Br. J. clin. Pharmac. (1990), **30**, 3S–12S

Synthetic peptides of the hamster β_2-adrenoceptor as substrates and inhibitors of the β-adrenoceptor kinase

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1 The β-adrenoceptor is one of a number of G protein-coupled receptors which have been proposed to contain seven transmembrane α-helices. The function of this receptor appears to be regulated by phosphorylation by a specific enzyme, the β-adrenoceptor kinase. Synthetic peptides which comprise each of the proposed intra- and extracellular domains of the β_2-adrenoceptor have been tested as potential substrates and inhibitors of the β-adrenoceptor kinase.
2 Two peptides which encompass the middle and terminal portions of the carboxyl tail of the receptor served as substrates by β-adrenoceptor kinase. The kinetics of the phosphorylation reaction, however, suggest that these peptides are 10^6-fold poorer substrates than the agonist occupied receptor.
3 A number of synthetic peptides also served as inhibitors of β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase. In particular, a peptide which comprised the first intracellular loop of the β_2-adrenoceptor (amino acids 56-74) inhibited most effectively with an IC_{50} of 40 μM.
4 These results suggest that multiple intracellular regions of the β-receptor may serve as potential sites of interaction with β-adrenoceptor kinase. Moreover, these regions may serve as potential targets for the development of specific inhibitors of β-adrenoceptor kinase which could be used to block homologous desensitization.

Keywords Receptors desensitization phosphorylation kinases inhibitors

Introduction

The β-adrenoceptor is a ubiquitous integral membrane glycoprotein which mediates stimulation of adenylyl cyclase via its agonist-promoted interaction with the stimulatory guanine nucleotide regulatory protein, G_s (Stiles *et al.*, 1984). Recent cloning of the gene for the hamster β_2-adrenoceptor suggests that the receptor structure contains seven transmembrane α-helices which are interconnected by intra- or extracellular loops (Dixon *et al.*, 1986). The extracellular amino-terminus contains two potential sites of *N*-linked glycosylation while the intracellular third loop and carboxyl terminus contain potential sites of regulatory phosphorylation. This overall topology has also been proposed for a large number of other G-protein coupled receptors including rhodopsin, the β_1-, α_1- and α_2-adrenoceptors, the M_1–M_5 mus-

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carinic receptors as well as a number of others (Dohlman *et al.*, 1987a; O'Dowd *et al.*, 1989).

Multiple G-protein coupled receptors also appear to be regulated by phosphorylation (Benovic *et al.*, 1988). These include the β_2-adrenoceptor which is phosphorylated *in vitro* by the cAMP-dependent protein kinase (Benovic *et al.*, 1985), by protein kinase C (Bouvier *et al.*, 1987) or by a more specific enzyme, the β-adrenoceptor kinase (Benovic *et al.*, 1986). Rhodopsin, the 'receptor' for light, also appears to be phosphorylated and regulated by a specific enzyme, rhodopsin kinase (Kühn, 1981). In addition, evidence suggests that the chick cardiac muscarinic receptor is phosphorylated in response to agonist-occupancy (Kwatra & Hosey, 1986) possibly by β-adrenoceptor kinase (Kwatra *et al.*, 1989) while the α_1-adrenoceptor appears to be phosphorylated in a feedback form of regulation by protein kinase C (Leeb-Lundberg *et al.*, 1985, 1987). Agonist-promoted phosphorylation may serve as a general mechanism for regulating receptor responsiveness. For the β_2-adrenoceptor, agonist-dependent phosphorylation by β-adrenoceptor kinase may be responsible for mediating rapid homologous desensitization of adenylyl cyclase activity (Benovic *et al.*, 1988).

While much work has focused on characterizing β-adrenoceptor phosphorylation by β-adrenoceptor kinase the sites of phosphorylation on the receptor remain poorly defined. Moreover, the mechanism(s) by which the kinase specifically phosphorylates the agonist-occupied receptor remains a mystery. In order to approach these issues in the present work we have used synthetic peptides derived from the β_2-adrenoceptor to 1) define the β-adrenoceptor kinase phosphorylation domains on the receptor, 2) localize potential regions of receptor/kinase interaction and 3) identify peptide substrates and inhibitors to characterize further the enzyme.

Methods

Materials

Most chemicals were from sources previously described (Benovic *et al.*, 1984, 1987a). Peptides were synthesized by tBOC chemistry on an Applied Biosystems 430A Synthesizer. Peptides were deblocked by HF treatment and were purified by reversed phase high performance liquid chromatography on a C18 column using a 0–50% acetonitrile gradient.

Purification and reconstitution of the β-adrenoceptor

The β_2-adrenoceptor from hamster lung was purified to apparent homogeneity by sequential affinity and high performance liquid chromatography as described (Benovic *et al.*, 1984). The purified receptor was reinserted into phosphatidylcholine vesicles as previously described (Cerione *et al.*, 1983). The protein-lipid pellets were resuspended in 20 mM Tris-HCl, pH 7.2, 2 mM EDTA and used as a substrate for the β-adrenoceptor kinase.

Purification of the β-adrenoceptor kinase

β-Adrenoceptor kinase was purified from bovine cerebral cortex by modification of a previously described procedure (Benovic *et al.*, 1987a). Briefly, 250 g of bovine cortex was homogenized and the resulting high speed supernatant fraction was precipitated with 13–26% ammonium sulphate. This material was initially chromatographed on an Ultrogel AcA34 column equilibrated with 5 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 15 μg ml^{-1} benzamidine, 0.2 mM phenylmethyl sulphonyl fluoride (buffer A). The peak activity was diluted 1:1 with buffer A containing 0.02% triton X-100 and applied to a DEAE Sephacel column. Elution was accomplished with a 0–80 mM NaCl gradient in buffer A containing triton X-100. The peak activity was then applied directly to a CM Fractogel column and eluted with a 0–100 mM NaCl gradient in buffer A containing triton X-100. The purified kinase was stored at 4° C.

Phosphorylation of the β-adrenoceptor by β-adrenoceptor kinase

Reconstituted β-adrenoceptor (0.5–5 pmol/incubation) was incubated with β-adrenoceptor kinase (10–100 ng) in a total volume of 10–40 μl which also contained 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM sodium phosphate, 0.5 mM ascorbic acid and 0.15 mM [γ-^{32}P]ATP (1–5 counts min^{-1} $fmol^{-1}$). Samples were incubated at 30° C for the time indicated in the figure legends. Some incubations also contained 50 μM (-)isoprenaline. Incubations were stopped by the addition of 50 μl of SDS sample buffer followed by electrophoresis on 10% homogeneous polyacrylamide gels. Phosphorylated β-adrenoceptor was visualized by autoradiography and the corresponding bands were excised and counted to determine the pmol of phosphate incorporated.

Phosphorylation of synthetic β-adrenoceptor peptides by β-adrenoceptor kinase

Synthetic peptides derived from the hamster lung β_2-adrenoceptor were incubated with β-adrenoceptor kinase (50–100 ng) in a buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.10 mM [γ-^{32}P]ATP (1–5 counts min^{-1} $fmol^{-1}$) at 30° C for the time indicated in the figure legends. The peptide concentrations varied from 0.6–5 mM. Peptides were separated from free ATP by one of several methods. In most experiments the reactions were stopped by the addition of 50 μl of SDS sample buffer or SDS urea sample buffer followed by electrophoresis on either a 15% homogeneous polyacrylamide gel or a 9% polyacrylamide gel containing 6.5 urea (see below). Following autoradiography the phosphorylated peptides were excised and counted to determine the pmol of phosphate incorporated. In some experiments, samples were separated by reversed phase high performance liquid chromatography on a C18 column (Kuenzel & Krebs, 1985). Following injection of the sample the column was washed with 30 ml of buffer (0.1 M sodium phosphate, pH 6.5, 0.1 M NaCl) before elution with a linear gradient from 0 to 50% acetonitrile. An alternative method involved direct application of the sample to phosphocellulose paper followed by extensive washing in 75 mM H_3PO_4 (Cook *et al.*, 1982). While this procedure gave comparable results for the CT-2 peptide, the CT-3 peptide did not appear to bind appreciably to the phosphocellulose paper.

Synthetic peptide inhibition of substrate phosphorylation by β-adrenoceptor kinase

Reconstituted β-adrenoceptor (0.2–1 pmol/incubation) was incubated with β-adrenoceptor kinase (10–30 ng) in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM sodium phosphate, 50 μM (-)isoprenaline, 0.10 mM [γ^{32}P]ATP (1–3 counts min^{-1} $fmol^{-1}$) at 30° C for 30 min. Synthetic β_2-adrenoceptor peptides were varied in concentration from 0 to 1 mM. Reactions were quenched by the addition of SDS sample buffer followed by gel electrophoresis. In some experiments urea treated rod outer segments (Shichi & Somers, 1978; Wilden & Kühn, 1982) were used as the substrate for β-adrenoceptor kinase. To define further the specificity of the peptide inhibition, phosvitin, casein (Benovic *et al.*, 1989) and the synthetic peptides CT-2 and CT-3 were used as substrates for β-adrenoceptor kinase.

Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) using 10 or 15% homogeneous slab gels. SDS sample buffer contained 8% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.5 and 0.003% bromphenol blue. SDS urea polyacrylamide gel electrophoresis was carried out using 6.5 M urea, 0.1% SDS, 100 mM H_3PO_4, pH 6.8 with Tris-HCl and 9% acrylamide (Swank & Munkres, 1971). SDS urea sample buffer contained 1% SDS, 8 M urea, 10 mM H_3PO_4, pH 6.8 with Tris-HCl, 1% β-mercaptoethanol and 0.003% bromphenol blue.

Results

Figure 1 presents the amino acid sequence and proposed topology of the hamster lung β_2-adrenoceptor. The synthetic peptides utilized in this study are highlighted and encompass the amino and carboxyl termini as well as the first, second and third extracellular and first, second and third intracellular loops.

Our initial studies focused on determining whether any of these 11 peptides could serve as substrates for β-adrenoceptor kinase. The results shown in Figure 2 demonstrate that two of the carboxyl terminal peptides (CT-2 and CT-3) are phosphorylated by β-adrenoceptor kinase. These peptides are, however, much poorer substrates than the agonist-occupied β_2-adrenoceptor even when present at a 100,000 fold higher concentration. Phosphoamino acid analysis of the phosphorylated peptides reveals that CT-2 contains solely phosphoserine while CT-3 contains predominantly phosphoserine with some phosphothreonine (data not shown). None of the other peptides tested is phosphorylated by β-adrenoceptor kinase, however, two of these peptides (CIII-2 and CT-1), which contain a consensus sequence for cAMP dependent protein kinase phosphorylation, serve as substrates for protein kinase A (Bouvier *et al.*, 1989).

The kinetics of β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase in the presence or absence of the β-adrenoceptor agonist isoprenaline are shown in Figure 3a. It is evident that the major agonist-promoted difference in the phosphorylation of the receptor is in the V_{max}, with the agonist-occupied receptor having an ~6 fold higher V_{max} (37 *vs* 6.6 nmol Pi min^{-1} mg^{-1}, Table 1). In contrast, the K_m of the receptor varies only 1.8-fold (0.16 *vs* 0.29 μM). Overall, the agonist-occupied receptor is an ~10

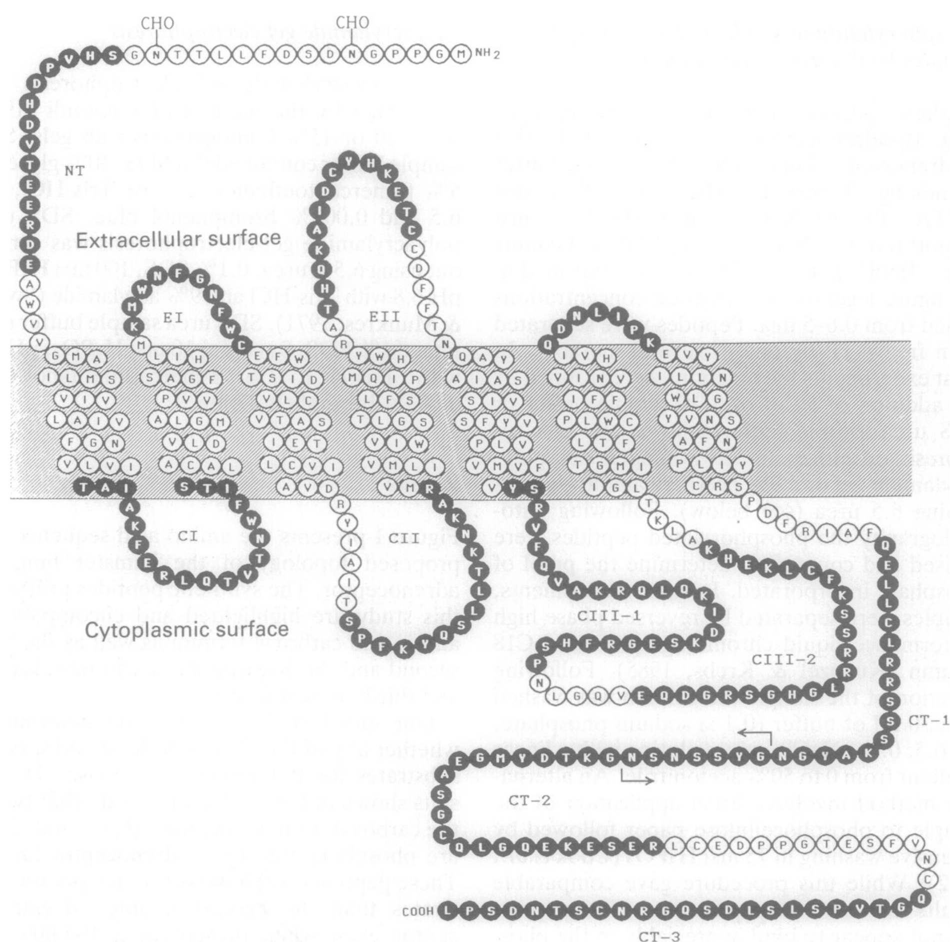

Figure 1 Model for the organization of the hamster β_2-adrenoceptor in the plasma membrane. The amino acid sequence and proposed structure of the hamster β_2-adrenoceptor in the plasma membrane (Dixon *et al.*, 1986). The synthetic peptides used in this study are bracketed and labelled: NT—amino terminus; CI, CII, CIII—first, second and third intracellular loops; EI, EII, EIII—first, second and third extracellular loops; CT-1, CT-2, CT-3—carboxyl terminus.

Table 1 β-Adrenoceptor phosphorylation of β_2-adrenoceptor and synthetic β_2-adrenoceptor peptides
Experimental conditions are as described in Figures 3a and 3b. The results for peptides CT-2 and CT-3 are presented as the mean ± s.e. mean from a total of six experiments. The results for β_2-adrenoceptor + ISO and β_2-adrenoceptor are from a single experiment

Substrate	K_m (*μM*)	V_{max} (*nmol min^{-1} mg^{-1}*)	V_{max}/K_m	*Ratio*
β_2-adrenoceptor + ISO	0.16	37	231	1
β_2-adrenoceptor	0.29	6.6	23	10^{-1}
CT-2	3080 ± 770	2.7 ± 0.7	8.8×10^{-4}	3.8×10^{-6}
CT-3	3900 ± 1280	2.5 ± 0.5	6.4×10^{-4}	2.8×10^{-6}

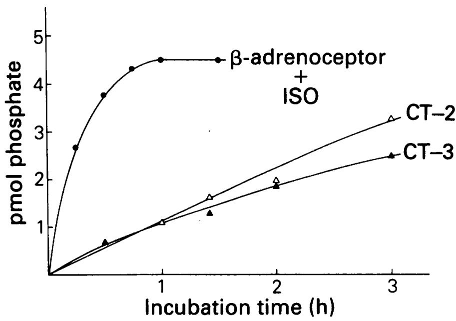

Figure 2 Time course of phosphorylation of β_2-adrenoceptor and synthetic peptides by β-adrenoceptor kinase. Reconstituted hamster lung β_2-adrenoceptor (~0.5 pmol) was phosphorylated by β-adrenoceptor kinase (~0.1 μg) in the presence of 50 μM (-)isoprenaline (●). Two synthetic carboxyl terminal peptides of the hamster lung β_2-adrenoceptor (CT-2 (△), CT-3 (▲)) were also phosphorylated by β-adrenoceptor kinase under identical conditions. Following the incubation period the reactions (20 μl) were stopped by addition of 50 μl of SDS sample buffer followed by electrophoresis on a 10% (β_2-adrenoceptor) or 15% (CT-2, CT-3) homogeneous polyacrylamide gel.

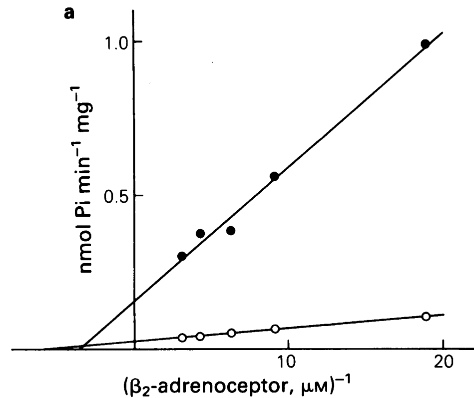

Figure 3a Kinetic parameters of the β-adrenoceptor kinase for β_2-adrenoceptors. Reconstituted β_2-adrenoceptor (0.05–0.3 μM) was incubated for 15 min at 30° C with purified β-adrenoceptor kinase (~20 ng) in the presence (○) or absence (●) of 50 μM (-)isoprenaline. Other conditions are outlined in **Methods**. Reactions were stopped by the addition of SDS sample buffer before electrophoresis on a 10% SDS polyacrylamide gel. ^{32}P-labelled receptor was determined by cutting and counting the dried gel following autoradiography.

fold better substrate than the unoccupied receptor as assessed by the V_{max}/K_m ratio. In contrast, the phosphorylation of the synthetic β_2-adrenoceptor peptides by β-adrenoceptor kinase have strikingly different kinetics as compared with the receptor (Figure 3b). The carboxyl peptide CT-2 is phosphorylated by β-adrenoceptor kinase with a K_m 3.1 ± 0.8 mM and a V_{max} 2.7 ± 0.7 nmol Pi min^{-1} mg^{-1} while CT-3 has a K_m 3.9 ± 1.3 mM and a V_{max} 2.5 ± 0.5. A comparison of the V_{max}/K_m ratios demonstrates that the agonist-occupied receptor is an ~3 × 10^5 fold better substrate than the carboxyl peptides (Table 1). The major difference between the substrates (receptor *vs* peptides) is in the K_m obtained, with a 10,000–24,000 fold difference.

Overall, these results demonstrate that the intact β_2-adrenoceptor serves as a much better substrate for β-adrenoceptor kinase than the peptides, suggesting that the secondary or tertiary structure of the receptor is important for kinase recognition. These results may also suggest that β-adrenoceptor kinase recognizes other regions of the receptor in addition to the phosphorylation sites at the carboxyl terminus.

In an attempt to address whether other regions of the β_2-adrenoceptor might interact with β-adrenoceptor kinase we tested the synthetic peptides as potential inhibitors of β_2-adrenoceptor phosphorylation. As shown in Table 2 a number of synthetic peptides are potent inhibi-

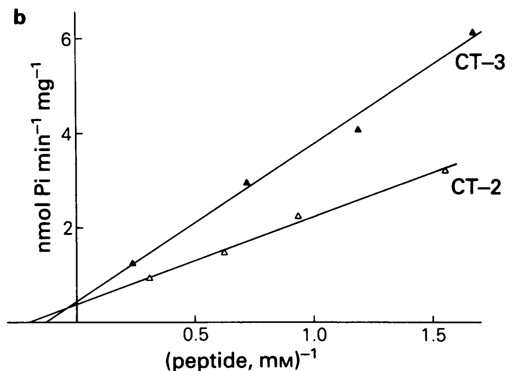

Figure 3b Kinetic parameters of the β-adrenoceptor kinase for synthetic peptides CT-2 and CT-3. The synthetic β_2-adrenoceptor peptides CT-2 (0.6–3.2 mM) and CT-3 (0.8–4.2 mM) were incubated for 60 min at 30° C with purified β-adrenoceptor kinase (94 ng). Other conditions are outlined in **Methods**. Reactions were stopped by the addition of SDS urea sample buffer before electrophoresis on a 9% SDS urea polyacrylamide gel. ^{32}P-labelled peptide was determined by cutting and counting the gel following autoradiography.

tors of β-adrenoceptor phosphorylation by β-adrenoceptor kinase. These include the first intracellular loop (CI) which has an IC_{50} ~40 μM as well as the second and third intracellular loops (CII, CIII-1 and CIII-2) and the carboxyl terminus (CT-1 and CT-2) which have IC_{50}s ranging

Table 2 Synthetic peptides of the hamster β_2-adrenoceptor as inhibitors of β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase

Reconstituted β_2-adrenoceptor was incubated for 30 min at 30° C with purified β-adrenoceptor kinase in the presence of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM sodium phosphate, 50 μM (-)isoprenaline and 0.10 mM [γ^{32}P]ATP. Synthetic β_2-adrenoceptor peptides were varied from 0 to 500 μM. Reactions were stopped by the addition of SDS buffer followed by polyacrylamide gel electrophoresis. ^{32}P-labelled receptor was determined by cutting and counting the dried gel following autoradiography

Peptide		IC_{50} (μM)
NT	(17–32)	ND
CI	(56–74)	40
EI	(97–106)	300
CII	(137–151)	240
EII	(177–190)	ND
CIII-1	(219–243)	76
CIII-2	(248–268)	208
EIII	(299–305)	ND
CT-1	(337–355)	320
CT-2	(353–381)	142
CT-3	(396–418)	ND

ND—no significant inhibition when tested at a concentration of ~500 μM.

from 76–320 μM. Several peptides do not inhibit the phosphorylation when tested at a concentration of 500 μM (NT, EII, EII, CT-3). These results suggest that β-adrenoceptor kinase may interact with multiple intracellular regions of the receptor, perhaps explaining the ~10,000 fold difference in K_m between the intact receptor and synthetic peptides for phosphorylation by β-adrenoceptor kinase (Table 1).

Since the first intracellular loop β_2-adrenoceptor peptide was the most potent inhibitor of β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase the specificity of this inhibition was studied further. As the length of the peptide decreases the ability to inhibit β-adrenoceptor kinase also decreases (Table 3). In particular shortening of the peptide from 15 to 11 amino acids results in a dramatic loss of the inhibition (IC_{50} increases from 62 to 1600 μM). This suggests that one or more of the four amino acids removed (A, I, Y, F) might be critical for the inhibition. In addition, several mutant peptides with amino acid substitutions have also been synthesized. A peptide with Leu^{64} and Asn^{69} both changed to alanine does not appear to affect the inhibition. Converting Lys^{60} and Arg^{63} to alanine also does not significantly affect the inhibition. In contrast, a peptide which has Glu^{62} changed to glutamine has significantly lower affinity as an inhibitor (IC_{50} from 62 to 280 μM) suggesting that the glutamic acid is important in kinase interaction. Also shown in Table 3 are results from studies with peptides derived from the first intracellular loops of the β_1-adrenoceptor, the α_2-adrenoceptor and rhodopsin. It is evident that these peptides do not significantly inhibit β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase. Interestingly the β_1-adrenoceptor peptide differs by only four amino acids (out of 15) from the β_2-adrenoceptor peptide. However, one of these amino acids is Glu^{62} which appears to be important for inhibition.

Although detailed studies have not been performed, several of the synthetic β_2-adrenoceptor peptides are also able to inhibit the phosphorylation of rhodopsin by β-adrenoceptor kinase with CI being the most potent. In addition,

Table 3 Comparison of first intracellular loop peptides as inhibitors of β-adrenoceptor kinase

Reconstituted β_2-adrenoceptor was incubated for 30 min at 30° C with purified β-adrenoceptor kinase in the presence of various concentrations of the peptides. Synthetic peptides were varied from 0 to 4000 μM. Reactions were performed as described in **Methods**

Peptide	*Sequence*	IC_{50} (μM)
β_2-adrenoceptor (56–74)	TAIAKFERLQTVTNYFITS	40
β_2-adrenoceptor (57–71)	AIAKFERLQTVTNYF	62
β_2-adrenoceptor (59–69)	AKFERLQTVTN	1600
β_2-adrenoceptor (60–66)	KFERLQT	2600
β_2-adrenoceptor (57–71, L→A, N→A)	AIAKFERAQTVTAYF	43
β_2-adrenoceptor (57–71, K→A, R→A)	AIAAFEALQTVTNYF	88
β_2-adrenoceptor (57–71, E→Q)	AIAKFQRLQTVTNYF	280
β_1-adrenoceptor	AIAKTPRLQTLTNLF	700
α_2-adrenoceptor	AVFTSRALKAPQNLF	>1000
Rhodopsin	VTVQHKKLRTPLNYI	>1000

further confirmation of the specificity of the peptide inhibition is the observation that the peptides did not inhibit β-adrenoceptor kinase mediated phosphorylation of non-receptor substrates such as casein and phosvitin. Moreover, the first, second and third intracellular loop peptides did not inhibit β-adrenoceptor kinase phosphorylation of the carboxyl terminal β-adrenoceptor peptides (data not shown).

Discussion

In this work we have utilized synthetic β_2-adrenoceptor peptides to characterize receptor phosphorylation by β-adrenoceptor kinase. Two carboxyl terminal peptides (353–381 and 396–418) which serve as specific substrates for β-adrenoceptor kinase were identified. These peptides are much poorer substrates than the intact receptor, however. Several synthetic peptides derived from presumed intracellular domains of the β_2-adrenoceptor effectively inhibit receptor phosphorylation by β-adrenoceptor kinase. These results suggest that β-adrenoceptor kinase interaction with multiple intracellular domains of the β_2-adrenoceptor serves to target the receptor for phosphorylation at the carboxyl terminus.

A role for the involvement of receptor phosphorylation in homologous desensitization was initially suggested by studies on intact frog erythrocytes where it was demonstrated that agonist-induced desensitization was associated with stoichiometric (approximately 2 mol Pi mol^{-1} β-adrenoceptor) phosphorylation of the β_2-adrenoceptor (Sibley *et al.*, 1985). Similar results have been observed in S49 lymphoma cells (Strasser *et al.*, 1986). Moreover, in mutant S49 cells which lack an agonist-promoted cAMP-dependent protein kinase response, receptor phosphorylation and desensitization are also observed. These results strongly implicated a cAMP-independent pathway in homologous desensitization.

The identification of a novel protein kinase which specifically phosphorylated only the agonist-occupied form of the β_2-adrenoceptor suggested a potential involvement in homologous desensitization (Benovic *et al.*, 1986). This kinase, termed the β-adrenoceptor kinase, is distinct from the cAMP-, cGMP-, Ca^{2+}/calmodulin- and Ca^{2+}/phospholipid-dependent protein kinases. β-Adrenoceptor kinase does not phosphorylate general kinase substrates such as histones. However, the agonist-occupied β_2-adrenergic, α_2-adrenergic (Benovic *et al.*, 1987b) and muscarinic acetylcholine receptors (Kwatra *et al.*, 1989) all serve as excellent substrates. Thus, β-adrenoceptor kinase may be involved in phosphorylating and regulating the activity of multiple adenylyl cyclase-coupled receptors.

An analogous regulatory pathway appears to modulate the retinal light-activated phosphodiesterase system. This system is composed of a receptor, rhodopsin, which is coupled through the G protein transducin to activation of a cGMP phosphodiesterase (Stryer, 1986). Light activation of rhodopsin also leads to phosphorylation of the receptor by a specific enzyme, rhodopsin kinase (Kühn, 1984). Rhodopsin phosphorylation serves as a stop signal for phosphodiesterase activation, however, this effect appears to require the interaction of an additional protein, variously termed arrestin, 48K protein or S-antigen, with the phosphorylated rhodopsin (Wilden *et al.*, 1986). The sites of phosphorylation on rhodopsin appear to be predominantly localized at the carboxyl terminus (Wilden & Kühn, 1982). However, additional evidence suggests that a region in loop V–VI may also be phosphorylated by rhodopsin kinase (McDowell *et al.*, 1985). Additional work utilizing synthetic rhodopsin peptides supports the notion that most of the phosphorylation sites are at the carboxyl terminus (Palczewski *et al.*, 1988).

The β_2-adrenoceptor can be phosphorylated *in vitro* by three distinct kinases, the cAMP-dependent kinase, protein kinase C and β-adrenoceptor kinase. The cAMP-dependent protein kinase phosphorylates the β_2-adrenoceptor to a stoichiometry of 2 mol mol^{-1} (Benovic *et al.*, 1985) presumably at the two consensus sequence sites (RRXS) located in the third intracellular loop and carboxyl tail of the receptor (Dixon *et al.*, 1986). Protein kinase C can also phosphorylate the β_2-adrenoceptor at sites similar to or identical with those phosphorylated by the cAMP-dependent kinase (Bouvier *et al.*, 1987). β-adrenoceptor kinase multiply phosphorylates the β_2-adrenoceptor to a stoichiometry of ~8 mol mol^{-1} (Benovic *et al.*, 1987a), however, the sites of phosphorylation remain poorly defined. Dohlman *et al.* (1987b) have demonstrated that carboxypeptidase Y treatment of reconstituted β-adrenoceptor kinase phosphorylated β_2-adrenoceptor, which removes the carboxyl terminal ~90 amino acids of the receptor, results in the loss of most, if not all, of the [^{32}P]phosphate from the receptor. Our results with synthetic peptides now further strengthen the assignment of the sites of β-adrenoceptor kinase catalyzed phosphorylation to the carboxyl terminus of the receptor.

The identification of peptides which inhibit

β-adrenoceptor kinase has important implications for elucidating further the mechanism of receptor phosphorylation and desensitization. Specific inhibitors of β-adrenoceptor kinase should enable a direct assessment of the role of β-adrenoceptor kinase in homologous desensitization. We have, in fact, recently demonstrated that heparin, which inhibits β-adrenoceptor kinase with an IC_{50} ~10 nM (Benovic *et al.*, 1989), inhibits β_2-adrenoceptor phosphorylation and desensitization at comparable concentrations in permeabilized A431 cells (Lohse *et al.*, 1989). Moreover, the first intracellular loop β_2-adrenoceptor peptide (57–71), which inhibits β-adrenoceptor kinase with an IC_{50} ~60 μM, also inhibits homologous desensitization in this system (Lohse *et al.*, 1989). These results provide direct evidence for the involvement of β-adrenoceptor kinase in homologous desensitization.

The finding that several β_2-adrenoceptor peptides inhibit β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase suggests that multiple regions of the receptor interact with the kinase. These regions may include the three intracellular loops (predominantly the first and third) as well as the carboxyl terminus. Further evidence for such speculation resides in the finding that the inhibition by these peptides appears to have substrate specificity. That is, the β_2-adrenoceptor peptides do not inhibit β-adrenoceptor kinase-catalyzed phosphorylation of non-receptor substrates such as the carboxyl terminal β_2-adrenoceptor peptides, casein and phosvitin.

One somewhat paradoxical observation is that while the first intracellular loop β_2-adrenoceptor peptide potently inhibits β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase, the corresponding peptide derived from either rhodopsin, the β_1-adrenoceptor or the α_2-adrenoceptor does not inhibit β_2-adrenoceptor phosphorylation. It is possible that different receptors might be phosphorylated by distinct receptor specific kinases. Clearly, this is true for the phosphorylation of rhodopsin where rhodopsin kinase is the enzyme involved. However, the β_1-adrenoceptor would be expected to serve as a substrate for β-adrenoceptor kinase although this has not been directly assessed. It is also possible that a particular peptide might only inhibit phosphorylation of the receptor from which it is derived, a hypothesis which has not been tested for the α_2- or β_1-adrenoceptors. Finally, it is also conceivable that β-adrenoceptor kinase might interact with different domains from different receptors. Consistent with this notion, β-adrenoceptor kinase most likely phosphorylates the third intracellular loop of the α_2-adrenoceptor as the carboxyl terminus of the α_2-adrenoceptor contains no serines or threonines (Kobilka *et al.*, 1987).

In summary, this work provides several new insights into the mechanism of receptor phosphorylation by β-adrenoceptor kinase. The kinase appears to interact with multiple intracellular regions of the β_2-adrenoceptor while the phosphorylation sites appear to be localized at the carboxyl terminus. This work also provides potential tools for characterizing further β-adrenoceptor kinase: synthetic peptides which serve as specific substrates of the enzyme as well as peptides which potently inhibit β_2-adrenoceptor phosphorylation by β-adrenoceptor kinase. Future work will utilize mutated forms of the β_2-adrenoceptor to delineate further the receptor domains involved in interaction and phosphorylation by β-adrenoceptor kinase.

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