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SYNTHETIC SEGMENTS OF THE MAMMALIAN βAR ARE PREFERENTIALLY RECOGNIZED BY cAMP-DEPENDENT PROTEIN KINASE AND PROTEIN KINASE C

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SUMMARY: Desensitization of the beta-adrenergic receptor has been correlated in some cell systems with receptor phosphorylation. Various kinases have been implicated in these phosphorylation processes, including both cAMP-dependent protein kinase and protein kinase C. In the present study, we have utilized the protein sequence information obtained from the cloning of the mammalian beta-adrenergic receptor to prepare synthetic peptides corresponding to regions of the receptor which would be predicted to act as possible substrates for these kinases in vivo. Two of these receptor-derived peptides were found to serve as substrates for these protein kinases. A peptide corresponding to amino acids 257-264 of the beta-receptor is the preferred substrate for the cAMP-dependent protein kinase, while protein kinase C showed a marked preference for phosphorylation of a peptide corresponding to residues 341-351 of the beta-adrenergic receptor.

The chronic stimulation of cells by beta-adrenergic agonists leads to an attenuation of their biological responsiveness upon rechallenge, resulting from the desensitization of the beta-adrenergic receptor (βAR) (1,2). Although other mechanisms have been proposed, recent studies suggest that the phosphorylation of the βAR may be directly involved in desensitization, although specific phosphate acceptor sites on the receptor have not yet been identified (3). A beta-adrenergic receptor-specific protein kinase has been postulated to specifically phosphorylate the agonist-occupied form of the receptor during the process of homologous desensitization, possibly within the serine-rich C-terminal region (4). The heterologous desensitization of the βAR in cells treated with dibutyryl cAMP or phorbol 12-myristate 13-acetate (TPA), coupled with the observed phosphorylation of the βAR by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) in vitro (5) has implicated receptor phosphorylation by PKA and PKC in heterologous desensitization (6,7), and at least two possible phosphorylation sites for PKC and PKA on the βAR have been noted (8).

In the present study, we have prepared synthetic peptides corresponding to serine containing sequences of the hamster β_2AR, and assessed the relative ability of these peptides to be phosphorylated by the two serine-specific kinases, PKA or PKC. We find that one of the peptides shows a marked affinity for phosphorylation by PKA, while another peptide is the preferred substrate for PKC. In contrast, a peptide corresponding to the C-terminal region of the βAR is not phosphorylated by either protein kinase.

Abbreviations: βAR, beta-adrenergic receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TPA, 12-myristate 13-acetate.

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MATERIALS AND METHODS

Peptide Synthesis: The βAR-derived peptides (sequences given in Table 1) were synthesized using a Sam II peptide synthesizer (Biosearch - San Rafael, CA) by the basic procedures of solid-phase synthesis (9) using 4-methylbenzhydralamine resin. After purification on a Whatman C-18 column, each peptide was determined to be homogeneous on the basis of amino acid and mass spectral analysis. The peptides are identified in the text by the corresponding amino acid number from the sequence of the hamster β_2AR (5).

Phosphorylation by Cyclic AMP-dependent Protein Kinase: The phosphorylation of the peptides by PKA was determined according to Erlichman, et al. (10). 26 ng of holoenzyme (Sigma) was incubated with various concentrations of the peptides in 20 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 100 mM DTT, 300 μg/ml BSA, 160 μM [$-^{32}P$] ATP (50-200 cpm/pmol), and 10 μM cyclic AMP at 23°C. The enzyme concentration was determined by [2,8-^3H] cAMP binding (11). The reactions were terminated in 2.5% trichloroacetic acid containing 25 mM sodium pyrophosphate. Aliquots were spotted on P81 phosphocellulose paper (Whatman) and washed with 75 mM H_3PO_4. Alternatively, peptide phosphorylation was assessed by ion-exchange chromatography (12), with identical results.

Phosphorylation by Protein Kinase C: Protein kinase C was the generous gift of Drs. M. Lee and R. Bell (Duke University) and was assayed by a modification of the method of Hannun, et al (13). Enzyme concentration was standardized against calf thymus histone type III-S. Peptides were incubated in 0.2 ml of 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 200 μM $CaCl_2$, 20 μg/ml phosphatidylserine reconstituted in 0.3% Triton X-100, 100 μM ATP (250-500 cpm/pmol), plus 10 μM TPA at 30°C. Peptide phosphorylation was measured as described above for phosphorylation by PKA.

Data analysis: The kinetic parameters were determined by computer-assisted least squares analysis, according to the Michaelis-Menten equation.

RESULTS

Four synthetic peptides were constructed, based upon the primary amino acid sequence of the mammalian β_2AR (Table 1). Each peptide was selected from a proposed intracellular domain of the protein containing serine residues C-terminal to basic amino acids. Peptides 248-255 and 257-264 are derived from

Table 1. Phosphorylation of synthetic peptides by protein kinases

		Protein Kinase Activity					
		PKA			PKC		
Peptide	Sequence	Km (μM)	Vmax (μMoles/min/mg)	Vmax/Km (min^{-1}/mg protein)	Km (μM)	Vmax (μMoles/min/mg)	Vmax/Km (min^{-1}/mg protein)
Kemptide	LRRASLG	14±4	3.7±.8	0.264	—	—	—
βAR 248-255	C(Nle)VEQDGRSG	—	—	—	—	—	—
βAR 257-264	C(Nle)GLRRSSKF	21±3	5.4±.5	0.257	154±23	2.2±.4	0.014
βAR 341-351	C(Nle)RRSSSKAYG	359±84	1.2±.1	0.003	4.1±5	5.0±.5	1.22
βAR 404-418	CLDSQGRN(Nle)STNDSPL	—	—	—	—	—	—

Peptides represent sequences derived from various regions of the hamster βAR. A norleucine (Nle) residue was inserted into each peptide in order to facilitate the detection of the peptide in the presence of other proteins (5). For peptides 248-255, 257-264, and 341-351, a Cys-Nle pair was simply attached to the N-terminus of the βAR sequence to form the synthetic peptide. In peptide 404-418, the Cys residue at position 411 in the native βAR has been replaced by a Nle. Phosphorylation reactions were performed for 3 min as described in Methods. Values shown represent the means of duplicates from two separate experiments. The background enzyme activity was determined at zero time and was subtracted from all values. --- indicates that a peptide was determined not to serve as a substrate for the enzyme at peptide concentrations up to 2 mM.

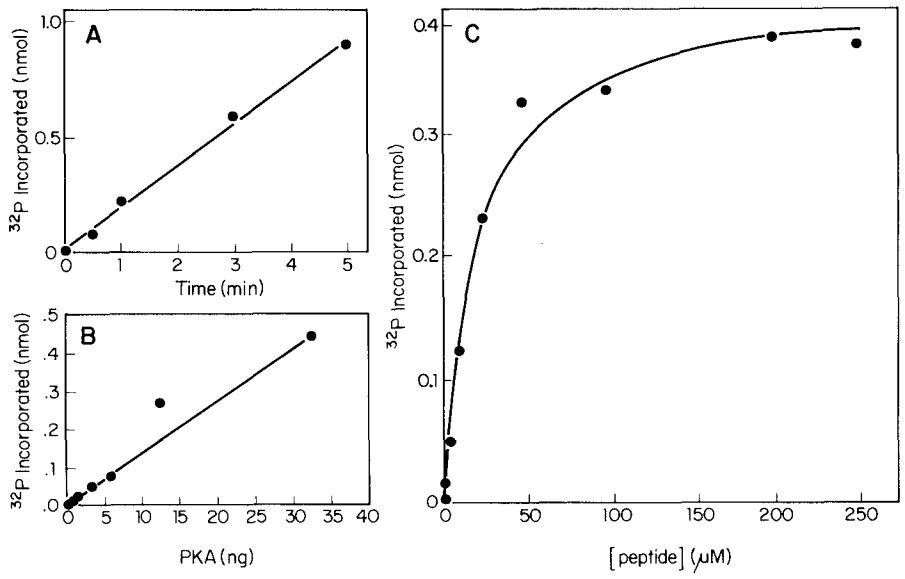

Figure 1. Characterization of the phosphorylation of peptide 257-264 by PKA.
(A) Time course of the phosphorylation reaction. 5 nmol of peptide 257-264 was reacted with 26 ng of PKA in 0.05 ml for the times shown. (B) Dependence of peptide phosphorylation on enzyme concentration. 10 nmol of peptide 257-264 was incubated with various amounts of PKA in 0.2 ml for 0.5 min. (C) Dependence of peptide phosphorylation on peptide concentration. The reaction was performed for 3 min at 23°C with 26 ng PKA in 0.05 ml. The curve was determined from the Michaelis-Menten equation. All data shown are the means of duplicate determinations, representative of 2-3 similar experiments.

the third putative intracellular loop and peptides 341-351 and 404-418 from the C-terminal domain, with 404-418 representing the C-terminus, of the βAR.

The ability of these peptides to be phosphorylated by either PKA or PKC was determined using an in vitro phosphorylation assay. As shown in Figure 1, phosphorylation of peptide 257-264 by PKA was linear with time over at least the first 5 minutes of the reaction (Figure 1A), and with enzyme concentration over the range of 10-150 ng/ml (Figure 1B). Based on this analysis, the subsequent phosphorylation of the peptide was measured at 3 min, using 130 ng/ml of PKA. Under these conditions, <15% of the total substrate present was converted to the phosphorylated product, permitting the determination of kinetic parameters from the data. The peptide phosphorylation was analyzed by the Michaelis-Menten equation, revealing a Km of 21 ± 3 μM and a Vmax of 5.4 ± 0.5 μmoles min^{-1} mg^{-1} (Figure 1C). These characterization experiments were repeated for the phosphorylation of the other peptides by both PKA and PKC, to determine conditions under which the reaction was linear with time and enzyme concentration and where <15 % of the peptide substrate was depleted during the course of the reaction (data not shown).

The synthetic peptides were phosphorylated by PKA and PKC, giving the parameters listed in Table 1. Two of the four peptides were recognized by PKA. Peptide 257-264 was phosphorylated with the kinetic parameters described above, giving a ratio of Vmax/Km of 0.257 min^{-1}/mg. Peptide 341-351 was a significantly poorer substrate for the enzyme, with a Vmax/Km of 0.003 min^{-1}/mg. The values obtained for peptide 257-264 were similar to those determined for the phosphorylation by PKA of Kemptide (Table 1), a synthetic peptide known to serve as a good substrate for PKA (14). Two additional peptides, 248-255 and 404-418, did not serve as substrates for PKA.

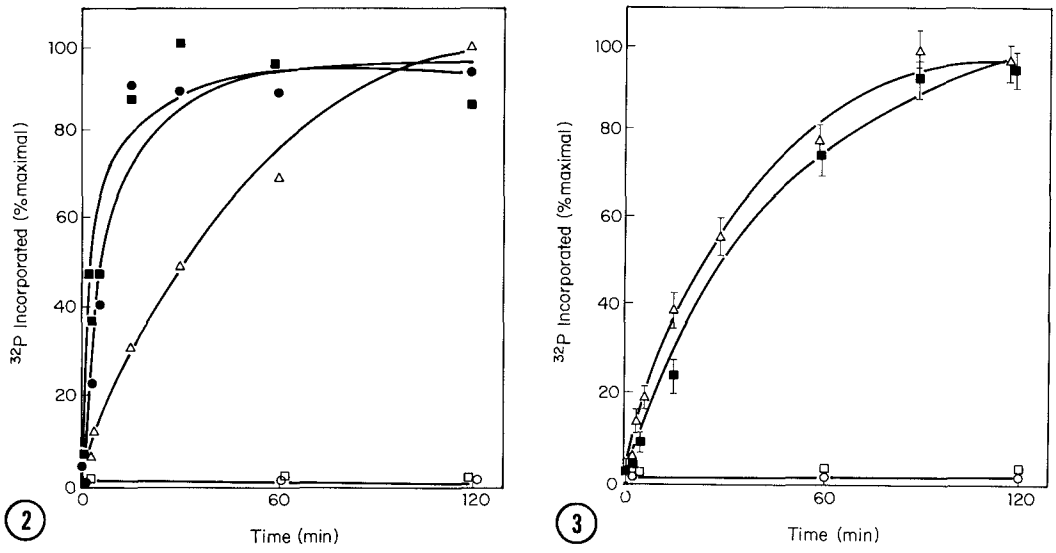

Figure 2. Time-dependent phosphorylation of synthetic peptides by PKA.
Synthetic peptides were phosphorylated with 26 ng PKA in 0.2 ml. Kemptide, 257-264, 341-351, 248-255, and 404-418 were present in the assay at 100 μM, 120 μM, 150 μM, 2 mM and 2 mM, respectively. Background values were determined in the absence of cAMP and represented <10% of the total phosphorylation. Each point represents the mean $\pm$SEM of duplicate determinations, representative of at least three experiments. The maximal phosphorylation (mole phosphate/mole peptide) was as follows: Kemptide, 0.5(●); 257-264, 0.6(■); 341-351, 0.4(△); 248-255, 0(□); 404-418, 0(○).

Figure 3. Phorbol ester-dependent phosphorylation of peptides by PKC.
Peptides 257-264, 341-351, 248-255, and 404-418 were assayed at 10 μM, 660 μM, 2 mM, and 2 mM, respectively, with 1.4 ng PKC. Nonspecific phosphorylation (10% of total) was measured with the inactive analog, 4- -phorbol in the presence of 100 μM EGTA and the absence of CaCl2. Each point represents the mean $\pm$SEM of duplicate determinations from two experiments. Maximal phosphorylation (mole phosphate/mole peptide) was as follows: 257-264, 0.003 (■); 341-351, 0.07 (△); 248-255, 0 (□); 404-418, 0(○).

Both peptides 257-264 and 341-351 were also phosphorylated by PKC (Table 1). However, in contrast to the results found with PKA, peptide 341-351 was the preferred substrate for PKC, having a Vmax/Km of 1.22 min^{-1} /mg, while peptide 257-264 was phosphorylated much less readily, with a Vmax/Km of 0.014 min^{-1}/mg. The kinetic parameters determined for 341-351 compare favorably with those of other known substrates for PKC (15-19). Neither the C-terminal peptide, 404-418, nor peptide 248-255 was recognized as a substrate by PKC. The PKA substrate, Kemptide, was also not recognized by PKC under the assay conditions employed in this study.

The time course of phosphorylation of the peptides by PKA and PKC was observed over a period of 120 minutes, with the results shown in Figures 2 and 3. The phosphorylation of 257-264 was linear throughout the first 5 minutes (Figure 1), with the phosphorylation reaching a plateau at 30 minutes (Figure 2). The maximal level of phosphorylation of 257-264 by PKA was 0.6 mole phosphate/mole peptide (Figure 2). These results were similar to that observed for Kemptide, which incorporated 0.5 mole phosphate/mole peptide (Figure 2). Peptide 341-351 was phosphorylated by PKA more slowly, with the maximal ^{32}P incorporation, 0.4 mole phosphate/mole peptide, occurring only after 120 minutes of incubation (Figure 2). No significant increase in phosphorylation over these maximal levels was evident at times up to six hours, or upon addition of more ATP or PKA, while the addition of more peptide resulted in increased phosphorylation

(data not shown). Thus, the plateau in phosphorylation observed in Figures 2 and 3 represented the maximal phosphate incorporation into the peptide substrate.

The time courses of phosphorylation of 257-264 and 341-351 by PKC were very similar, reaching a plateau after 90 min, despite the observed differences in the relative affinities of the two substrates for the enzyme (Figure 3). However, the preference of the enzyme for 341-351 over 257-264 was reflected in the maximal level of phosphorylation of the two peptides (0.07 mole phosphate/mole 341-351 compared to 0.003 mole phosphate/mole 257-264). These levels of phosphate incorporation are similar to those reported for other peptide substrates of PKC (15-18). No subsequent peptide phosphorylation could be detected in the presence of additional enzyme or ATP, indicating that the peptide substrate was limiting under these conditions (data not shown).

DISCUSSION

The results of the present study indicate that synthetic peptides corresponding to segments of the primary amino acid sequence of the mammalian β_2AR are recognized as substrates by both PKA and PKC. These peptides, 257-264 and 341-351, were derived from serine-rich amino acid segments of two regions of the receptor which would be postulated to be exposed intracellularly according to the model proposed for the mammalian β_2AR (8). Both of these peptides contain double arginine residues N-terminal to either two (257-264) or three (341-351) serine residues, followed by a lysine. This pattern of serine residues surrounded by basic amino acids is similar to the sequence of known phosphorylation sites on other protein and peptide substrates for PKA (12, 20). Recent studies have shown that a minimum peptide substrate requirement for PKC includes a serine or threonine located two positions N-terminal to a lysine or arginine (15-19). By these criteria, both peptides 257-264 and 341-351 would be predicted to serve as substrates for PKC. The presence of basic amino acids proximal to serine or threonine residues is also apparent in peptides 248-255 and 404-418, each of which contains a single arginine residue N-terminal to 1-3 serine residues. However, neither of these peptides serves as a substrate for either protein kinase.

The preference of PKC for peptide 341-351 over 257-264 could reflect either the presence of the additional serine residue in 341-351 or the proximity of the arginine residues to the N-terminus of peptide 341-351. The characteristics which serve to enhance the phosphorylation of 341-351 by PKC also appear to decrease its phosphorylation by PKA relative to 257-264. This difference in phosphorylation by the two kinases may reflect the different physiological substrate specificities of the two enzymes.

The direct determination of a physiological role for these peptide segments within the native βAR lies beyond the scope of the present study. However, it is interesting to speculate that recent data implicating both PKA and PKC in the phosphorylation of the βAR which correlates with heterologous desensitization may reflect the ability of these peptide regions in the native βAR to serve as substrates for these kinases. By utilizing synthetic peptides corresponding to proposed intracellular regions of the βAR, we have demonstrated the suitability of these peptides as substrates for these two protein kinases. The data suggest a potential specificity of phosphorylation sites on the βAR, with PKA preferentially recognizing a region of the protein between the fourth and fifth hydrophobic helices and PKC primarily phosphorylating a region located closer to the C-terminus of the receptor. Future studies utilizing cell-based phosphorylation of the native βAR protein will be necessary to delineate the physiological effects of the phosphorylation of the βAR by these kinases.

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