in the presence of 0.3–0.5 mM Mg^{2+}. Exposure of the cells expressing the wild-type βAR to 50 nM epinephrine caused a 2–3-fold increase in the K_{act} for epinephrine, with no significant change in the maximal stimulation observed (Fig. 1A). Thus, at concentrations around the K_{act}, epinephrine-stimulated adenylyl cyclase activity was desensitized by 50% in response to this treatment, similar to that previously observed for the endogenous βAR in S49 lymphoma cells. As previously reported for S49 lymphoma cells and DDT_1MF-2 smooth muscle cells (8, 9), detection of this heterologous desensitization in L cells required the presence of relatively low concentrations (<1 mM) of free Mg^{2+} ions in the adenylyl cyclase assays, and the phenomenon was obscured by the presence of >5 mM Mg^{2+}.

To assess the effects of removal of potential sites of phosphorylation by various kinases on this heterologous desensitization process, mutant βARs were constructed in which various potential kinase substrate domains were deleted (11). D(259–262)βAR removes a potential cA·PK site located in the third intracellular loop. Another potential cA·PK site in the C-terminal domain of the βAR was removed to form D(343–348)βAR, and these two deletions were combined in D(259–262)D(343–348)βAR. The Ser/Thr-rich C-terminal tail of the βAR was truncated in T(354)βAR, in which amino acid residues 354–418 were deleted. Treatment of cell lines expressing these mutant receptors with 50 nM epinephrine for 5–7 min gave the results shown in Fig. 1, B–E, and summarized in Table 1. D(343–348)βAR showed a 40% desensitization in response to epinephrine treatment, similar to that observed for the wild-type receptor (Fig. 1B). Desensitization of the epinephrine response in cells expressing T(354)βAR was slightly less than that observed for the wild-type βAR but was nonetheless significant (32%) (Fig. 1C). In contrast, D(259–262)βAR and D(259–262)D(343–348)βAR failed to show an agonist-mediated desensitization response under these conditions (Fig. 1, D and E). Exposure to epinephrine did not cause a significant decrease in either forskolin or PGE_1 stimulation of adenylyl cyclase in cells expressing the wild-type βAR or any of the mutant receptors (Table 1).

It should be emphasized that it was necessary to use low concentrations of epinephrine in the pretreatment to reveal the differences in heterologous desensitization among the various mutant receptors. Treatment of cells expressing the wild-type or mutant βAR with higher concentrations of agonist was previously shown to cause homologous desensitization of the receptor, characterized by a decrease in the responsiveness of adenylyl cyclase to β-adrenergic agonists and by sequestration/

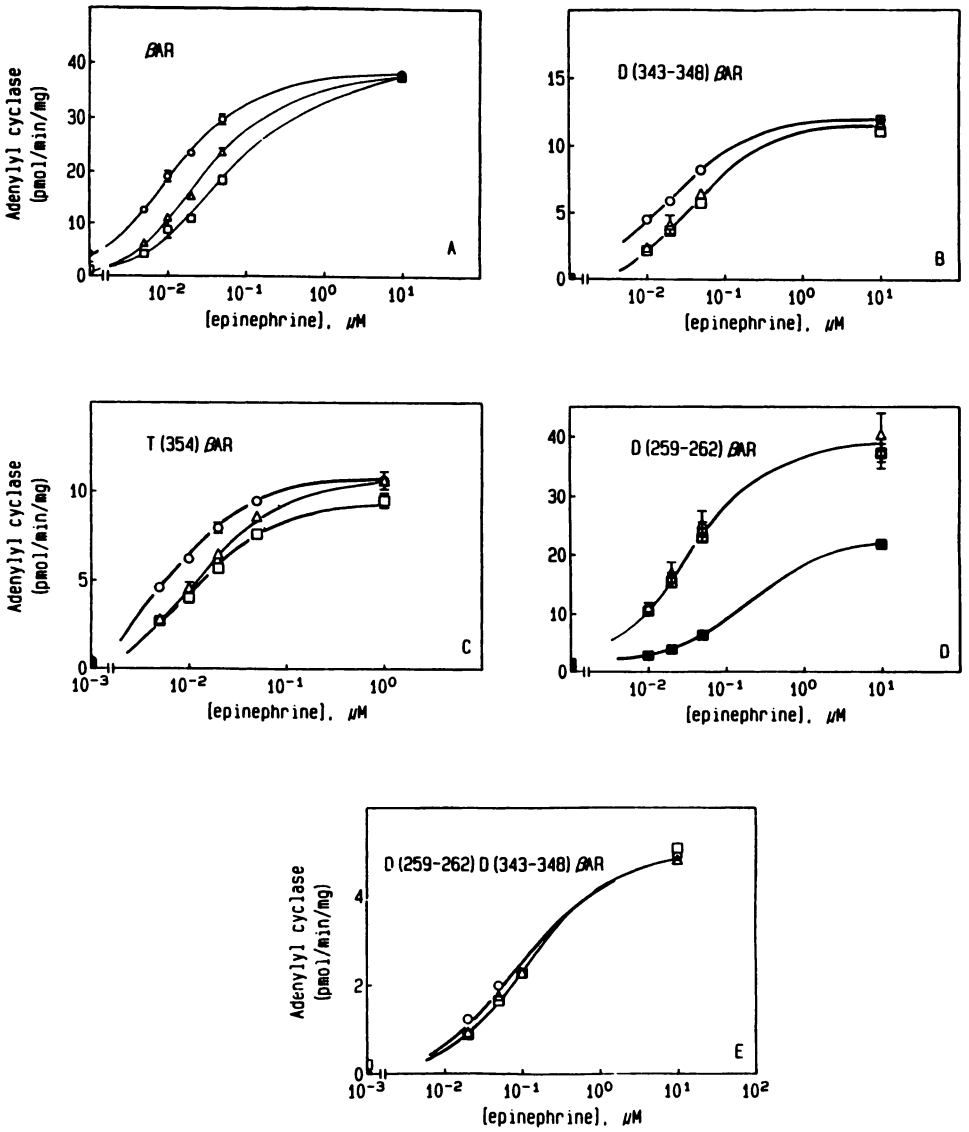

Fig. 1. Epinephrine- and PGE_1-induced desensitization of β-adrenergic stimulation in L cell mutants. Cells expressing βAR (A), D(343–348)βAR (B), T(354)βAR (C), D(259–262)βAR (D), or D(259–262)D(343–348)βAR (E) were treated for 7 min at 37° with 50 nM epinephrine (□), 1 μM PGE_1 (△), or carriers (0.1 mM ascorbate, 1 mM thiourea, 0.02% ethanol) (○). In D, D(259–262)βAR was also treated with 1 μM epinephrine for 7 min (■). Membranes were prepared as described in Materials and Methods and were assayed for adenylyl cyclase activity at various epinephrine concentrations. The final free Mg^{2+} concentration for all experiments was 0.3 mM. The values shown are means ± standard deviations of triplicate determinations from a representative experiment. Where the standard deviation is not visible, the values were less than the size of the symbol.

TABLE 1

Desensitization of epinephrine and PGE_1 stimulations of adenylyl cyclase in response to treatment of intact L cells with either hormone

As in the experiments shown in Fig. 1, cells expressing βAR, D(343–348)βAR, T(354)βAR, or D(259–262)βAR were treated with 50 nM epinephrine or 1 μM PGE_1. Membranes were prepared and adenylyl cyclase was measured. Desensitization was assessed at EC_{50} concentrations of each hormone and is defined as [1 − (pmol of cAMP formed in membranes from treated cells/pmol of cAMP formed in membranes from untreated cells)] × 100. The values presented are the means ± standard deviations for the number of experiments shown in parentheses. Representative experiments are shown in Fig. 1.

Receptor	Epinephrine pretreatment		PGE_1 pretreatment	
	Epinephrine desensitization	PGE_1 desensitization	Epinephrine desensitization	PGE_1 desensitization
	%			
βAR	50.0 ± 11 (3)	ND[a]	38.5 ± 9 (5)	32.0 ± 10 (5)
D(343–348)βAR	41.6 ± 3 (4)	5.6 ± 8 (2)	35.4 ± 4 (7)	30.3 ± 9 (5)
T(354)βAR	32.3 ± 2 (3)	9.0 ± 3 (3)	27.4 ± 3 (3)	26.5 ± 5 (4)
D(259–262)βAR	6.4 ± 12 (3)	0.2 ± 20 (3)	10.9 ± 13 (4)	26.8 ± 8 (4)

[a] ND, not determined.

internalization of the receptor (11). This homologous desensitization in response to high agonist concentrations was also observed in the present study. As shown in Fig. 1D, treatment of cells expressing D(259–262)βAR with 1 μM epinephrine for 7 min resulted in a decrease in the V_{max} for epinephrine-stimulated adenylyl cyclase activity, accompanied by an increase in the K_{act}. Thus, although D(259–262)βAR showed no heterologous desensitization in response to low concentrations of epinephrine, this mutant receptor underwent marked homologous desensitization when treated with higher concentrations of the agonist.

Cells expressing βAR, D(343–348)βAR, or T(354)βAR were also desensitized in response to PGE_1 (Fig. 1 and Table 1). Pretreatment of these cells with 1 μM PGE_1 for 5–7 min resulted in a 2-fold increase in the K_{act} for epinephrine stimulation of adenylyl cyclase, corresponding to a 35–40% desensitization at

epinephrine concentrations near the K_{act} (Fig. 1, A–C). In contrast, epinephrine stimulation of adenylyl cyclase was not reduced in cells expressing D(259–262)βAR or D(259–262)D(343–348)βAR after exposure to 1 μM PGE_1 (Figure 1, D and E). However, treatment with PGE_1 induced a 25–30% decrease in the level of PGE_1-stimulated adenylyl cyclase activity in all of these cell lines (Table 1), indicating that desensitization of the PGE_1 receptor was not affected by the mutations in the βAR. There was no significant decrease in forskolin stimulation of adenylyl cyclase following the PGE_1 treatment of any of the cell lines expressing the wild-type or mutant βAR (data not shown).

cA·PK-induced desensitization in cell-free membrane preparations. To assess the involvement of cA·PK in heterologous desensitization, membranes from cell lines expressing the wild-type or mutant βAR were treated with highly purified cA·PK_c and adenylyl cyclase activities stimulated by epinephrine were measured. The effects of these treatments on the epinephrine stimulation of adenylyl cyclase in membranes containing the wild-type and mutant βAR are shown in Fig. 2, with the results summarized in Table 2. Exposure to cA·PK_c caused a 2–3-fold increase in the K_{act} for epinephrine stimulation of adenylyl cyclase by the βAR, D(343–348)βAR, and T(354)βAR. This cell-free reduction in epinephrine stimulation by adenylyl cyclase was blocked by 10 μM Wiptide, a specific inhibitor of cA·PK_c (data not shown) (17). In contrast, cA·PK_c had no effect on adenylyl cyclase stimulation by D(259–262)βAR. Thus, the wild-type βAR, D(343–348)βAR, and T(354)βAR showed a 45–50% desensitization of epinephrine-stimulated adenylyl cyclase activity in the presence of cA·PK_c, whereas D(259–262)βAR was not desensitized under these conditions. cA·PK_c had no effect on either the PGE_1 or forskolin stimulation of adenylyl cyclase in membranes from any of the cell lines studied.

Discussion

The molecular mechanisms involved in desensitization of the βAR are varied and complex. Several different kinases have been implicated in the various types of desensitization, including cA·PK and protein kinase C in heterologous desensitization and βAR kinase in homologous desensitization (2–5). However, the physiological roles for these and other kinases in the desensitization processes have not been firmly established, nor have the structural parameters and functional effects of the presumptive phosphorylations been determined. Recently, genetic approaches, both through the use of somatic cell mutants and through oligonucleotide-directed mutagenesis of receptors, have yielded some insights into these processes (7–14). The observation that cAMP causes heterologous desensitization of the βAR in the wild-type S49 lymphoma cell line but not in the somatic kin^- mutant of these cells, which lacks cA·PK, demonstrated that cA·PK is essential for heterologous desensitization to occur (8). Demonstration that the heterologous desensitization seen in intact cells could be mimicked by the addition of cA·PK_c to membranes provided further support for the notion that the kinase acts directly on the βAR (9).

In the present study, we have used site-directed mutagenesis of the βAR to gain further insight into the molecular mechanisms of heterologous desensitization, by assessing the effects of deletion of the two cA·PK consensus substrate sites from the protein. Deletion of a cA·PK consensus site in the C-terminal domain of the βAR (residues 343–348, of which Ser^{346} or Ser^{347} would be the predicted site for phosphorylation by cA·PK) or deletion of the Ser/Thr rich C-terminal tail had no effect on heterologous desensitization of the βAR induced by 50 nM epinephrine or 1 μM PGE_1. In contrast, deletion of the cA·PK site in the third intracellular loop of the βAR (residues 259–262, of which Ser^{262} would be the predicted site for phos-

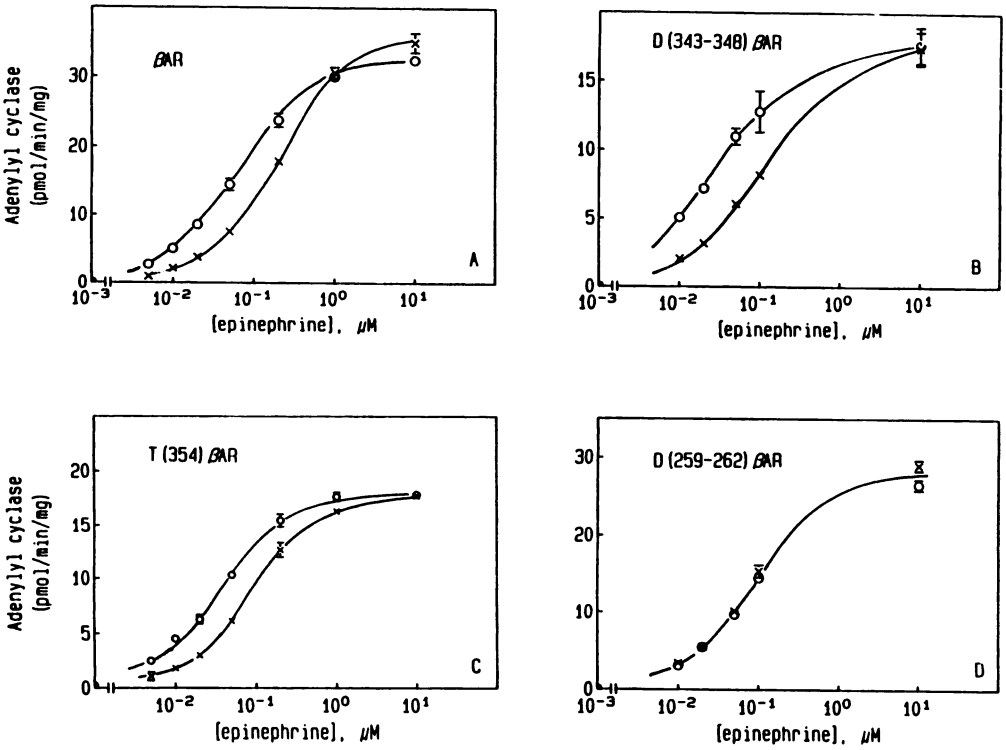

Fig. 2. cA·PK_c-induced desensitization of epinephrine stimulation in membranes from L cells expressing the wild-type or mutant βARs. Membranes were prepared from untreated L cells expressing βAR (A), D(343–348)βAR (B), T(354)βAR (C), or D(259–262)βAR (D), and adenylyl cyclase activities were measured at various epinephrine concentrations with (×) or without (○) cA·PK_c, as described in Materials and Methods for cell-free desensitization experiments. The values shown are the means ± standard deviations of triplicate determinations from a representative experiment.

TABLE 2

Cell-free desensitization of epinephrine and PGE_1 stimulation of adenylyl cyclase in response to treatment of L cell membranes with cA·PK_c

Washed membranes from untreated cells were assayed either with or without cA·PK_c, as described for cell-free experiments in Materials and Methods. Desensitization was measured as described in the legend to Table 1, at EC_{50} concentrations of epinephrine and PGE_1. A negative value for percentage of desensitization indicates that the adenylyl cyclase activities of the treated membranes were greater than the control activities. Values given are the means ± standard deviations for the number of experiments shown in parentheses. Representative experiments are shown in Fig. 2.

Receptor	Epinephrine desensitization	PGE_1 desensitization
	%	
βAR	50.7 ± 3 (5)	7.5 ± 11 (4)
D(343–348)βAR	45.7 ± 11 (3)	−13.9 ± 14 (2)
T(354)βAR	50.0 ± 9 (4)	−3.9 ± 12 (4)
D(259–262)βAR	−18.7 ± 16 (3)	−15.7 ± 10 (3)

phorylation by cA·PK) prevented this heterologous desensitization response to epinephrine and PGE_1.

In agreement with these results from intact cells, the addition of cA·PK_c to membranes from cells expressing βAR, D(343–348)βAR, or T(354)βAR resulted in desensitization of the responsiveness to epinephrine, whereas this desensitization was not observed with D(259–262)βAR. Both the specificity of the effect of the kinase on epinephrine stimulation (i.e., no effect on either forskolin or PGE_1 activity) and the magnitude of the effect (2–3-fold increase in the K_{act} for epinephrine) were consistent with the desensitization observed in intact cells. Together, the results of the intact cell and cell-free experiments suggest that heterologous desensitization is mediated by cA·PK, that it requires the cA·PK consensus site in the third intracellular loop of the βAR, and that neither the truncation of a large part of the C-terminal tail of the receptor nor the deletion of the cA·PK consensus site in the C-terminal domain of the receptor affects this process.

The desensitization of the βAR observed in response to PGE_1 provides an independent means of evaluating heterologous desensitization of the βAR without interference from homologous mechanisms. The similar characteristics of the desensitization processes observed in response to PGE_1 and epinephrine suggest that exposure to 50 nM epinephrine probably induces little homologous desensitization of the βAR, although the differences in the magnitudes of these effects may be attributable to a slight homologous component occurring due to epinephrine. The lack of any significant desensitization of D(259–262)βAR upon exposure to 50 nM epinephrine or 1 μM PGE_1 suggests that this domain is essential for heterologous desensitization. In contrast, the mechanism for homologous desensitization is not affected by this mutation, as evidenced by the 70% desensitization observed in response to 1 μM epinephrine and the normal sequestration and desensitization responses to 10 μM isoproterenol previously observed for this mutant receptor (11).

It is interesting that PGE_1-stimulated adenylyl cyclase activity was unaltered by either cA·PK_c treatment of membranes or by treatment of intact cells with epinephrine, whereas this activity was desensitized in response to treatment of intact cells with PGE_1. Thus, the reduction in PGE_1 stimulation after intact cell treatment may be attributed to a cA·PK-independent PGE_1-induced homologous desensitization of the PGE_1 receptor in this system. The lack of heterologous desensitization of PGE_1-stimulated adenylyl cyclase following treatment of intact cells with epinephrine or incubation of membranes with cA·PK_c suggests that cA·PK-mediated desensitization need not always be heterologous with respect to all receptors that stimulate adenylyl cyclase and demonstrates the potential for receptor specificity within this pathway. Previous studies have shown the PGE_1 receptor to be susceptible to cA·PK-mediated heterologous desensitization in S49 and DDT_1MF-2 cells (7–9). An understanding of the molecular basis for the absence of heterologous desensitization of the PGE_1 receptor in L cells awaits the availability of structural information on this receptor protein.

The requirement for residues 259–262 of the βAR for cA·PK-mediated desensitization to occur might indicate that phosphorylation of the receptor on Ser^{262} by cA·PK is a critical step in this desensitization process. Attempts in several laboratories to directly identify the specific site(s) of phosphorylation during any of the desensitization processes have thus far proven unsuccessful. Analysis of the phosphorylation of synthetic peptides corresponding to various Ser-rich regions of the βAR by cA·PK showed that the peptide corresponding to residues 257–264 was a better substrate for cA·PK than was the peptide corresponding to residues 341–351 of the receptor (18), consistent with the present identification of this region of the receptor as the critical domain for cA·PK-mediated desensitization. Further support for the functional significance of Ser^{262} arises from the observation that an analogous consensus cA·PK site is present near the C-terminus of the third intracellular loop in all of the β_1 and β_2-adrenergic receptors whose sequences have been determined (10, 19–21). This site is not conserved with other receptors that couple to different G proteins, consistent with the observation that this heterologous desensitization process appears to be specific for G_s-associated receptors. The β_1 and β_2AR are the only G_s-coupled receptors whose sequences are currently known, and it will be interesting to see whether this cA·PK consensus site is conserved among all G_s-coupled receptors. In contrast, the consensus site in the C-terminal tail of the β_2AR, which is not essential for heterologous desensitization, is not present in the avian or mammalian β_1AR (19, 20).

Residues 259–262 are located on a hydrophilic loop of the βAR that has been demonstrated immunohistochemically to be exposed cytoplasmically and, hence, would be accessible to cA·PK in intact cells (22). A model that has been developed for the βAR based on its structural similarity to rhodopsin would predict this region to form the third intracellular loop of the receptor protein (23). Deletion of residues 259–262 does not affect the ability of the receptor to bind ligands or to couple to adenylyl cyclase, indicating that this mutation does not adversely affect the conformation of the receptor (24). However, it is interesting to note that a larger deletion in this region, encompassing residues 258–270, severely attenuates the ability of the receptor to couple to G_s, suggesting that the amino acid residues at the C-terminus of this third intracellular loop are important for achieving the active conformation of the βAR (25). Thus, although residues 259–262 are not themselves required for G_s coupling, it seems likely that phosphorylation of Ser^{262} by cA·PK could perturb the conformation of this loop in such a way as to interfere with the ability of the receptor to couple to the G_s/adenylyl cyclase system, resulting in receptor desensitization. Significantly, neither the deletion of residues

259–262 nor of residues 258–270 affected the ability of the receptor to undergo the rapid agonist-mediated sequestration that is characteristic of homologous desensitization, consistent with our current observation that this region of the receptor is important for heterologous, but not homologous, desensitization (12).

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