Volume 224, number 2, 325–330 FEB 05319 November 1987

Phosphorylation of high- and low-molecular-mass atrial natriuretic peptide analogs by cyclic AMP-dependent protein kinase

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Received 6 October 1987

Synthetic high- and low-molecular-mass atrial peptides were phosphorylated in vitro by cyclic AMP-dependent protein kinase and [^{32}P]ATP. From a series of atrial peptide analogs, it was deduced that the amino acid sequence, Arg^{101}–Ser^{104} of atriopeptin was required for optimal phosphorylation. Phosphorylated AP(99–126) was less potent than the parent atriopeptin in vasorelaxant activity and receptor-binding properties. These results indicate that the presence of a phosphate group at the N-terminus of AP(99–126) decreases the interaction of the peptide with its receptor and, as a consequence, decreases bioactivity. These observations are in contrast to those of Rittenhouse et al. [(1986) J. Biol. Chem. 261, 7607 7610] who reported that phosphorylation of AP(101–126) enhanced the stimulation of Na/K/Cl cotransport in cultured vascular smooth muscle cells.

Atrial natriuretic peptide; Phosphorylation; Protein kinase

1. INTRODUCTION

The vasorelaxant, natriuretic and diuretic properties of atrial natriuretic peptide enable it to play an important role in the regulation of blood pressure and fluid volume [1,2]. The circulating form of the peptide, AP(99–126), consists of 28 amino acids, and is derived from the carboxyl-terminus of a 126-amino-acid prohormone, AP(1–126), stored in specific granules in the heart [3]. A disulfide bridge links two cysteine residues at positions 105 and 121 of the peptide to form a ring structure which is required for biological activity [4–6].

Peptides and proteins containing the sequence, Arg-Arg-X-Ser(P), often serve as phosphorylatable substrates for cyclic AMP-dependent protein kinase [7]. We and others [8,9] have observed that this recognition sequence is also present in AP(99–126):

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99
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-
        Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-
        Gly-Cys-Asn-Ser-Phe-Arg-Tyr
                              126
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Based on this observation, we wished to determine the sequence specificity for phosphorylation of various atrial peptide analogs by cyclic AMP-

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Abbreviations: AP, atrial natriuretic peptide; HPLC, high performance liquid chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

dependent protein kinase, and the biological properties of phosphorylated AP(99–126).

2. MATERIALS AND METHODS

2.1. *Materials*

The catalytic subunit of cyclic AMP-dependent protein kinase was obtained from Sigma. [γ-^{32}P]ATP was purchased from New England Nuclear. Adenosine 5′-triphosphate (ATP) and adenosine 5′-*O*-(3-thiotriphosphate) were obtained from Boehringer Mannheim. Rat atriopeptin(99–126) was purchased from Peninsula Laboratories. Electrophoresis reagents and molecular mass standards were from Bio-Rad.

2.2. *Peptide synthesis*

Atrial peptide analogs were synthesized by the Merrifield method using PAM resin [10] for peptide acids, and mBHA resin [11] for peptide amides. Peptides were cleaved from the resins with liquid HF/anisole (9:1, v/v) for 60 min at 0°C. Free peptides were purified by reverse-phase HPLC on a Waters μBondapak C_{18} column using a linear gradient of 15–40% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min for 25 min. Disulfide bond formation was accomplished by air oxidation in 0.1 M ammonium bicarbonate (pH 7) at a peptide concentration of 0.1 mg/ml. The cyclized product was purified on a μBondapak column, and the integrity was verified by amino acid and gas-phase sequence analyses.

2.3. *Preparation of atrial peptide prohormone*

The atrial peptide prohormone used in the [^{32}P]phosphate-labelling studies consisted of amino acid residues 24–152 of the rat preprohormone coding sequence [12]. The gene was expressed in *E. coli* (Olins, P.O. et al., in preparation). The prohormone was extracted from the cells using 1 M acetic acid, and purified by reverse-phase and ion-exchange chromatography [13].

2.4. *^{32}P labelling of high- and low-molecular-mass atrial peptides*

Low- (0.6 μg) and high- (3 μg) molecular-mass atrial peptides were incubated at 30°C for 30 min in 0.03 ml reaction mixtures containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, and the catalytic subunit of cyclic AMP-dependent protein kinase (15 U). The reactions were terminated by the addition of EDTA to a final concentration of 10 mM. The samples were subjected to SDS-polyacrylamide gel electrophoresis [14] on a 15% polyacrylamide gel containing 0.1% SDS. The gel was stained, dried and subjected to autoradiography in order to detect ^{32}P-labelled peptides.

2.5. *Preparation of phosphorylated and thiophosphorylated AP(99–126)*

Phosphorylated AP(99–126) was prepared essentially by the method of Rittenhouse et al. [8]. Briefly, AP(99–126) (750 μg/0.1 ml) was incubated for 60 min at 30°C in the presence of 15 mM $MgCl_2$, 62.5 mM Mes, pH 6.5, 0.24 mM EGTA, 1.5 mM ATP and the catalytic subunit of cyclic AMP-dependent protein kinase (3000 U). The reaction was terminated by the addition of EDTA to a final concentration of 20 mM. Thiophosphorylated AP(99–126) was similarly prepared except adenosine 5′-*O*-(3-thiotriphosphate) was substituted for ATP in the incubation. Phosphorylated and thiophosphorylated AP(99–126) were purified by reverse-phase HPLC. The reaction mixtures were injected onto a Vydac C_{18} column (10 mm i.d. × 25 cm; 5 μm/300 nm) equilibrated with water containing 0.05% trifluoroacetic acid. A linear gradient of 0–23% acetonitrile containing 0.05% trifluoroacetic acid was developed for 17 min at a flow rate of 2 ml/min. Phosphorylated and thiophosphorylated atrial peptides eluted during isocratic conditions of 23% acetonitrile. Both the gradient and flow rate were maintained by a Waters HPLC system consisting of two model 510 pumps connected to an automated gradient controller. Column effluent was monitored at 220 nm by a Waters model 481 spectrophotometer. Column fractions containing the phosphorylated or thiophosphorylated AP(99–126) were pooled, lyophilized and stored at −70°C until use.

2.6. *AP receptor-binding and bioactivity assays*

Receptor-binding properties of atrial peptides were studied by measuring the displacement of ^{125}I-labelled AP(99–126) from the rabbit lung membrane receptor [15]. Atrial peptides were

assayed for vasorelaxant activity using norepinephrine-contracted rabbit thoracic aortic strips [2].

3. RESULTS AND DISCUSSION

3.1. *Sequence requirements for atrial peptide phosphorylation*

A series of atrial peptide analogs (table 1) were incubated with cyclic AMP-dependent protein kinase in the presence of [^{32}P]ATP. The incubation mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography to detect ^{32}P-labelled peptides (fig.1). [^{32}P]Phosphate was incorporated into the circulating form of atriopeptin, AP(99–126), which contains the protein kinase recognition sequence, Arg^{101}-Arg^{102}-Ser^{103}-Ser^{104}. In contrast, AP(103–126), which lacks the two arginines of the recognition sequence, was not phosphorylated. Interestingly, AP(102–126) which retains only a portion of the protein kinase recognition sequence, Arg^{102}-Ser^{103}-Ser^{104}, showed low levels of [^{32}P]phosphate incorporation. Other analogs of atrial peptides in which the recognition sequence was disrupted by deletion or substitution for other amino acids were not phosphorylated. These results indicate that the sequence Arg^{101}-Arg^{102}-Ser^{103}-Ser^{104} is required for the optimal phosphorylation of atrial peptides, and the results are consistent with Ser^{104} being the site of phosphorylation [8].

An analog of the atrial peptide prohormone was also phosphorylated by cyclic AMP-dependent protein kinase, as shown in fig.1. Recently, Bloch et al. [9] have reported that a similar prohormone analog, AP(2–126), was phosphorylated, presumably at the Ser^{104} position, by the same enzyme in vitro. In addition, they studied the secretion of proatrial natriuretic factor (proANF) from cultured rat cardiocytes, and found that approx. 35% of the secreted proANF was phosphorylated. However, incorporation of phosphate was localized to the amino-terminal portion of the prohormone, and not in residues 99–126, the amino acid sequence of the mature peptide. It is not known whether cyclic AMP-dependent protein kinase or an alternative protein kinase was responsible for the phosphorylation. Moreover, it is still unclear whether phosphorylation of proANF plays a role in its storage or secretion from atrial cardiocytes.

3.2. *Biological properties of phosphorylated and thiophosphorylated atrial peptides*

To determine the effect of phosphorylation on the biological activities of atrial peptides, AP(99–126) was phosphorylated by incubation with cyclic AMP-dependent protein kinase and ATP, and purified by reverse-phase HPLC. Fig.2 shows HPLC profiles of the crude and purified phosphorylated AP(99–126). Phosphorylated AP(99–126) competed with ^{125}I-labelled AP-(99–126) for binding to rabbit lung atrial peptide binding sites (fig.3). The relative potency for phosphorylated AP(99–126) binding to the lung receptors was 0.28 vs the parent peptide. Phosphorylated AP(99–126) had the ability to relax norepinephrine-contracted rabbit aorta strips, and the relative potency vs AP(99–126) was determined to be 0.26 which correlated well with the receptor-binding data.

We considered the possibility that the phosphate group on AP(99–126) might be susceptible to phosphatase digestion during assay, and that this

Table 1

N-terminal amino acid sequences of the low-molecular-mass atrial peptide analogs used in the phosphorylation studies

Analog	Sequence
	99 102 105
AP(99–126)	S -L -R -R -S -S -C -F -G ...
S^{101},L^{102},R^{103,104} AP(101–125)-NH_2	S -L -R -R -C -F -G ...
AP(102–126)	R -S -S -C -F -G ...
R^{103} AP(103–125)-NH_2	R -S -C -F -G ...
R^{103,104} AP(103–126)	R -R -C -F -G ...
AP(103–126)	S -S -C -F -G ...

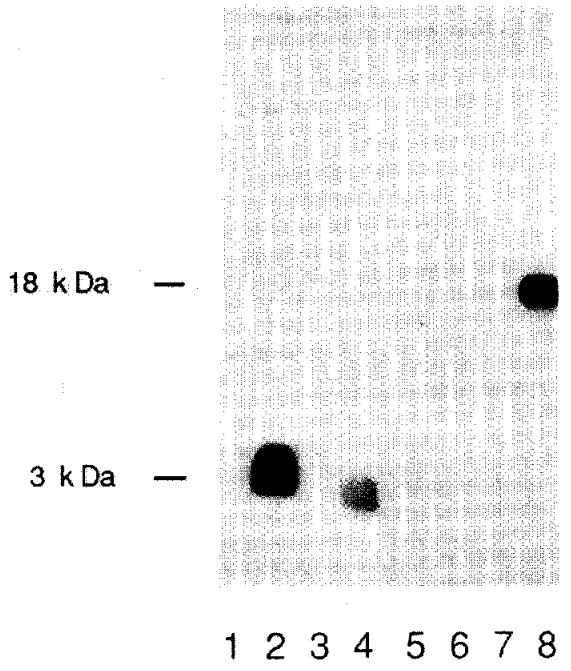

Fig.1. Incorporation of [^{32}P]phosphate into low- and high-molecular-mass atrial peptide analogs. Atrial peptides were incubated with [^{32}P]ATP and cyclic AMP-dependent protein kinase as described in section 2 and the incubation mixtures were resolved by SDS-polyacrylamide gel electrophoresis. An autoradiograph of the stained and dried gel is shown, where the peptide substrates are as follows – lanes: 1, no substrate; 2, AP(99–126); 3, $S^{101}L^{102}R^{103,104}$AP(101–125)-$NH_2$; 4, AP(102–126); 5, R^{103}AP(103–125)-NH_2; 6, $R^{103,104}$AP(103–126); 7, AP(103–126); 8, AP prohormone analog.

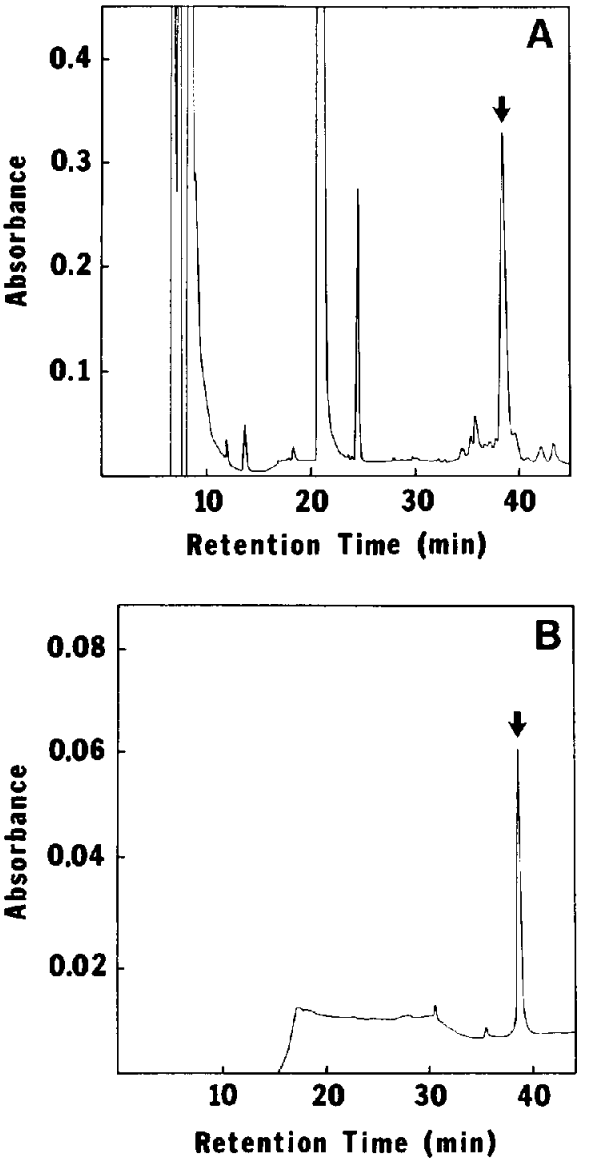

Fig.2. HPLC profiles of the crude and purified phosphorylated AP(99–126). (A) Sample of incubation mixture containing approx. 90 μg phosphorylated AP(99–126). (B) Sample (20 μg) of purified phosphorylated AP(99–126). Arrow denotes the absorbance peak of phosphorylated AP(99–126).

might account for the residual bioactivity observed with the phosphorylated peptide. To rule this out, thiophosphorylated AP(99–126) was prepared using adenosine 5′-*O*-(3-thiotriphosphate) and cyclic AMP-dependent protein kinase. Thiophosphorylated compounds have been reported to be less susceptible to the action of phosphatases (review [16]). Fig.4 shows HPLC profiles of the crude and purified thiophosphorylated AP(99–126). Thiophosphorylated AP(99–126) had receptor-binding (fig.5) and vasorelaxant properties similar to the phosphorylated AP(99–126). The thiophosphorylated peptide had relative potencies of 0.21 and 0.26 for receptor-binding and vasorelaxant activities, respectively. The results are summarized in table 2.

Thus, the presence of a phosphate group in the N-terminal region of AP(99–126) appears to

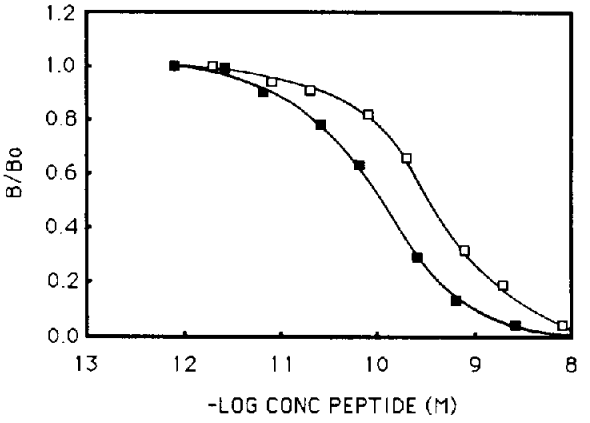

Fig.3. Competitive binding of phosphorylated AP(99–126) to rabbit lung membranes. The membranes were equilibrated with 90 pM ^{125}I-AP(99–126) and varying concentrations of unlabelled AP(99–126) (■) and phosphorylated AP(99–126) (□). Values of specific binding measured in the presence of unlabelled peptide (B) were expressed as a fraction of the specific binding measured in the absence of competitor (B_0). Data points represent the mean of two determinations.

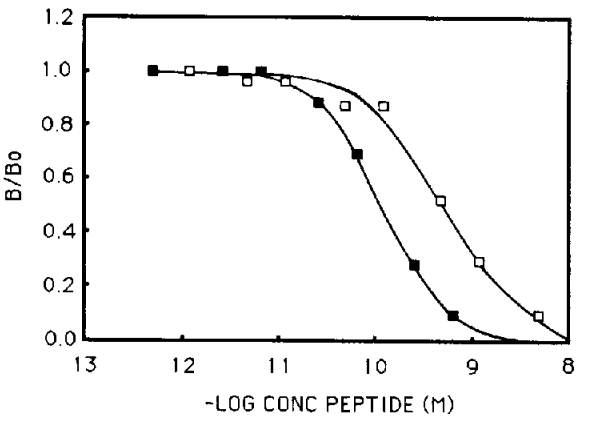

Fig.5. Competitive binding of thiophosphorylated AP(99–126) to rabbit lung membranes. The membranes were equilibrated with 90 pM ^{125}I-AP(99–126) and varying concentrations of unlabelled AP(99–126) (■) and thiophosphorylated AP(99–126) (□). Values of specific binding measured in the presence of unlabelled peptide (B) were expressed as a fraction of the specific binding measured in the absence of competitor (B_0). Data points represent the mean of two determinations.

decrease the interaction of the peptide with the atrial peptide receptor, and decreases biological activity. There are a number of possible explanations for this. Binding of the peptide to the atrial peptide receptor could be sterically hindered by the presence of a phosphate group. If the atrial peptide binding site contained a negatively charged amino acid residue, a phosphorylated atrial peptide might

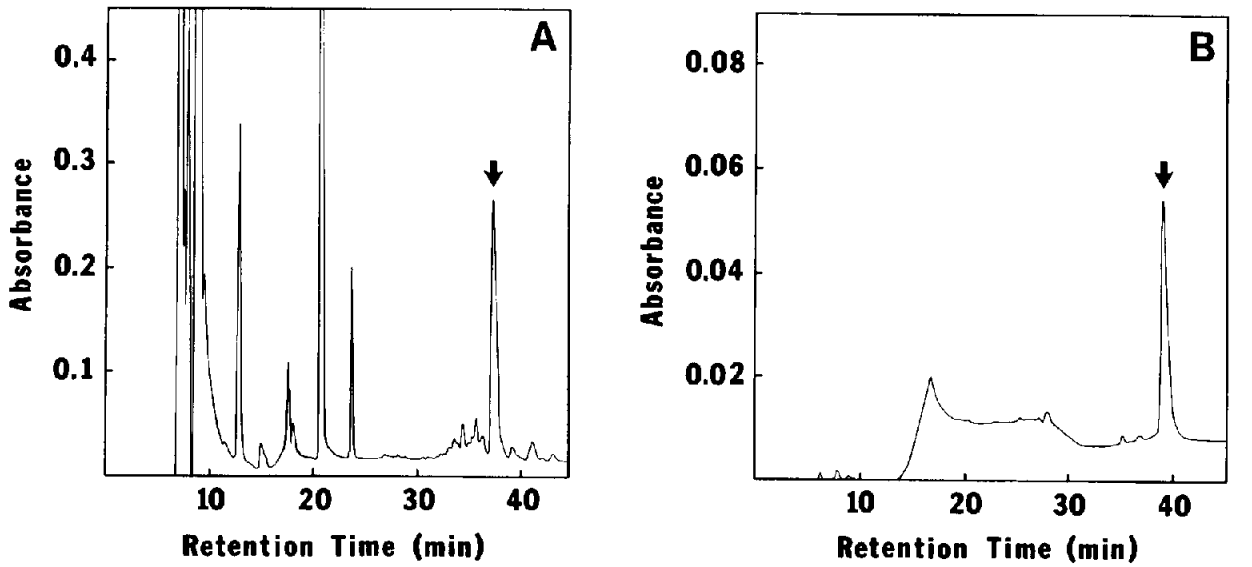

Fig.4. HPLC profiles of the crude and purified thiophosphorylated AP(99–126). (A) Samples of incubation mixture containing approx. 90 μg thiophosphorylated AP(99–126). (B) Sample (20 μg) of purified thiophosphorylated AP(99–126). Arrow denotes the absorbance peak of thiophosphorylated AP(99–126).

Table 2

Relative potencies of phosphorylated and thiophosphorylated AP(99–126) for receptor-binding affinity to rabbit lung membranes and vasorelaxant activity on norepinephrine-contracted rabbit aorta strips

Peptide	Receptor binding	Vasorelaxant activity
AP(99–126)	1.00	1.00
Phosphorylated AP(99–126)	0.28	0.26
Thiophosphorylated AP(99–126)	0.21	0.26

be repelled. Alternatively, if the positively charged arginine residues at the amino-terminus of the peptide were involved in binding to the atrial peptide receptor, a phosphate group might neutralize these.

Although our results showed that phosphorylated atrial peptides were approx. 4-fold less potent in receptor-binding and vasorelaxant properties, Rittenhouse et al. [8] have reported that phosphorylated AP(101–126) and AP(99–126) were more effective than the corresponding dephospho forms in stimulating Na/K/Cl cotransport in cultured vascular smooth muscle cells. The reason for the discrepancy between these observations is unclear, except that different atrial peptide analogs were tested in different biological assays. Nevertheless, phosphorylation of atrial peptides alters their receptor-binding and biological activities, and may therefore have an important role in modulating their action at the target tissue or perhaps in feedback control mechanisms.

In summary, we have shown that Arg-Ser-Ser is the minimal sequence requirement for phosphorylation of atrial peptides by cyclic AMP-dependent protein kinase. Phosphorylated atrial peptides have decreased biological activity due to the interference of the phosphate group with receptor-binding interactions. From these and other in vitro studies, evidence is accumulating that phosphorylation may play a regulatory role in the storage, secretion and biological activity of these factors. It still remains to be determined whether atria, plasma or target tissues contain phosphorylated atrial peptides.

ACKNOWLEDGEMENTS

We wish to thank N.R. Siegel, M.G. Jennings and C.E. Smith for the amino acid and peptide sequence analyses.

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