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Phosphorylation of Ser^{360} in the third intracellular loop of the α_{2A}-adrenoceptor during protein kinase C-mediated desensitization

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Abstract

The α_{2A}-adrenoceptor undergoes desensitization in response to protein kinase C activation. Using mutagenesis and recombinant expression we sought to determine the specific sites within the receptor which are phosphorylated by protein kinase C and are responsible for this desensitization. Ser/Thr in the third intracellular loop were substituted with Ala to create mutant receptors T272A, S258A, S324A and S360A. These mutations had no effect on ligand binding or functional coupling to inositol phosphate accumulation and intracellular Ca^{2+} release. Three of the four mutant receptors displayed wild-type desensitization (~60% loss of function) in response to 0.1 μM phorbol-12-myristate-13-acetate (PMA) exposure for 2 min. However, the S360A mutant had only ~24% desensitization. In whole cell phosphorylation studies, S360A failed to undergo detectable PMA promoted phosphorylation. We conclude that protein kinase C-mediated desensitization of α_{2A}-adrenoceptor function is primarily due to phosphorylation of Ser at amino acid 360. This thus represents one mechanism by which these receptors undergo regulation by heterologous means, such as pathologic processes which activate protein kinase C or crosstalk with other receptors.

Keywords: Desensitization; Phosphorylation; Protein kinase C; Mutation

1. Introduction

Like many other G protein-coupled receptors, the α_2-adrenoreceptors display a high degree of dynamic regulation due to homeostatic and pathologic processes, as well as persistent agonist exposure (Manji et al., 1997; Hein, 2001; Eason and Liggett, 1992; Liang et al., 1998). Desensitization of α_2-adrenoceptor function from short-term agonist exposure, termed homologous desensitization, has been found to be due to phosphorylation of receptors by G protein-coupled receptor kinases (Eason et al., 1995; Jewell-Motz and Liggett, 1996). We have recently also shown (Liang et al., 1998) that the α_{2A}-subtype is phosphorylated by protein kinase C, and that this event results in a marked uncoupling of receptor signaling to inositol phosphate accumulation and intracellular Ca^{2+} release. In this report, we utilize site-directed mutagenesis of the α_{2A}-adrenoceptor to determine the phosphorylation sites within the receptor responsible for protein kinase C-mediated desensitization.

2. Materials and methods

2.1. Mutagenesis

Table 1 shows the location of the six potential protein kinase C phosphorylation sites in the third intracellular loop that were considered for mutagenesis. We have recently shown that when S232 is mutated to Ala the resulting receptor is significantly impaired at baseline (Liang et al., 2001). In addition, others have shown that constitutive receptor activation occurs when T373 is mutated (Ren et al., 1993). Since baseline coupling of these two mutated receptors is markedly altered, these were not considered for the current study. For the other locations, Ala was substituted for Ser or Thr as indicated to construct the mutant α_{2A}-adrenoceptors denoted T272A, S258A, S324A and S360A. Mutagenesis was carried out with human wild-type cDNA as a template using methods previously described (Small et al., 2000b). Each receptor was epitope tagged at the amino-

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Table 1
Potential protein kinase C phosphorylation sites in the third intracellular loop of α_{2A} adrenoceptor

Sequence	Amino acid location	Note
KRR**T**RV	T227	
PP**S**RRG	S232	coupling defect
PER**S**AG	S258	
RA**S**QVK	S324	
KA**S**RWR	S360	
KRF**T**FV	T373	constitutively active receptor

Bolded **S** or **T** represent the potential sites. Mutations at two residues alter baseline coupling and these were not studied.

terminus with the influenza hemagglutinin nonapeptide as described (Jewell-Motz et al., 2000) and ultimately subcloned into the expression vector pBC12BI. The integrity of the constructs was verified by sequencing.

2.2. Transfection and cell culture

For stable expression of the wild-type and mutant α_{2A}-adrenoceptors, Chinese hamster ovary (CHO) cells in monolayers at ~30% confluence were cotransfected using a calcium precipitation method (Liang et al., 1998). Cells were transfected with 3 μg of pSV_2neo, which provides for G-418 resistance, and 20 μg of the wild-type or mutant constructs. Cells were selected in 1000 μg/ml G418, and clones were screened for expression of the wild-type or mutant α_{2A}-adrenoceptor using a [^{3}H]yohimbine binding assay as described below. African green monkey kidney (COS-7) cells were transfected using a DEAE-dextran method as described (Eason et al., 1995). Cells were maintained in medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3. Inositol phosphate assays

Inositol phosphate accumulation in whole cells was determined as described previously (Small et al., 2000a). Briefly, nearly confluent CHO cells stably expressing wild-type and mutant α_{2A}-adrenoceptors were incubated with [^{3}H]myo-inositol (5 μCi/ml) in media without fetal calf serum for 16–18 h at 37 °C in a 5% CO_2 atmosphere. Cells were then washed and incubated with warm PBS for 30 min, followed by incubation with 20 mM LiCl in phosphate buffered saline (PBS) for 30 min at 37 °C in a 5% CO_2 atmosphere. Cells were then treated with vehicle alone (basal) or 10 μM epinephrine for 5 min; for desensitization conditions, cells were first treated with 0.1 μM phorbol-12-myristate-13-acetate (PMA) for 2 min, then 10 μM epinephrine for 5 min. The reactions were terminated by aspiration of the media and addition of 0.4 M perchloric acid for 10 min at 25 °C. After neutralization of the extracts with KOH and $KHCO_3$, they were loaded on Dowex AG1-X8 resin formate columns, and total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

2.4. Radioligand binding assays

Expression levels of the wild-type and mutant α_{2A}-adrenoceptor were determined by radioligand binding with [^{3}H]yohimbine. Confluent cells expressing wild-type or mutant receptors in monolayers were washed three times with cold PBS and membranes were prepared by hypotonic lysis in cold buffer (5 mM Tris, 2 mM EDTA, pH 7.4) and scraping with a rubber policeman, followed by centrifugation at 42,000 × g for 10 min at 4 °C. The crude membrane pellets were then resuspended in a buffer which contained 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA. Saturation and competition binding assays were carried out as described previously (Eason and Liggett, 1996). Reactions were terminated by dilution with the cold 10 mM Tris, pH 7.4, followed by vacuum filtration over glass fiber filters.

2.5. Intact cell receptor phosphorylation

Whole cell phosphorylation studies were carried out in a manner similar to that described previously (Jewell-Motz et al., 2000). For these studies, COS-7 cells were transiently transfected in order to achieve expression levels of 10–15 pmol/mg of receptor. Briefly, cells expressing the wild-type or the indicated α_{2A}-adrenoceptor at equal expression levels were incubated with 0.5 mCi/ml [^{32}P]orthophosphate in 6-well plates for 1 h at 37 °C in 5% CO_2. Cells were then incubated with vehicle or 0.1 μM PMA for 5 min, washed twice with cold PBS, and scraped in a solubilization buffer consisting of 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 50 mM NaF, 10 mM sodium pyrophosphate, with the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml aprotinin, and 1 μg/ml pepstatin A. Particulates were solubilized at 4 °C for 1 h on a rotating wheel and non-solubilized material removed by centrifugation at 40,000 × g for 15 min at 4 °C. The supernatant was incubated with a 1:200 dilution of a monoclonal antibody to the HA-epitope (12CA5, Boehringer Mannheim, Indianapolis, IN) and Protein A/Sepharose CL 4B beads (Amersham Pharmacia, Chicago, IL) with 2% (w/v) bovine serum albumin for 3 h at 4 °C. The beads were washed three times in the above solubilization buffer, and the immune complexes were dissociated by incubation of the beads in SDS sample buffer at 37 °C for 1 h. Proteins were fractionated on 12.5% SDS-polyacrylamide gels and detection and quantitation of radioactive bands carried out on the dried gels using a Molecular Dynamics PhosphorImager™.

2.6. Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} release was quantitated by monitoring fluorescence of FURA-2 loaded cells as recently described (Dorn et al., 1997). Fluorescence ratios were converted to

Table 2
Radioligand binding parameters of the wild-type and mutated α_{2A}-adrenoceptors expressed in CHO cells

Receptor	[^{3}H]yohimbine		K_i		
	B_{max} (fmol/mg)	K_d (nM)	UK 14303 (nM)	Epinephrine (nM)	Phentolamine (nM)
WT	1508 ± 108	3.4 ± 0.76	128 ± 19	279 ± 83	10.3 ± 0.9
T227A	1314 ± 124	3.1 ± 0.26	100 ± 16	295 ± 81	6.9 ± 1.1
S258A	1428 ± 86	3.7 ± 1.17	128 ± 8	314 ± 125	11.9 ± 2.1
S324A	1183 ± 79	2.4 ± 0.24	74 ± 7	136 ± 23	5.7 ± 0.4
S360A	1529 ± 157	2.5 ± 0.14	138 ± 20	151 ± 5	6.9 ± 0.3

Shown are means ± S.E. of three or four experiments. B_{max}, receptor density; K_d, equilibrium constant; K_i, dissociation constant.

Ca^{2+} assuming a K_d for calcium binding to FURA-2 of 224 nM (Grynkiewicz et al., 1985). Loaded cells in 3.0 ml aliquots (~4×10^6 cells) at 37 °C were added to cuvettes and after baseline measurements were obtained, increases in intracellular Ca^{2+} in response to 10 μM of the α_2-adrenoceptor agonist 5-bromo-6-(2-imidazolin-2-yl-amino) quinozaline (UK14304) were determined over the ensuing 2 min. In some cases, cells were pre-incubated with 0.1 μM PMA for 1 min prior to addition of agonist.

2.7. Data analysis

Data are represented as mean ± S.E. Comparisons of results were by a two-way *t*-test, with significance imparted when $P < 0.05$. Curve fitting was carried out using GraphPad Prism Software (GraphPad, San Diego, CA). Desensitization was defined as follows:

$$\text{desensitization} = \left[1 - \frac{R_{PMA}}{R_{ctrl}}\right] \times 100\%$$

where R represents the agonist-stimulated response (either inositol phosphate accumulation or intracellular Ca^{2+} release) under control (R_{ctrl}) or after PMA exposure (R_{PMA}).

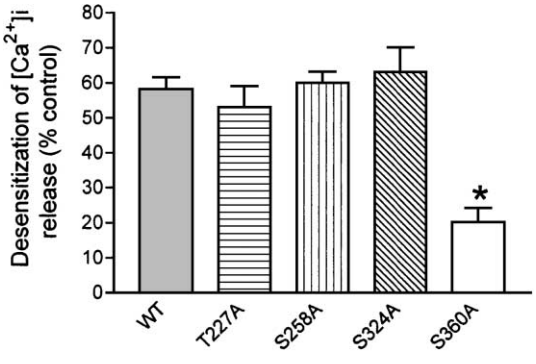

Fig. 1. Protein kinase C-promoted desensitization of wild-type and mutated α_{2A}-adrenoceptor stimulation of intracellular Ca^{2+} release. The mutant α_{2A}-adrenoceptors have potential protein kinase C phosphorylation sites substituted by Ala as indicated in Table 1 and were expressed in CHO cells. Cells were exposed to 0.1 μM PMA and agonist (UK14304) stimulated intracellular Ca^{2+} release determined as described in Section 2. Wild-type α_{2A}-adrenoceptors, and the T227A, S258A and S324A receptors all underwent equivalent degrees of desensitization. The S360A receptor underwent significantly less desensitization than the wild-type receptor (23 ± 3 vs. 57 ± 3%). Shown are mean results from five experiments. * $P < 0.01$.

3. Results

Stable cell lines were obtained expressing each of the indicated mutant α_{2A}-adrenoceptors depicted in Table 1. For the current studies, cell lines were chosen which had similar levels of expression (~1100–1500 fmol/mg). The results of radioligand binding studies are shown in Table 2. As can be seen, the K_d for [^{3}H]yohimbine as determined in saturation binding isotherms was not altered by the mutations. In addition, the affinities for the agonists epinephrine and UK14304, and the antagonist phentolamine, were the same for the four mutant receptors compared to the wild-type receptor.

To assess protein kinase C promoted desensitization of the α_{2A}-adrenoceptor, agonist-stimulated intracellular Ca^{2+} release and inositol phosphate accumulation were determined in whole cells treated with vehicle alone, or 0.1 μM PMA, for 2 min. The results for intracellular Ca^{2+} release are shown in

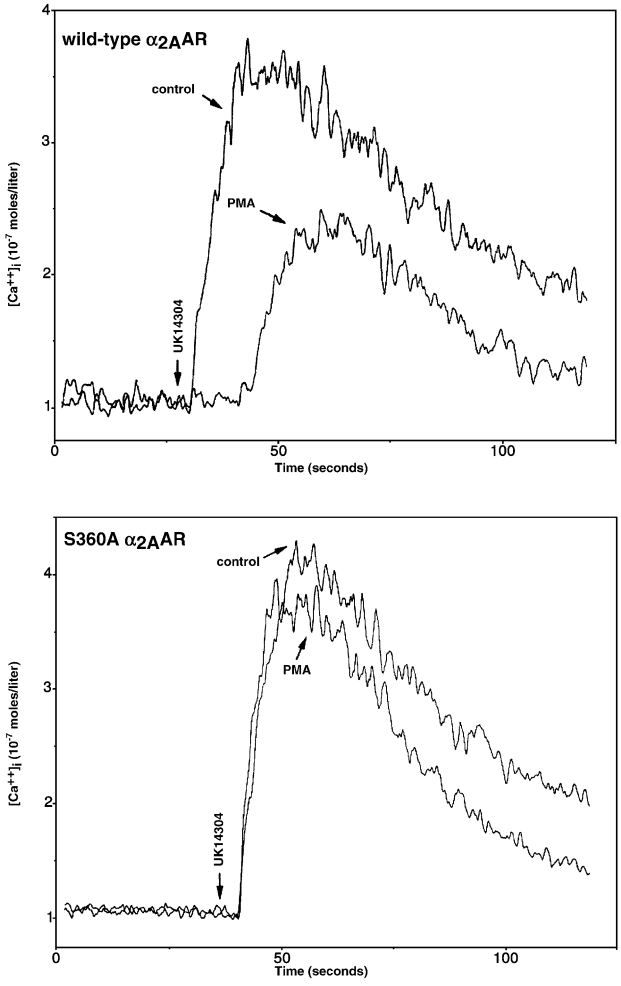

Fig. 2. The mutant S360A α_{2A}-adrenoceptor has impaired protein kinase C-mediated desensitization of intracellular Ca^{2+} release. Shown are agonist (UK14310) stimulated Ca^{2+} transients from a representative study of the wild-type and the S360A mutant α_{2A}-adrenoceptor in the absence (control) and presence of 0.1 μM PMA. See Table 2 for mean data.

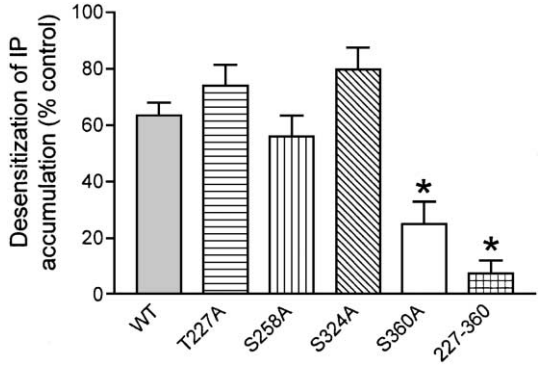

Fig. 3. Protein kinase C-promoted desensitization of wild-type and mutated α_{2A}-adrenoceptor stimulation of inositol phosphate accumulation. The mutant α_{2A}-adrenoceptors have potential protein kinase C phosphorylation sites substituted by Ala as indicated in Table 1 and were expressed in CHO cells. Cells were exposed to 0.1 μM PMA, and agonist (epinephrine) stimulated inositol phosphate accumulation determined as described in Section 2. The wild-type and the mutant T227A, S258A and S324A receptors all underwent equivalent degrees of desensitization. The S360A and the combination 227–360 receptors underwent significantly less desensitization than wild-type α_{2A}-adrenoceptor (25 ± 8.1 and 7 ± 4.6 vs. $64 \pm 4.5\%$). Shown are mean results from five experiments. * $P < 0.01$.

Figs. 1 and 2 and Table 2. For this response, PMA exposure resulted in ~60% decrease in agonist stimulated intracellular Ca^{2+} release for the wild-type α_{2A}-adrenoceptor, as well as the T272A, S258A, and S324A receptors. In contrast, the S360A receptor underwent only $23 \pm 3\%$ desensitization ($P < 0.01$ vs. wild-type receptor, Figs. 1 and 2). In CHO cells, the intracellular Ca^{2+} evoked by α_2-adrenoceptor activation is due to G_i $\beta\gamma$ activation of phospholipase C, leading to inositol 1,4,5-trisphosphate (IP_3) generation and its consequent binding to an IP_3 receptor and release of Ca^{2+} from intracellular stores (Dorn et al., 1997). We thus expected that the defective PMA-promoted desensitization of intracellular Ca^{2+} signaling observed with the S360A mutant would also be seen when inositol phosphate accumulation was measured. As is shown in Fig. 3, this turned out to be the case. The wild-type α_{2A}-adrenoceptor underwent $63 \pm 4.5\%$ desensitization of agonist-promoted inositol phosphate accumulation in response to PMA pretreatment. Three of the mutant receptors displayed desensitizations between 56% and 79%, which were not statistically different from the wild-type desensitization. In contrast, the S360A receptor

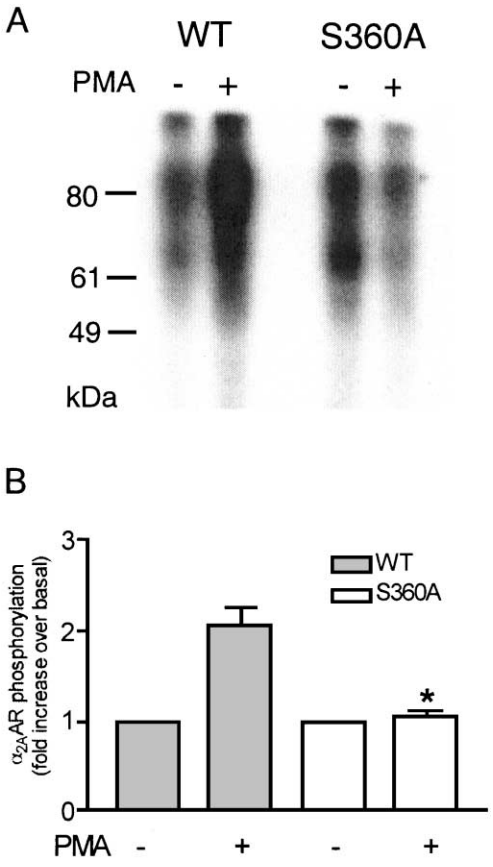

Fig. 4. The mutant S360A α_{2A}-adrenoceptor fails to undergo protein kinase C-promoted receptor phosphorylation. In (A), a representative autoradiogram is shown. In (B), the mean results from three experiments are summarized. * $P < 0.01$.

underwent only $24.8 \pm 8.1\%$ desensitization of inositol phosphate accumulation due to PMA pretreatment ($P < 0.01$ vs. wild-type receptor). These results are quantitatively very similar to the intracellular Ca^{2+} accumulation studies shown in Fig. 1 (Table 3).

These signaling studies strongly indicate that the site of protein kinase C phosphorylation which has functional relevance within the α_{2A}-adrenoceptor is S360. To confirm that S360 is indeed phosphorylated by protein kinase C, whole cell phosphorylation studies were carried out. Cells were incubated with [^{32}P]orthophosphate, exposed to vehicle

Table 3
Desensitization of wild-type and mutated α_{2A}-adrenoceptor expressed in CHO cells

Receptor	Calcium release			Inositol phosphate turnover		
	− PMA (nM)	+ PMA (nM)	Desensitization (%)	− PMA (% over basal)	+ PMA (% over basal)	Desensitization (%)
WT	431 ± 80	206 ± 5	57 ± 3.5	46.0 ± 4.5	16.8 ± 2.6	63 ± 4.5
T227A	413 ± 51	196 ± 40	53 ± 6.0	26.5 ± 3.5	6.5 ± 1.5	74 ± 7.4
S258A	668 ± 67	253 ± 37	60 ± 3.2	43.1 ± 4.0	19.6 ± 4.2	56 ± 7.3
S324A	534 ± 102	213 ± 67	63 ± 7.0	39.9 ± 4.9	10.3 ± 3.3	79 ± 8.3
S360A	344 ± 37	266 ± 36	23 ± 3.2[a]	32.7 ± 2.3	24.5 ± 2.8	24 ± 8.1[a]

Data shown are means ± S.E.M. from experiments described in Figs. 2 and 3.
[a] $P < 0.01$.

or 0.1 μM PMA for 5 min, and receptor purified by immunoprecipitation using an antibody to the HA epitope tag which was engineered at the amino-terminus of each receptor. As shown in Fig. 4, an ~2-fold increase in phosphorylation of wild-type α_{2A}-adrenoceptor was evident with PMA exposure. In contrast, no detectable phosphorylation over baseline was observed with the S360A mutant receptor. Finally, we were intrigued by the limited degree of remaining desensitization (~24%) present with the S360 mutant and wondered whether there could be small contributions by the other potential protein kinase C sites. Since PMA-promoted phosphorylation was completely abolished in the S360A mutant, no additional information could be expected by studying combination mutations in phosphorylation assays. However, functional studies offered such an opportunity. We therefore constructed one receptor (denoted "272–360") encompassing all four mutations. In response to PMA exposure, this receptor underwent $7 \pm 4.6\%$ ($P < 0.01$ vs. wild-type) desensitization of agonist-promoted inositol phosphate accumulation (Fig. 3).

4. Discussion

In this work, a phosphorylation site within the α_{2A}-adrenoceptor has been identified which confers the major functional effect of protein kinase C on receptor desensitization. We concentrated on the third intracellular loop of the receptor, which we and others have previously shown to contain major determinants of G protein coupling or binding (Dalman and Neubig, 1991; Eason and Liggett, 1995, 1996). The α_{2A}-adrenoceptor couples via the G_i/G_o family of G proteins, and some functions, such as inhibition of adenylyl cyclase, are mediated by the $G_{\alpha i}$ subunits. In CHO cells, inhibition of adenylyl cyclase is a highly efficient coupling pathway and overexpression results in substantial receptor reserve (Liang et al., 2001). Consequently, desensitization is difficult to quantitate when examining inhibition of adenylyl cyclase as the signal. The released $\beta\gamma$ from G_i heterotrimers activates phospholipase C-β, resulting in generation of IP_3 and diacylglycerol. The former, binding to an IP_3 receptor, acts to release intracellular Ca^{2+}. The above pathway appears to have less amplification and is of lower efficiency than the adenylyl cyclase pathway, and receptor overexpression of 1–2 pmol/mg does not result in substantial receptor reserve (Liang et al., 1998). Consequently, measurements of inositol phosphate accumulation and intracellular Ca^{2+} release provides for a sensitive way to quantitate functional impairment of α_2-adrenoceptor provoked by modifications such as receptor phosphorylation.

We showed that substitution of Ser at amino acid position 360 ablated protein kinase C mediated phosphorylation of the receptor. Consistent with phosphorylation at this site having functional relevance, the S360A mutant had markedly diminished PMA-promoted desensitization. Interestingly, this mutant did display some degree of desensitization. However, the extent amounted to only a 24% decrease in receptor function after PMA pretreatment as compared to 60–80% desensitization with the wild-type, or the other mutant, α_{2A}-adrenoceptors. We considered that there may be some small contribution(s) from the other potential sites. However, the large number of unique receptor mutant combinations of 2–4 altered sites, and the relatively small residual level of desensitization makes it unfeasible to attempt to delineate which of these, if any, can be attributed to this remaining desensitization. Nevertheless, since the mutant lacking all four sites had a desensitization of only ~10%, we must conclude that there is likely a minor contribution of one or more of these other sites to protein kinase C-promoted desensitization. In a recent study (Liang et al., 2001) we found that substitution of the Ser at position 232 to Ala markedly altered baseline coupling to inositol phosphate stimulation and decreased PMA-promoted receptor phosphorylation. So, we cannot exclude a somewhat atypical role for S232 in receptor coupling and/or protein kinase C-mediated modulation of α_{2A}-adrenoceptor function.

The Ser at amino acid 360 which we have identified as a protein kinase C phosphorylation site lies within the carboxy-terminal region of the large third intracellular loop of the α_{2A}-adrenoceptor, ~16 amino acids from the sixth transmembrane spanning domain. By the use of synthetic peptide inhibitors, it has been shown that this region is important for binding of the receptor to G_i (Dalman and Neubig, 1991). Similarly, chimeric mutagenesis studies have revealed that this region is necessary for fully functional G_i coupling (Eason and Liggett, 1996). The current work is thus entirely consistent with these concepts, in that this post-translational modification by protein kinase C phosphorylation in this critical G protein-coupling region indeed results in significant functional uncoupling.

In summary, we have shown that a Ser at amino acid position 360 within the carboxy terminal region of the third intracellular loop of the α_{2A}-adrenoceptor is phosphorylated by protein kinase C. This phosphorylation results in desensitization of agonist-promoted receptor function as delineated by inositol phosphate accumulation and intracellular Ca^{2+} release. Since many signaling pathways culminate in activation of protein kinase C, this is one mechanism by which desensitization of α_{2A}-adrenoceptor function by other receptors occurs (Liang et al., 1998). In addition, a host of pathologic processes are accompanied by protein kinase C activation (Meier and King, 2000; Naruse and King, 2000), which based on the current study could subsequently act to depress α_{2A}-adrenoceptor function by this heterologous mechanism.

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