Parathyroid Hormone-related Protein Antagonizes the Action of Parathyroid Hormone on Adult Cardiomyocytes

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Ventricular cardiomyocytes have been identified as target cells for parathyroid hormone (PTH). A structurally related peptide hormone, parathyroid hormone-related peptide (PTH-rP), is expressed in the heart. In the present study, it was investigated whether PTH-rP can mimic or modify effects of PTH on cardiomyocytes. The investigated effect was induction of creatine kinase (CK) activity, which is associated with cardiac hypertrophy.

PTH and PTH-rP have a similar secondary structure within the active domain 28–34, with exception of amino acid 29. At this position the hydrophilic glutamine in the PTH molecule corresponds to hydrophobic alanine in the PTH-rP molecule. Synthetic PTH or PTH-rP peptides covering domain 28–34 and recombinant full-length PTH(1–84) were used. PTH(28–48) (100 nM) induced CK activity within 24 h (123 ± 3% means ± S.D., n = 4). PTH-rP(7–34) (1 nM to 1 μM) failed to induce CK activity in cardiomyocytes. Given simultaneously, PTH-rP (1 μM) reduced the stimulation of CK activity by PTH(1–84), PTH(1–34), and PTH(28–48) by 94 ± 7, 79 ± 8, and 69 ± 14%, respectively (means ± S.D., n = 4). In contrast, PTH-rP(7–34) was sufficient to stimulate proliferation of chicken chondrocytes. Thus, PTH-rP exerts different effects on cardiomyocytes and classical target cells for PTH.

A synthetic hybrid peptide was synthesized, [Ala29]PTH(28–48), in which alanine replaced glutamine at position 29, as in the PTH-rP molecule. In contrast to PTH(28–48), this mutated peptide [Ala29]PTH(28–48) had no intrinsic activity but antagonized the effect of PTH(1–84) and PTH(28–48) on cardiomyocytes. The results demonstrate that on cardiomyocytes the effect of PTH can be antagonized by PTH-rP. This antagonism seems due to a hydrophobic replacement at position 29.

Cardiac myocytes have been identified as target cells for parathyroid hormone (PTH)1 (1, 2). We found recently that PTH exerts a hypertrophic effect on adult cardiomyocytes, characterized by an increase in protein synthesis and a selective induction of cytosolic creatine kinase (CK) (3). Parathyroid hormone-related peptide (PTH-rP) is a peptide hormone structurally related to PTH. Both peptide hormones have a strong homology in the N-terminal part of the molecule and can bind to the same receptor (4). In contrast to PTH, which is synthesized in the parathyroids, PTH-rP is expressed in many tissues including the heart (5, 6). Direct effects of PTH-rP on myocardial cells have not been investigated before. The aim of the present study was to compare the effects of PTH-rP and PTH on cardiomyocytes with their effects on classical target cells for PTH, i.e. chondrocytes. Recombinant full-length PTH and commercially available synthetic peptides, either covering the protein kinase C-activating domain and the N-terminally located adenylate cyclase activating domain of PTH, or N-truncated peptides, covering exclusively the protein kinase C-activating domain, were used.

Our study focused on the midregional part of the PTH molecule, covering amino acids 28–34. This part of the molecule has been identified as the core of a protein kinase C-activating domain of PTH and PTH-rP (7–11). The secondary structure of the two peptide hormones in this region is very similar (12), consisting of a helical motif. In PTH, the hydrophobic amino acids are placed on one side and the hydrophilic amino acids on the other side (Fig. 1). In PTH-rP, the exception to this rule is that the hydrophobic alanine is located at position 29, i.e. in a hydrophilic environment. The role of this amino acid in the structure-function relationship of the two peptide hormones was analyzed specifically in this study. For this purpose two mutated proteins were synthesized: mutant [Ala29]PTH(28–48), in which the hydrophilic glutamine at position 29 of the PTH molecule is replaced by the hydrophobic alanine, and [AnS29]PTH(28–48), in which glutamine is replaced conservatively by the hydrophilic asparagine.

Isolated cardiomyocytes from the ventricular myocardium of the adult rat were used as an experimental model. In this preparation other cells are absent and the cardiomyocytes are mechanically quiescent. The parameter under investigation was the induction of cytosolic CK, a characteristic feature of the hypertrophic response of cardiomyocytes to PTH stimulation (3).

To compare the effects of PTH-rP on cardiomyocytes with those of classical target cells, we also used primary cultures of chicken derived chondrocytes. This cell system has been used previously to identify the protein-kinase C-dependent domain of PTH (9). PTH stimulates the proliferation of chicken chondrocytes, and incorporation of [3H]thymidine was determined as a measure of DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Ventricular heart muscle cells were isolated from 200–250-g male Wistar rats as described previously (13). Isolated cells were suspended in serum-free culture medium and plated at a density of 4 × 10^5 cells/60-mm culture dish (Falcon 3004). The culture dishes had been preincubated overnight with 4% fetal calf serum in medium 199. The basic culture medium consisted of modified glutamine-free medium 199 with Earle's salts, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (14). To prevent growth of
nonmyocytes, media were also supplemented with 10 μM cytosine β-β-arabinofuranoside.

Four hours after plating, cultures were washed twice with culture medium to remove round and non-attached cells. The remaining cultures consisted of ~95% rod-shaped cells. Following this washing procedure, experimental media were added in which the cells were incubated at 37°C for the times indicated. The experimental media consisted of the basic culture medium (control) and the following additions, as indicated: PTH peptides (human PTH(1–84), bovine PTH(1–34), human PTH(28–48), human [Asn29]PTH(28–48), human [Ala29]PTH(28–48) at position 29, glutamine is replaced by alanine which is at position 29 in PTH-rP).

Chondrocytes were isolated from sterna of 16-day-old embryonic chicks as described previously (9). Chondrocytes were seeded into microfeder plates with 96 wells (6-mm diameter; 14,000 cells/cm²) and a 200-μl volume of medium 199 containing 10% fetal calf serum. After 17 h, the medium was replaced by 200 μl of serum-free medium 199. After 4 days the cultures were used for determination of DNA synthesis.

Analysis of Creatine Kinase Activity—Specific activities of the cytosolic enzyme creatine kinase were determined as follows: Cultures were first washed twice with phosphate-buffered saline (composition in mM: 137 NaCl, 2.6 KCl, 1.5 KH₂PO₄, and 8.1 Na₂HPO₄; pH 7.4). After addition of creatine kinase assay-buffer (composition in mM: 5 magnesium acetate, 0.4 EDTA, 2.5 dithioerythritol, 50 Tris/HCl, and 250 sucrose; pH 6.8) to the dishes, the cells were scraped off, homogenized, and frozen until use at −14°C. For analysis these samples were thawed, and the resulting suspension was sonicated and centrifuged at 12,000 × g for 2 min. The supernatants were used for enzyme analysis. The activity of creatine kinase was determined according to Gerhardt (15) using standard ultraviolet methods. Protein contents were determined according to Bradford (16).

The distribution of the cytosolic isoenzymes of creatine kinase, MM, MB, and BB, was analyzed as described previously (3). The supernatants were applied to 1-ml DEAE-cellulose columns that had been equilibrated with SAE-buffer (composition in mM: 20 NaCl, 5 magnesium acetate, 0.4 EDTA, and 100 Tris/HCl; pH 7.9). The CK-MM isoenzyme eluted directly with this buffer, the CK-MB isoenzyme with change of NaCl concentration to 40 mM and pH to 6.4, the CK-BB with change of NaCl concentration to 250 mM and pH 6.4.

Assay for Thymidine Incorporation in Chondrocytes—The rate of DNA-synthesis was assayed in monolayer cultures of chondrocytes by the incorporation of [³H]thymidine into perchloric acid-predictable material as described previously (9). Briefly, chondrocyte cultures were incubated for 4 h with the appropriate effector and 1 μCi of [³H]thymidine. Subsequently, medium was removed and the cells were washed twice with 200 μl of phosphate-buffered saline. Then cells were lysed by 100 μl of 2% (w/v) Nonidet P-40 and 2% (w/v) sodium dodecyl sulfate treatment and perchloric-insoluble material was precipitated by adding an equal volume of 2% (w/v) perchloric acid in presence of 1% herring sperm DNA as carrier. After storage for 10 min at −20°C, the precipitated material of each well was transferred to glass fiber filters with a Scatron-Assemiautomatic cell harvester. Filters were dried at 80°C for 20 min, transferred to scintillation vials, and 2 ml of scintillation mixture were added. Radioactivity of the samples was determined with a β-counter.

Peptide Synthesis and Purification—The PTH mutants [Asn²⁹]PTH(28–48) and [Ala²⁹]PTH(28–48) were assembled with a Milligen 9500 automatic peptide synthesizer on Fmoc-Sert-butyl/Na-vasyn PA500 resins. Fmoc amino acids were used in a 3-fold excess and activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and diisopropylethylamine in equal molar ratios. Aspartic acid was t-butyl-protected, asparagine and histidine with trityl, arginine with pentamethyl-chroman. After completion of the synthesis, the dried resins were treated for 3 h with trifluoroacetic acid containing 3% trifluorobutylamine and 2% water (10 mg/ml resin). The resins were removed by filtration and washed several times with acetic acid, and the combined filtrate and washings were evaporated to near dryness. The peptides were precipitated by the addition of t-butyl methylthylether and collected by centrifugation. After two resuspensions in ether and centrifugations, the precipitates were dried in a vacuum. The crude peptides were dissolved in water/acetonitrile 1:1 and passed over 6-ml octadecyI disposable extraction columns. After rinsing the columns with acetonitrile, most of the acetonitrile of the combined eluents was evaporated. The samples were lyophilized and the resulting crude peptides analyzed by high performance liquid chromatography. Purification was carried out by preparative reverse-phase high performance liquid chromatography (C-18) with water/acetonitrile gradients containing 0.5% trifluoroacetic acid. The purified peptides were lyophilized and characterized by amino acid analysis and fast atom bombardment mass spectrometry.

Materials—Falcon tissue culture dishes were purchased from Becton-Dickinson (Heidelberg, Germany). Boehringer Mannheim (Mannheim, Germany) was the source for the medium 199 and fetal calf serum. PTH(1-34), PTH(28-48), PTH-rP(1-34), and PTH-rP(7-34) were obtained from Sigma (Deisenhofen, Germany). Recombinant full-
length PTH(1–84) was synthesized according to Ref. 17. All other chemicals were of analytical grade.

Statistics—Data are given as means ± S.D. from n different culture preparations. Statistical comparisons were performed by one-way analysis of variance and use of the Bonferroni test for post hoc analysis (18). Differences with p < 0.05 were regarded as significant.

RESULTS

Effects of PTH and PTH-rP on Creatine Kinase Activity of Cardiomyocytes—The specific activity of cytosolic CK was determined in 24-h-old cultures in presence of full-length PTH (PTH(1–84)) or PTH(1–34)). In classical target cells for PTH, the latter is a full biological agonist. Both PTH variants increased CK activity of cardiomyocytes dose-dependently. Full-length PTH exerted its maximal effect at a 10-fold lower concentration than PTH(1–34); the maximal effects were identical (Fig. 2). PTH(1–84) and PTH(1–34) cover two distinct functional domains: the N-terminal located adenylate cyclase activating domain and the protein kinase C-activating domain located in the 28–34 region. For clarity, a schematic figure of the PTH and PTH-rP variants used in these experiments is given in Fig. 3. To avoid possible side effects of the N-terminally located adenylate cyclase activating domain of PTH or PTH-rP, N-truncated PTH or PTH-rP peptides, i.e. PTH(28–48) or PTH-rP(7–34), were also used. Compared to control conditions, PTH(28–48) (1 μM) increased the activity of CK to 141% (Fig. 4). In contrast, PTH-rP(7–34) (1 nM to 1 μM) had no effect. When PTH-rP(7–34) was added simultaneously with PTH(28–48) (100 nM) to the cultures, PTH-rP(7–34) decreased dose-dependently the stimulation of CK by PTH(28–48), with a two-thirds reduction at 100 nM PTH-rP(7–34) (Fig. 5). PTH(28–48) and PTH-rP(7–34) differ in the length of adjacent C-terminal amino acids. To exclude effects of these C-terminally located amino acids PTH(1–34) and PTH-rP(1–34) were also tested. As shown in Fig. 6, 1 μM PTH-rP(1–34) antagonized the induction of CK through 100 nM PTH(1–34) too.

The question was investigated whether the three cytosolic isoenzymes of creatine kinase, i.e. CK-MM, CK-MB, and CK-BB, were differently induced in cells treated with PTH(28–48). PTH(28–48) (300 nM) increased only the activity of CK dimers containing the B isoform: The CK-MB activity was increased to 175%, and the CK-BB activity to 118%. CK-MM activity was not significantly enhanced (Fig. 7). Simultaneous addition of PTH-rP(7–34) (1 μM) abolished the induction of CK-MB and CK-BB through 100 nM PTH(28–48).

Effect of PTH-rP on DNA Synthesis of Chondrocytes—In contrast to the inability of PTH-rP peptides to induce CK activity in cardiomyocytes, PTH-rP peptides significantly induced DNA synthesis in chondrocytes. PTH-rP(1–34) and PTH-rP(7–34) stimulated DNA synthesis to a similar extent, by 88% and 82%, respectively (Fig. 8).

Effects of Mutated PTH Peptides on Cardiomyocytes—The ability of PTH(28–48) to induce CK activity was compared to the ability of mutated peptides. The conservative mutant, [Asn29]PTH(28–48), stimulated CK activity of cardiomyocytes.
in a dose-dependent manner (118 ± 4% at 100 nM). In contrast, the non-conservative mutant, [Ala29]PTH(28–48), did not induce CK (Fig. 9). It was investigated by addition of PTH(28–48) simultaneously with either [Ala29]PTH(28–48) or [Asn29]PTH(28–48), whether these mutants were able to antagonize the effect of PTH(28–48) on CK activity. [Ala29]PTH(28–48), which lacked intrinsic activity, antagonized the effect of PTH(28–48) in a dose-dependent manner. At 1 μM, a three-fourths inhibition was observed (Fig. 10). [Asn29]PTH(28–48) did not inhibit CK-induction by PTH(28–48). When a phorbol ester, which directly activates protein kinase C, was used to induce CK activity through a receptor-independent route, the mutant [Ala29]PTH(28–48) had no antagonistic effect (Fig. 11).

It was further investigated whether PTH-rP peptides and the mutated antagonistic PTH peptide are able to antagonize CK induction of naturally occurring full-length PTH(1–84) on cardiomyocytes as well. Induction of CK activity by PTH(1–84) was antagonized by either PTH-rP(1–34) or [Ala29]PTH(28–48) to 94 ± 8 and 82 ± 5%, respectively (Fig. 12).

**DISCUSSION**

In the present study the question was investigated whether PTH-rP, a peptide expressed in myocardial tissue, can mimic or modulate the ability of PTH to induce cytosolic CK in ventricular cardiomyocytes. The main finding of the present study is
that PTH and PTH-rP have comparable effects on classical target cells, i.e. chondrocytes, but PTH-rP cannot mimic the action of PTH in cardiomyocytes. PTH stimulates cytosolic CK activity of cardiomyocytes by inducing the fetal type B isoform. In contrast, PTH-rP had no such effect.

The present study has revealed for the first time that PTH-rP may act indirectly on cardiomyocytes, even if lacking a direct effect. PTH-rP (1-34) and PTH-rP (7-34) were found to antagonize the ability of PTH (28-48) and full-length PTH (1-84) to induce cytosolic CK. When at position 29 a hydrophobic residue (alanine) was introduced into the PTH (28-48) molecule, a mutant peptide was obtained that was functionally equivalent to PTH-rP (7-34). It antagonized the effect of full-length PTH (1-84) and PTH (28-48) but lacked intrinsic activity. In case of a hydrophilic replacement at position 29, the activity of PTH (28-48) was not altered. The results show that position 29 is essential for the function of the core domain of PTH, possibly due to the formation of a hydrogen bond. The results also suggest that PTH-rP competes with PTH for the same binding site but lacks intrinsic activity due to this structural differences within the core of the functional domain.

The antagonism between PTH (28-48) and peptides with hydrophobic replacement at position 29 are not due to adverse effects at the intracellular key step (3) for the induction of CK, i.e. protein kinase C. In experiments where protein kinase C was stimulated directly by a phorbol ester, [Ala²⁹]PTH (28-48) had no antagonistic effect. This finding supports the hypothesis that the antagonism between the peptides is due to a competition at sarcoplasmic binding sites.

It has been reported that PTH and PTH-rP differ also in other effects on adult cardiomyocytes; PTH but not PTH-rP was found to increase intracellular calcium (19). In the murine osteoblastic cell line MCT3T3-E1 (20) and in human placenta (21), PTH-rP was also found unable to mimic the action of PTH. These studies did not investigate, however, whether PTH-rP antagonized the effects of PTH on the investigated cell types and did not identify the structural cause for a difference in the action between the two peptide hormones.

In conclusion, the results of this study demonstrate a marked difference for the effects of PTH and PTH-rP on cardiomyocytes but not on chondrocytes. The results offer the opportunity to design heart-specific antagonists of PTH similar to [Ala²⁹]PTH (28-48) used in this study. Our results further indicate that PTH peptides, covering the 28–34 region of PTH, are full biological agonists in respect to CK induction on cardiomyocytes. In contrast, PTH-rP, which is expressed in myocardial tissue itself, seems to function as a paracrine modulator of the hypertrophic effects of PTH in cardiac muscle.

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