Parathyroid hormone improves contractile performance of adult rat ventricular cardiomyocytes at low concentrations in a non-acute way

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Received 16 September 2008; revised 6 January 2009; accepted 19 January 2009; online publish-ahead-of-print 24 January 2009

Time for primary review: 42 days

Aims In patients with congestive heart failure, plasma parathyroid hormone (PTH) levels are positively associated with cardiac function. PTH, used to mobilize stem cells from the bone marrow after myocardial infarction, causes an increased left ventricular ejection fraction. The aim of this study was to investigate whether low but plasma-relevant concentrations of PTH directly influence the contractile properties of cardiomyocytes.

Methods and results Isolated adult rat ventricular cardiomyocytes were exposed to PTH(1–34) or full-length PTH at picomolar concentrations for 24 h. Cell shortening was measured at 2 Hz as a cellular correlate of inotropic responsiveness. Intracellular calcium was measured in Fura-AM-loaded cells. PTH(1–3) (20–200 pM) and full-length PTH (200 pM) increased cell shortening within 24 h. PTH had no effect on cell size, but resting and peak systolic calcium concentrations were elevated. The beneficial effect of PTH was mediated via its cAMP/protein kinase A-activating domain and attenuated by addition of a protein kinase A inhibitor. In contrast, PTH peptides representing a protein kinase C-activating domain but not a cAMP/protein kinase A-activating domain or peptides that represent none of these domains had no effect on cell shortening. The effect of PTH on cell shortening was strong at low concentrations of extracellular calcium but declined at higher calcium concentrations. PTH downregulated the expression of the calcium sensing receptor, a receptor known to antagonize the action of PTH on calcium transport. Furthermore, PTH antagonized the angiotensin II-induced loss of cell function.

Conclusion Low concentrations of PTH improve cell shortening by increasing calcium load at rest. By this mechanism cardiomyocytes compensate reduced extracellular calcium levels as they occur in patients with heart failure.

KEYWORDS Heart function; Calcium sensing receptor; Inotropy

1. Introduction

Ventricular cardiomyocytes are well known as potential target cells of parathyroid hormone (PTH).1–3 PTH has been identified as a pro-hypertrophic agonist.4 Clinical data supported these findings as cardiac hypertrophy is a common finding in patients with end-stage renal disease or hyperparathyroidism in which PTH levels are elevated.5–8 Acute effects of PTH on cardiomyocytes required rather high concentrations of PTH, and it remained an open question whether cardiac effects of PTH really play a role in cardiac disease stages except in patients with end-stage renal disease or hyperparathyroidism. The finding that microvascular endothelial cells express PTH-related protein (PTHrP), a structurally related peptide, has led to the impression that PTHrP rather than PTH is the natural ligand of cardiac PTH/PTHrP receptors.9 PTH and PTHrP display a strong homology in their N-terminal part. This suggests that they act in a similar way on cardiomyocytes. However, detailed analysis of the effects of PTH and PTHrP on cardiomyocytes has also revealed specific effects for each of the two hormones.10

There is rising interest to re-address the question whether chronic elevated plasma levels of PTH influence cardiac physiology. First, end-stage heart failure is often accompanied by reduced plasma calcium concentrations leading to a subsequent increase in plasma PTH. Such a mechanistic link was hypothesized by McCarty in 2005.11 In heart failure, a rise in angiotensin II and subsequently in aldosterone levels must be assumed. The latter is known to increase calcium excretion, which will lead to a corresponding activation of PTH.12 A slight reduction in plasma...
sodium has been demonstrated in patients with end-stage heart failure, too. An other cause for a mild but significant increase in plasma PTH seems to be vitamin D deficiency due to less outdoor activity. The increase in PTH concentration may be small and within the so-called physiological range in patients with heart failure but normal kidney function in contrast to patients with end-stage renal disease or hyperparathyroidism, as mentioned above, but it is nevertheless significantly elevated. Thus, it needs to be analysed whether small increases in plasma PTH affect cardiac performance. Second, application of PTH has been introduced in the treatment of patients with osteoporosis, due to its anabolic effects on osteoblasts. Therefore, a better understanding of chronic effects of PTH on cardiac cells must be established to judge about possible cardiac side effects of chronically applied PTH. Finally, PTH has been shown to mobilize bone-derived stem cells. PTH improved post-infarct recovery, at least under experimental conditions. Nevertheless, it is difficult to decide at present whether this effect of PTH depends on direct cardiac effects evoked by PTH or whether it is indirectly mediated by stem-cell mobilization. Again, a better understanding of long-term effects on cardiac performance would help to understand the potential of PTH under these conditions.

In a previous study, we demonstrated that PTH exerts a pro-hypertrophic effect on cardiomyocytes. We were also able to demonstrate acute effects of PTH on β-adrenoceptor stimulation-dependent inotropic responses. However, it has never been investigated whether PTH modifies contractile performance of cardiomyocytes when applied in a chronic way to these cells. Therefore, the renewed interest in cardiac-specific effects of PTH has prompted us to determine long-term effects of PTH on the contractile behaviour of cardiomyocytes. In the present study, we hypothesized that PTH improves contractile function of cultured adult rat ventricular cardiomyocytes exposed to PTH for 24 h and that PTH counterbalances the effect of reduced calcium and sodium as it occurs in end-stage heart failure.

2. Methods

2.1 Cell culture

Adult ventricular rat cardiomyocytes were isolated from male Wistar rats by collagenase digestion of the hearts as previously described in greater detail. Rats were housed under standardized conditions with free access to food and water. The investigation conforms to the Guide of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Briefly, hearts were excised under deep ether anaesthesia, transferred rapidly to ice-cold saline and mounted on the cannula of a Langendorff perfusion system. Heart perfusion and subsequent steps were all performed at 37°C. First, hearts were perfused in the non-circulating mode for 5 min at 10 mL/min (perfusate in mM: NaCl 110, KH2PO4 1.2, KCl 1.2, MgSO4 1.2, NaHCO3 25, glucose 11, gassed with 5% CO2–95% O2). Thereafter, perfusion was continued with recirculation of 50 mL of the above perfusate supplemented with 0.06% (w/v) crude collagenase and 25 μM CaCl2 at 5 mL/min. After 30 min, ventricular tissue was minced and incubated for 20 min in recirculating medium with 1% (w/v) bovine serum albumin under 5% CO2–95% O2. Gentle trituration through a pipette released cells from the tissue chunks. The resulting cell suspension was filtered through a 78 μm nylon mesh. The filtered material was washed twice by centrifugation (3 min, 25 g) and resuspended in the above perfusate, in which the concentration of CaCl2 was increased stepwise to 0.2 and 0.5 mM. After further centrifugation (3 min, 25 g), the cells in the pellet were suspended in serum-free culture medium (medium 199 with Earle’s salts, 5 mM creatine, 2 mM carnitine, 5 mM taurine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 μM/β-D-arabinofuranoside). The calcium concentration in medium 199 is 1.8 mmol/L. Cells were attached to cell culture dishes by pre-coating of the culture dishes with 4% (vol/vol) fetal calf serum. On average, the initial number of rod-shaped cells was ~4 x 10^3 cells per dish. The cells were washed 2 h after preparation, and the remaining cells were incubated for 24 h with agonists as indicated in the Results section.

2.2 Determination of cell shortening

Cell shortening was determined as described before in greater detail. Briefly, cells were used after 24 h. All cell-shortening experiments were performed at room temperature. Only rod-shaped cells that contracted regularly were used for quantification. Cell length was analysed using a line-camera. Cells were stimulated at 2 Hz for 1 min via two AgCl electrodes with biphasic electrical stimuli composed of two equal, but opposite rectangular 50 V stimuli at 0.5 ms duration. Every 15 s cell shortening was recorded. The median of four cell shortenings per cell was used as the average cell shortening of the individual cell.

2.3 Quantification of calcium transients

To measure cytosolic [Ca2+]i, cardiomyocytes were loaded at 37°C with 5 μM Fura-2AM for 30 min and then washed for further 30 min as described before. Alternating excitation of the fluorescence dye at wavelengths of 340/380 nm of Fura-2 was performed with a Video-Imaging-System (Till Photonics) adapted to the microscope.

2.4 Determination of cell sizes

Myocyte growth was determined on phase-contrast micrographs recorded on tape using a CCD video camera as described before. Cell volumes were calculated by the following formula: volume = (radius)^2 × π × length, assuming a cylindrical cell shape.

2.5 Real-time RT–PCR

In order to quantify the expression of the calcium-sensing receptor in cardiomyocytes, cells were homogenized and RNA was extracted to obtain total cellular RNA. Aliquots (1 μg) were used for real-time polymerase chain reaction using the I-Cycler (Bio-Rad, Germany) and SYBR green as the fluorescence signal. The expressions of the calcium-sensing receptor and β-actin as control. Each sample was run in duplicate. The primer sequence is shown as follows: HPRT: forward CCAGCGTCGTGATTAGTGAT; reverse: AGGTCTGCCATAGTCGTCATC. 

2.6 Determination of protein kinase A activation

In order to determine protein kinase A activity in cells exposed to PTH cells were incubated with the hormone or antagonists for 10 min. Then media were removed from plates, the plates were washed with ice-cold PBS and 1 mL of a lysis buffer (composition in mM: MOPS 20, β-glycerolphosphate 50, sodium fluoride 50, sodium vanadate 1, EGTA 5, EDTA 2, NP40 1%, dithiothreitol 1, benzamidine 1, phenylmethanesulphonylfluoride 1, leupeptin 10 μg/
PTH improves contractile performance

mL, aprotinin 10 μg/mL was added. Cells were scraped using a rubber policeman, transferred to a microcentrifuge tube, sonicated \( 3 \times 20 \) s, and centrifuged. The remaining supernatant was used to determine the protein content and measure protein kinase A activity. Kinase activity was measured using the non-radioactive PKA kinase activity assay kit (assay Designs, Inc., Ann Arbor, USA). Samples were measured exactly according to the manufacturer’s suggestions.

2.7 Statistics

Results are expressed as means ± standard deviation (SD) or standard error of the mean (SEM) as indicated. Differences were analysed by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis. A value of \( P < 0.05 \) was regarded as significant.

3. Results

3.1 Long-term effect of parathyroid hormone on cardiomyocyte cell shortening

In order to address the question whether chronic exposure of cardiomyocytes to PTH(1–34) modifies the contractile performance, adult rat ventricular cardiomyocytes were exposed to various concentrations of PTH(1–34) for 24 h. Thereafter, cells were paced at 2 Hz and cell shortenings were recorded. A bell-shaped concentration curve was observed. PTH(1–34) at 20 pM was sufficient to increase cell shortening. A maximal effect was obtained at 200 pM (Figure 1A). On average, PTH (200 pM) increased cell shortening normalized to diastolic cell lengths by 16.1 ± 3.6% from 6.72 ± 0.16% of diastolic cell length to 7.80 ± 0.25% of diastolic cell length. In absolute values PTH(1–34) increased the mean shortening amplitude by 16.1 ± 4.9% from 5.77 ± 0.14 μm to 6.70 ± 0.28 μm. During the whole study the effect of PTH(1–34) on cell shortening was analysed in five different preparations (221 cells in total). The mean increase of cell shortening normalized to diastolic cell length was 11.3 ± 1.8% (range: 6.5–16.1%). Figure 1B gives a representative single-cell recording. Experiments were also repeated in the presence of rec-hPTH(1–84) in order to address the question whether N-terminal PTH-peptides used for this study represent the bioactivity of full-length PTH. Rec-hPTH(1–84) increased cell shortening by 10.6 ± 1.9% \( [n = 48 \text{ cells from two preparations; not significantly different from PTH(1–34)}] \).

The effect of PTH(1–34) at 200 pM was analysed in more detail. PTH(1–34) did not evoke any effect on cell length, cell width, or cell volume (Figure 2A). However, in addition to an increased cell shortening, the maximal contraction velocity and the maximal relaxation velocity were significantly accelerated by 33.3 and 26.9%, respectively. In absolute values contraction velocity increased from 123 ± 3 to 164 ± 7 μm/s and relaxation velocity from 119 ± 4 to 151 ± 5 μm/s (Figure 2B). Finally, we addressed the question whether PTH influences the intracellular calcium concentration. Cardiomyocytes exposed to PTH(1–34) for 24 h displayed higher resting calcium as well as a higher peak calcium. Calcium transients were not different (Figure 2C). PTH-dependent calcium absorption by mouse cortical ascending limbs is negatively regulated by the calcium-sensing receptor.22 Thus, we hypothesized that PTH may increase resting calcium by down-regulating calcium-sensing receptors. Indeed, PTH(1–34) at 200 pM decreased the steady-state mRNA expression of the calcium-sensing receptor by 48 ± 13% \( (n = 3, P = 0.019) \).

3.2 Signal transduction pathways involved in the parathyroid hormone effect

PTH is known to activate either adenyl cyclase or phospholipase C, leading to a subsequent activation of protein kinase A (PKA) or protein kinase C (PKC). We first addressed the question whether an activation of PKA is required for the PTH effects described above. For this purpose experiments were repeated in the presence of H89 (1 μM), an inhibitor of PKA. When the cells were exposed to H89 for 24 h basal contractile activity was already reduced (Figure 3). Moreover, PTH(1–34) was unable to increase cell shortenings in the presence of H89 (Figure 3). These data suggested that PTH exerts the above-mentioned effects via an activation of the adenyl cyclase-activating domain. This hypothesis was further verified in experiments in which cells were exposed to PTH(7–34). This peptide lacks the adenyl cyclase-activating domain but still represents an intact PKC-activating domain. As expected, PTH(7–34) was unable to increase cell shortening (Figure 3). These data strongly argue for a cAMP/PKA-dependent effect and against a potential role for PKC. This was further verified in experiments in which cardiomyocytes were exposed to N-truncated PTH peptides with an intact PKC-activating domain or a point mutation that destroys bioactivity of the PKC-activating domain.23 PTH(28–48)-Asn29, representing an intact PKC-activating domain but no adenyl cyclase-activating domain did not perform a significant effect on cell shortening (control: 10.60 ± 1.02; PTH: 11.16 ± 1.53%; \( n = 52 \) cells,

![Figure 1: Effect of PTH(1–34) on cell shortening. Cardiomyocytes were exposed to parathyroid hormone for 24 h and cell shortening was measured thereafter at 2 Hz. (A) Concentration response curves for cell shortening expressed after normalization to diastolic cell lengths (left) or as absolute values (right). (B) Representative single-cell recording. Data are means ± SEM from \( n = 36 \) cells. *\( P < 0.05 \) vs. untreated controls.](https://example.com/figure1.png)
$P = 0.07$ vs. control). PTH(28–48)-Ala29, representing a peptide with neither a PKC-activating domain nor an adenyl cyclase activating domain, did not exert any effect either ($10.97 \pm 1.53; n = 52$ cells, $P = 0.229$ vs. control). Collectively, these data strongly suggest that PTH(1–34) affects cell shortening via its adenyl cyclase/protein kinase A-activating domain.

The latter finding was confirmed by biochemical determination of protein kinase A activity. Cells were exposed to PTH(1–34), PTH(7–34), H89, or combinations of them for 10 min. Thereafter cells were harvested and the protein kinase A activity was measured. As expected, PTH(1–34) increased protein kinase A activity significantly by $26.3 \pm 11.9\%$ (Figure 3B). Neither PTH(7–34), nor H89 or PTH(1–34) in the presence of H89 increased protein kinase A activity. Isoprenaline (10 nM) was used as a positive control. The $\beta$-adrenergic agonist increased protein kinase A activity earlier (within 5 min) and to a significant higher amount ($+39.9 \pm 7.9\%, n = 4$) than PTH. These data confirmed the findings drawn from the cell-shortening experiments.

### 3.3 Effect of parathyroid hormone on maximal cell shortening

All experiments mentioned above characterize the effect of PTH on basal cell shortening. As next, we addressed the question whether PTH modifies the maximal induced cell shortening. Cardiomyocytes were again exposed to PTH(1–34) (200 pM) for 24 h. Then the cells were exposed to a buffer that contained either 1.1, 1.8, or 2.3 mM calcium. As expected, cell shortening increased by increasing extracellular calcium concentrations. However, cells pre-exposed to PTH(1–34) displayed a significant increase in cell shortening at low calcium concentration but not at high calcium concentrations (Figure 4A). As found for maximal
calcium-induced cell shortening, cells pre-exposed to PTH(1–34) for 24 h did not display a further increase in isoprenaline-induced cell shortening, too. As expected, isoprenaline increased cell shortenings of untreated control cells as well as in those pre-exposed to PTH. However, in cells pre-exposed to PTH no further increase was observed above the level of isoprenaline in untreated cells (Figure 4B).

3.4 Effect of parathyroid hormone on cell shortening in modified media

As indicated in the introduction, PTH concentrations are slightly elevated in patients with end-stage heart failure. Plasma concentration of calcium and sodium is slightly reduced in these patients.13 In order to understand the relevance of the observed effect of PTH under these conditions, we performed experiments in modified media that completely mimicked this situation. Extracellular calcium was reduced from 1.1 to 0.9 mM and extracellular sodium was reduced from 143 to 137 mM. Cells exposed to PTH(1–34) for 24 h developed an increased cell shortening in the presence of normal calcium and sodium that was improved under conditions of slightly reduced calcium and sodium that represent more directly the changes in electrolyte balance of patients with heart failure. Under these
conditions, PTH(1–34) increased cell shortening by 17.7% (Figure 5).

3.5 Effect of parathyroid hormone on angiotensin II-induced contractile failure

The same cell-culture system was recently used to investigate the effect of angiotensin II (Ang II, 100 nM) on cell shortening. As activation of the renin–angiotensin system is a key process leading to heart failure, we finally addressed the question whether PTH is able to antagonize this effect. As expected, Ang II reduced basal cell shortening by 29.7 ± 1.4% (Figure 6). Pre-exposure of cardiomyocytes to PTH increased cell shortening by 14.2 ± 3.8% and completely antagonized the Ang II-dependent negative contractile effect (Figure 6).

4. Discussion

In patients with heart failure various factors limit the contractile activity of cardiomyocytes and impair normal heart function. Among others, an elevated plasma Ang II level, electrolyte imbalance as characterized by lower free calcium and sodium, and as a result of this electrolyte imbalance slightly increased plasma values of PTH are observed. Although these alterations are well known and documented it is less clear which of these changes are part of an adaptive mechanism that allows the heart to maintain normal pump activity and which of these are directly linked to the loss of function. This study was aimed to clarify the role of constitutively elevated PTH levels as they occur in patients with heart failure. We hypothesized that cardiomyocytes develop an altered function if they are constitutively exposed to PTH levels in the range of those found in plasma of patients with heart failure but without established renal disease. Indeed, the current study shows that if cardiomyocytes are exposed to low concentrations of PTH for 24 h they develop an improved mechanical response to electrical stimulation. Moreover, this effect was observed at low concentrations of PTH. At higher concentrations PTH has been shown to evoke a pro-hypertrophic effect which was evoked via its PKC-activating domain. The newly described effect of picomolar concentrations of PTH on cardiomyocytes' function seemed to be cAMP/PKA-dependent. Although we did not directly measure cAMP levels, we showed that the effects of PTH described in this study require an intact cAMP/PKA-activating domain and were insensitive to peptides representing a PKC-activating domain. In a former study, we have also directly measured the accumulation of cAMP in adult rat ventricular cardiomyocytes in response to PTH(1–34). The data of this former study indicated a 1.50-fold increase in total cAMP within 5 min that was nevertheless low compared with isoprenaline (29.5-fold) and that did not reach the level of significance. We also found a small increase in contraction amplitudes (10 nM PTH: +7.2%, n.s.) that declined at higher concentrations (1 μM). The data of our new study suggest that the response of cardiomyocytes to cAMP-dependent signalling is slow and requires a longer time period to get significant, such as 24 h as has been used in this study. Noteworthily, the latter represents more precisely conditions of heart failure in which cardiomyocytes are exposed constitutively to small elevated plasma PTH levels.

Thus, our data suggest that slightly elevated concentrations of plasma PTH in patients with heart failure represent an adaptive response. It is in line with this assumption that patients treated for heart failure of less-advanced severity had higher circulating levels of PTH than patients with more severe heart failure.

The question whether PTH modifies the contractile response of cardiomyocytes has been addressed before. However, in most of those experiments it was investigated whether PTH modifies the contractile response in an acute way. In some conditions of heart failure in which PTH is elevated to much higher values such as hyperparathyroidism or in patients with renal dysfunction requiring dialysis, PTH is considered as an agent that depresses cardiac function. As expected from these studies a marked improvement of left ventricular function after parathyroidectomy was observed in a haemodialysis patient with secondary hyperparathyroidism and left ventricular dysfunction. Other studies on patients with primary hyperparathyroidism have demonstrated a long-lasting increased risk of myocardial hypertrophy and heart failure.

In contrast, experiments on cardiomyocytes exposed to PTH in an acute way have not developed a clear picture. In addition the question whether cardiomyocytes chronically exposed to PTH respond in a similar way has never been considered. Our study is different from studies published previously on PTH-dependent changes in cardiomyocytes’ function as the cells were exposed at least for 24 h to PTH. Such experiments have been performed before, but in those cases hypertrophy-related parameters were investigated rather than functional data. The study suggests that PTH improves cardiac function as long as the concentration is too low to activate the PKC-dependent pathways. The latter one seems to be related to myocardial hypertrophy and heart failure. On the mechanistic basis this seemed to be achieved by increased intracellular calcium. Similarly, peripheral blood mononuclear cells are characterized by elevations in intracellular calcium under conditions of aldosteronism that go along with reduced plasma calcium. This was termed ‘calcium paradox’ to indicate that lower plasma calcium leads to more plasma PTH that then increase intracellular calcium. The fall in plasma ionized calcium concentration seems to be the consequence of Ang II-dependent induced aldosteronism leading to an increased urinary and faecal excretion of calcium. Alternatively, aldosterone may increase calcium channel number or open probability directly. However, PTH is able to increase intracellular calcium via activation of voltage-dependent calcium channels in cardiomyocytes. Therefore, it is likely to speculate that the effect of PTH on diastolic and peak systolic calcium in cardiomyocytes is mediated by this pathway. However, the described acute effect of PTH on voltage-dependent calcium currents was independent of cAMP/PKA and full-length PTH evoked a stronger effect than PTH (1–34). Thus, we proved another possibility that might explain the effect of PTH on resting calcium. Here we
demonstrate that PTH downregulates the steady state mRNA of the calcium-sensing receptor in cardiomyocytes. This receptor antagonizes PTH-dependent calcium transport at least in mouse cortical ascending limbs. This indicates that on the cellular level stimulation of the calcium-sensing receptor is able to antagonize the action of PTH. Therefore, its downregulation may explain at least in part the increase in intracellular calcium observed in cells exposed to PTH. It is in line with these assumptions that in patients with congestive heart failure elevated PTH levels are associated with a better left ventricular ejection fraction and resting cardiac output.

Acknowledgements

We thank the expert technical assistance of Peter Volk and Nadine Woitasky.

Conflict of interest: none declared.

Funding

Supported by grants from the Deutsche Forschungsgemeinschaft (DFG), from the Excellence Cluster Cardio-Pulmonary Sciences (ECCPS), and from the Rhö́n-Klinikum Giessen-Marburg.

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