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Relaxation of smooth muscle by cardiodilatin/atrial natriuretic peptide is inhibited by cAMP-dependent phosphorylation

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Cardiodilatins/atrial natriuretic peptides (CDD/ANP) exhibit a common amino acid sequence: Arg^{101}-Arg^{102}-Ser^{103}-Ser^{104}. Cyclic AMP-dependent phosphorylation of Ser^{104} of atrial peptides with [γ-^{32}P]ATP enables rapid identification of cardiac hormones. The biological activity of in vitro phosphorylated cardiodilatin (CDD-28/α-hANP) is dramatically altered compared to the unphosphorylated peptide: the vasorelaxant effect of cardiodilatin 28 is inhibited upon phosphorylation.

Atrial natriuretic peptide; Phosphorylation; Cardiodilatin; cyclic AMP; Biological activity; (Vascular smooth muscle)

1. INTRODUCTION

The role of a particular family of atrial peptides as regulators of blood pressure [1,2] by stimulation of natriuresis, diuresis and vascular smooth muscle relaxation [3–6] has been generally acknowledged for several years. These peptides are designated atrial natriuretic factor (ANF) or cardiodilatin (CDD) [5]. The peptides are derived from a common precursor whose amino acid sequence is well established [6]. The largest fraction of peptides extracted from porcine atria using the method of Mutt [7], which includes heating, is cardiodilatin 126 (CDD-126). As a side fraction, CDD-88 was extracted. CDD-88 is bioactive [8] and comprises amino acids 38–126 of CDD-126. The final 28 amino acids of these peptides correspond to human α-ANP [9]. Both bioactive peptides (CDD-88, CDD-28) show a common amino acid sequence: Arg^{101}-Arg^{102}-Ser^{103}-Ser^{104}. Phosphorylation of a serine residue by cAMP-dependent protein kinase (cPK) requires a recognition sequence of Arg-Arg-X-Ser [10]. It has recently been reported [11] that two synthetic forms of CDD consisting of amino acids Gly^{96}–Tyr^{126} and Arg^{101}–Tyr^{126} are phosphorylated by cAMP-dependent protein kinase at Ser^{104}. The low K_m value of the longer peptide of 0.5 μM demonstrates extraordinary substrate susceptibility. Furthermore, it was demonstrated by the same authors that the $Na^+/K^+/Cl^-$ co-transport in cultured vascular smooth muscle cells was enhanced after phosphorylation of the peptide Arg^{101}–Tyr^{126}. Because of the vasorelaxant properties of CDD-28 and CDD-88 we investigated the effects of cAMP-dependent phosphorylation of both peptides on smooth muscle relaxation. The observation that the peptides of the atrial natriuretic peptide family are phosphorylated cAMP-dependently offers the possibility for rapid detection of these peptides in crude tissue extracts, serum or urine, thus facilitating their preparation.

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2. MATERIALS AND METHODS

Aortae were prepared from New Zealand rab-

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bits. The tension was measured isometrically in an organ bath on helical strips by standard methods [12]. Incubation of the strips was performed in physiological salt solution as in [5]. Initially, strips were pretreated with 10^{-6} M norepinephrine followed by complete relaxation. For the assay another contraction was elicited by 10^{-7} M norepinephrine. When constant force levels had been obtained, peptides were added. Preparation of cAMP-dependent protein kinase (EC 2.7.1.37) from bovine cardiac muscle was as described earlier [13]. Isolation of CDD-88 from bovine atria [14] was carried out in our laboratory; CDD-28 (α-hANP) was a gift from Bissendorf Peptides Co. For the phosphorylation reaction, peptides were incubated in Eppendorff tubes at 30°C in a solution containing 100 mM Mops buffer (pH 6.8), 10 mM Mg acetate and 12–13 μg catalytic subunit of cAMP-dependent protein kinase (100 μl reaction volume). The reaction was started by the addition of 10 μl of 2 mM ATP, unless otherwise stated. In the case of radioactive labelling [γ-^{32}P]ATP from NEN was included (0.5 mCi/μmol ATP). Controls were treated identically except that no catalytic subunit was present. The use of plastic tubes was necessary because of undesired peptide absorption to glass. Aliquots were separated by high-performance liquid chromatography on a reverse-phase column (TSK-ODS 120T). The column was developed by using a linear gradient of acetonitrile (5 – 80%) containing 0.01 N HCl using a device from Waters Associates. Absorption was measured at 210 nm. Fractions of 1.5 ml were eluted and aliquots (0.5 ml) were counted in a liquid scintillation counter with 2 ml water.

3. RESULTS AND DISCUSSION

Cyclic AMP-dependent phosphorylation of CDD-28 and CDD-88 was tested by assaying 9 μg CDD-28 and 13 μg CDD-88 phosphorylated with 12 μg cPK as described in section 2. After 15 min reaction at 30°C, 70 μl of the reaction volume was applied onto a TSK-ODS 120T column. The column was eluted with a linear gradient of acetonitrile (5–80%) containing 0.01 N HCl. A typical elution pattern of a phosphorylated mixture of CDD-28 and CDD-88 is demonstrated in fig.1A (ATP is not retained and elutes in front). CDD-28 was eluted at 42% acetonitrile followed by elution of CDD-88 (48% acetonitrile). Both peaks contained incorporated radioactivity indicating phosphorylation of a serine residue by the catalytic subunit of cAMP-dependent protein kinase (cPK). The specific phosphorylation of atrial natriuretic peptides provides a useful tool for rapid identification of the peptide hormones as may be observed in fig.1B. Here, an aliquot of a crude extract from bovine atria (15 μg protein) was phosphorylated in the presence of 13 μg cPK for 20 min and 80 μl of the reaction volume was applied onto the column. As can be seen in fig.1B, a peak of radioactivity is eluted at 48% acetonitrile indicating the presence of CDD-88.

The bioactivity of atrial natriuretic peptide hormones is identified by means of biotests with vascular smooth muscles. In this assay the bioactive substances exhibit vasorelaxation. The vasorelaxant effect of unphosphorylated and phosphorylated CDD-28 and CDD-88 was tested using rabbit aorta strips in an organ bath solution. After contraction, when constant force levels had been achieved, peptides were added at a final concentration of 0.3–0.6 $\times$ 10^{-9} M. A typical experiment showing the effects of unphosphorylated and phosphorylated CDD-28 on contracted aortic muscle strips is depicted in fig.2. In comparison to phosphorylated CDD-28 (broken line) the application (arrow) of unphosphorylated CDD-28 (continuous line) causes pronounced relaxation; thus relaxation of vascular smooth muscle is strongly inhibited upon application of phosphorylated CDD-28. The inset in fig.2 summarizes a number of experiments with unphosphorylated and phosphorylated CDD-28 and CDD-88 performed with different smooth muscle preparations. As a measure of the bioactivity the rate of tension decline (force decay between 2 and 10 min after application of the peptide) was plotted vs the logarithm of the time (100% tension prior to application of peptides). The rate of relaxation was calculated from the slopes. The slopes are not true rate constants but only estimates of the relative relaxation per time period. Application of 0.3–0.6 $\times$ 10^{-9} M phosphorylated CDD-28 strongly depresses relaxation by a factor of about four compared to the unphosphorylated peptide. Relaxation caused by 0.3–0.6 $\times$ 10^{-9} M unphosphorylated CDD-88 is less compared to that observed with un-

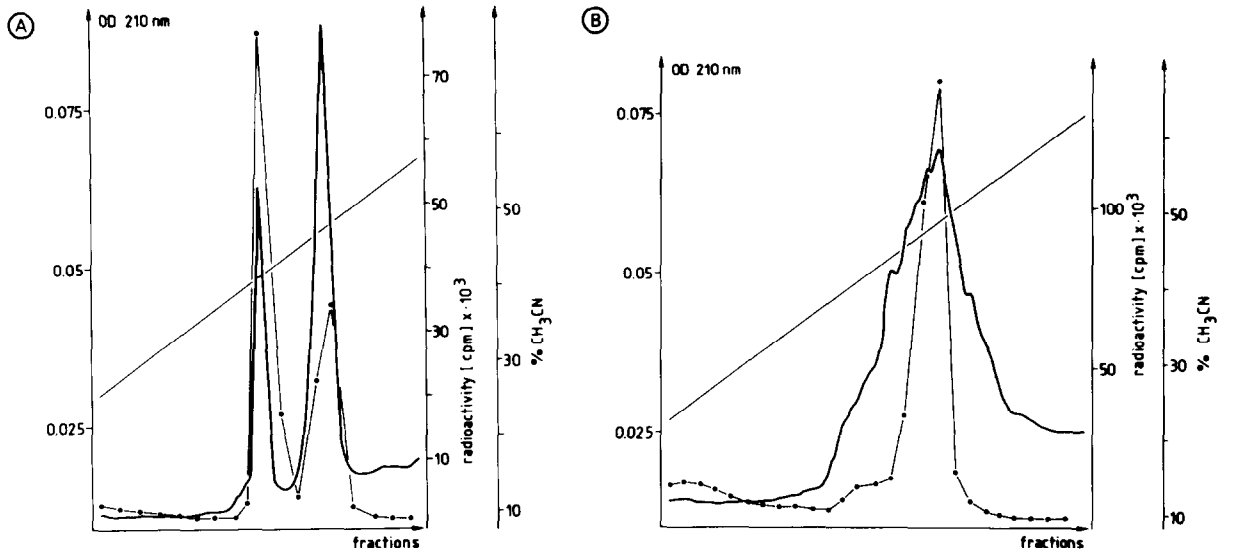

Fig.1. Elution pattern of phosphorylated CDD-28 and CDD-88 (A) and phosphorylated crude bovine atrial extract (B). Cyclic AMP-dependent phosphorylated aliquots of CDD-28 and CDD-88 were separated by reverse-phase HPLC on a TSK-ODS-120T column (300 × 7.8 mm i.d.). Solvent system: a, 0.01 M HCl; b, 0.01 M HCl in 80% aqueous acetonitrile; flow rate, 1.5 ml/min. The gradient is shown by the continuous line. Absorbance (▬) was measured at 210 nm (0.1 absorbance unit range). For detection of radioactivity (●—●) 0.5 ml aliquots per fraction were counted.

phosphorylated CDD-28. Phosphorylation of CDD-88, however, has no significant effect on the relaxation rate. This result is not unexpected, as CDD-88 is extracted from atrial tissue and to our knowledge so far, does not represent a circulating peptide [15].

Studies on primary cultures of neonatal rat cardiocytes [16] demonstrate that cAMP-dependent phosphorylation of Ser^{104} in the 126 amino acid precursor (proANP) occurs to an insignificant extent. Thus, it seems unlikely that the physiological mechanism of cAMP-dependent phosphorylation

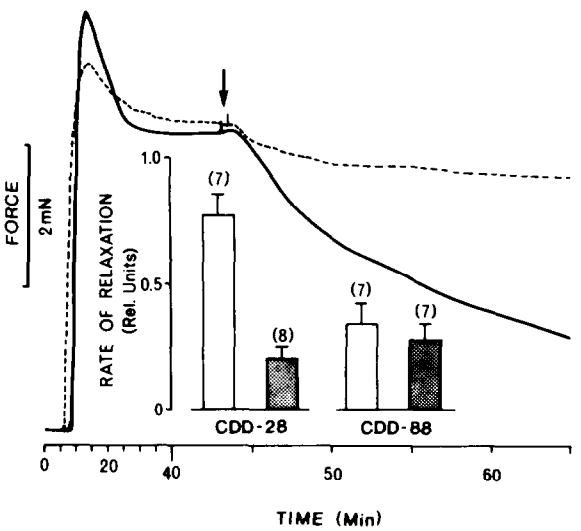

Fig.2. Effect of cAMP-dependent phosphorylation of CDD-28 and CDD-88 on vascular smooth muscle relaxation. Rabbit aortic muscle strips were precontracted by 10^{-7} M norepinephrine. Upon plateau force levels being achieved, peptides were added (arrow) at a final concentration of 0.3–0.6 × 10^{-9} M (unphosphorylated CDD-28, continuous line; phosphorylated CDD-28, broken line). The inset summarises a number of experiments showing the estimates of the rate of relaxation ± SE for unphosphorylated (open bars) and phosphorylated (stippled bars) CDD-28 and CDD-88, respectively. In order to ensure complete phosphorylation of CDD-28 and CDD-88 the concentration of co-substrate in the reaction volume was doubled and the incubation time was prolonged to 40 min.

controls storage or excretion of the peptide hormone. However, cAMP-dependent phosphorylation may be involved in regulation of the biological activity upon secretion. It has been reported that the effect of CDD-28/α-hANP on vascular smooth muscles exhibits regional selectivity [17,18]. The heterogeneity in vasorelaxation seems not to arise solely as a result of differences in the regional distribution of high-affinity receptors for CDD/ANP. In addition, differences in the distribution of particulate guanylate cyclase may influence the vasorelaxant activity ([19] and references cited therein). Modification of the hormone itself appears to determine whether vessels exhibit relaxation to CDD/ANP. Our observations show decreased smooth muscle relaxation after cAMP-dependent phosphorylation of CDD-28/α-hANP. Thus, one may suggest that the expression of a specific peptide activity on a target tissue may be controlled by a fine tuning mediated by a phosphorylation-dependent modification step of the hormone.

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