Parathyroid hormone induces protein kinase C but not adenylate cyclase in adult cardiomyocytes and regulates cyclic AMP levels via protein kinase C-dependent phosphodiesterase activity

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Adult ventricular cardiomyocytes have been identified as target cells for parathyroid hormone (PTH) but little is known about its signal transduction in these cells. In the present study the influence of PTH on cyclic AMP accumulation and the activity of protein kinase C (PKC) in cardiomyocytes was evaluated. A mid-regional synthetic fragment of PTH, PTH-(28–48), which exerts a hypertrophic effect on cardiomyocytes, increased the activity of membrane-associated PKC in a dose-dependent manner (1–100 nM). Activated membranous PKC was dependent on Ca^{2+} and sensitive to an inhibitor of Ca^{2+}-dependent isoforms of PKC. When adenylate cyclase was stimulated by the addition of isoprenaline, a β-adrenoceptor agonist, PTH-(28–48) antagonized cyclic AMP accumulation. This antagonistic effect of PTH-(28–48) could be mimicked by activation of PKC with a phorbol ester and inhibited by isobutylmethylxanthine, a phosphodiesterase inhibitor. An *N*-terminal synthetic fragment, PTH-(1–34), which includes an adenylate cyclase-activating domain, did not stimulate the accumulation of cyclic AMP in cardiomyocytes. The results demonstrate that in adult cardiomyocytes PTH (1) is able to stimulate PKC, (2) is not able to cause accumulation of cyclic AMP and (3) functionally antagonizes the effect of β-adrenoceptor stimulation to increase cellular cyclic AMP concentrations via PKC-dependent phosphodiesterase activity.

INTRODUCTION

Cardiac myocytes have been identified as target cells for parathyroid hormone (PTH) [1–3]. In a recent study from our laboratory it was demonstrated that PTH exerts a hypertrophic effect on adult ventricular cardiomyocytes, characterized by an increase in protein synthesis and selective induction of cytosolic creatine kinase [4]. This hypertrophic effect could be mimicked by a phorbol ester and partially abolished by staurosporine, suggesting that activation of protein kinase C (PKC) is involved in the signal transduction.

In bone- and kidney-derived cells, i.e. well-known classic target cells of PTH, the mechanism of signal transduction of PTH has been investigated in great detail. In these cells, PTH activates both adenylate cyclase [5] and PKC [6,7]. For cardiomyocytes, little is known about the second-messenger pathways activated by PTH. In cultures of neonatal cardiomyocytes from rats, PTH was found to activate adenylate cyclase [1,8], but no such effect was found when papillary muscles from adult rats were used [9]. The possible action of PTH on PKC in heart cells has not yet been examined experimentally.

The aim of the present study was to evaluate the possible effects of PTH on PKC and adenylate cyclase in adult ventricular cardiomyocytes. The analysis was focused on the domains of the PTH molecule that have been found to activate PKC and adenylate cyclase in classic PTH-target cells. In these cell types, the N-terminal region of the hormone including the first two amino acids stimulates adenylate cyclase [5], and a mid-regional part, including amino acids 28–34, stimulates the activity of PKC [10,11].

Therefore two commercially available synthetic fragments of PTH were used: PTH-(1–34), which contains both active domains and is therefore capable of stimulating adenylate cyclase and PKC; PTH-(28–48), which contains only the mid-regional domain known to stimulate PKC. Both fragments have a hypertrophic effect on adult cardiomyocytes [4].

Isolated cardiomyocytes from the ventricular myocardium of the adult rat were used as an experimental model. In this model the metabolic response of the myocardial cell can be investigated independently of the influence of other cell types and, as these cells are mechanically quiescent, independently of direct and indirect effects on cell contractility.

MATERIALS AND METHODS

Cell culture

Ventricular heart muscle cells were isolated from 200–250 g male Wistar rats as described previously [12]. Isolated cells were suspended in serum-free culture medium and plated at a density of 4×10^5 elongated cells/60 mm culture dish (Falcon type 3004). The culture dishes had been preincubated overnight with 4% fetal calf serum in medium 199. The basic cell culture medium consisted of modified glutamine-free medium 199 with Earle's salts, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 100 units/ml penicillin and 100 μg/ml streptomycin [13]. To prevent growth of non-myocytes, media were also supplemented with 10 μM cytosine β-D-arabinofuranoside.

At 4 h after plating, cultures were washed twice with culture medium to remove round and unattached cells. The remaining cultures consisted of more than 95% rod-shaped cells. After 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: fragments of PTH [bovine PTH-(1–34), human PTH-(28–48)], phorbol 12-

Abbreviations used: IBMX, isobutylmethylxanthine; PKC, protein kinase C; PTH, parathyroid hormone; PMA, phorbol 12-myristate 13-acetate.

myristate 13-acetate (PMA) and isoprenaline. Ascorbic acid (100 μM) was added to culture media as an antioxidant. Under all conditions, cardiomyocytes remained mechanically quiescent.

Accumulation of cyclic AMP

Adenylate cyclase activity was measured as cyclic AMP accumulation in the cultures over a period of 5 min under the specified experimental conditions. The cells were incubated with modified Tyrode's solution (pH 7.4) containing 125 mM NaCl, 1.2 mM KH_2PO_4, 2.6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes and, in some of the experiments, 1 mM 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. All experiments were performed in the presence of adenosine deaminase (5 units/ml), as adenosine released from the cells may exert an antagonistic effect on adenylate cyclase by interacting with the A_1-adenosine receptor [14]. Experiments were terminated by the addition of 1 ml of 1.2 M $HClO_4$ to the contents of the culture dishes. The cells were scraped off and centrifuged for 1 min at 12000 *g*. The supernatant was neutralized and quickly frozen in liquid nitrogen. The pellet was redissolved in 0.1 M NaOH, and its protein content determined by the method of Bradford [15] with BSA as standard. The frozen supernatants were freeze-dried and redissolved in 100 μl of 0.1 M Hepes (pH 7.4). Cyclic AMP content of these samples was determined using a protein-binding assay (Amersham-Buchler, Braunschweig, Germany).

Measurement of PKC activity

The specific activity of PKC was determined in the membrane fraction of cardiomyocytes using the method of Charkravarthy et al. [16] which allows measurement of the activity of the enzyme in its native membrane-associated state. With this method the active membrane-associated fraction is determined; *in vitro* stimulators of the enzyme are not used. Cultures were incubated for the time indicated, rinsed twice with ice-cold PBS and finally covered with ice-cold hypotonic lysis buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF, pH 7.5). The swollen cells were scraped off the dishes and lysed by vigorous mixing at room temperature for 2 min. The lysates were centrifuged at 4 °C for 5 min at 1000 *g* to sediment any unlysed cells and the nuclei. The postnuclear supernatants were centrifuged at 4 °C for 20 min at 35000 *g* to sediment the cell membranes. The supernatants from this step were discarded and the pelleted membranes suspended in 200 μl of assay buffer (50 mM Tris/HCl buffer, pH 7.5, 2 μM $CaCl_2$, 10 mM $MgCl_2$, 200 μM PMSF, 2 mM NaF, 200 μM sodium pyrophosphate, 200 μM sodium vanadate), and vigorously triturated through a pipette tip to produce a homogeneous suspension.

PKC activity in this fraction was measured using the specific substrate peptide PLSRTLSVAAKK [17]. The assay mixture contained 50 μl of membrane suspension (10 μg of protein) in assay buffer and 10 μl of 900 μM substrate peptide (in 50 mM Tris/HCl buffer, pH 7.5). The total volume was adjusted to 90 μl with 50 mM Tris/HCl buffer (pH 7.5). The reaction was started by the addition of 10 μl of 500 μM [^{32}P]ATP (0.5 μCi/tube). The reaction was stopped after 10 min by the addition of 10 μl of 5% acetic acid. The tubes were centrifuged at 12000 *g* for 5 min at 4 °C and then placed on ice. Supernatant (50 μl) was passed through DEAE-Sepharose columns. A stepwise salt gradient (20, 40, 250 and 1000 mM NaCl) was used to elute the phosphorylated substrate peptide within the 250 mM fraction. Radioactivity in each fraction was counted. To calculate the amount of radioactivity incorporated specifically into the substrate peptide, blanks without the substrate peptide were also measured and subtracted. In the range 1–10 μg of membrane protein, the reaction was linear ($r = 0.97$, $n = 7$).

Extraction of nuclear proteins from cardiomyocytes

Nuclear extracts from cardiomyocytes were prepared as described by Dignam et al. [18]. Cells were washed twice with ice-cold PBS, scraped off and centrifuged at 4 °C and 1000 *g*. The pellet was redissolved in the hypotonic lysis buffer (as used for preparation of the membrane fraction) and again centrifuged at 4 °C and 1000 *g*. The pellet was redissolved again in hypotonic lysis buffer, stirred for 30 min at 4 °C and centrifuged for 30 min at 25000 *g*. The supernatant was dialysed against buffer containing 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM dithiothreitol overnight at 4 °C. The dialysate was centrifuged at 25000 *g* for 20 min. The supernatant represented the nuclear extract. Aliquots containing 1 mg/ml were frozen and stored at −14 °C until use.

PAGE

Samples for electrophoretic analysis were precipitated by adding 10% (v/v) trichloroacetic acid for 1 h at 4 °C. Samples were centrifuged at 12000 *g* and the pellets redissolved in sample buffer [62.5 μM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycine, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) Bromophenol Blue] as described by Laemmli [19]. Electrophoresis was carried out on SDS/10% polyacrylamide gels [19]. The gels were stained with Coomassie Blue R 250 and dried. Autoradiographs were obtained by exposing gels to Kodak X-Omat AR film.

Cell contractions

Maximal cell shortening [dL/L (% of cell length)] was determined as described previously [20]. Cells were incubated in modified Tyrode's solution and stimulated for 15 min with isoprenaline and PTH fragments. At the end of the preincubation period the cultures were placed on a microscopic stage maintained at 37 °C. Keeping the microscopic visual field in the middle, two AgCl electrodes were immersed to a distance of 5 mm into the fluid. Biphasic electrical stimuli composed of two equal but opposite rectangular 60 V stimuli of 0.5 ms duration were applied at a frequency of 0.5 Hz. The phase-contrast micrograph was recorded on tape using a CCD-video camera. The contractions of single cells were determined from consecutive frozen video frames magnifying the cell's picture 500-fold on a video monitor screen.

To assess the onset of spontaneous cell contractions, cultures were incubated for 3 days under serum-free conditions, as described previously [13]. On the third day, isoprenaline, PTH fragments or both were added. After 1 h, the rate of spontaneous contraction was determined.

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 [21]. Differences with $P < 0.05$ were regarded as significant.

Materials

Falcon tissue-culture dishes were purchased from Becton-Dickinson (Heidelberg, Germany). Boehringer-Mannheim (Mannheim, Germany) was the source of medium 199 and

fetal calf serum. PTH peptides, isoprenaline, PMA and cell culture supplements were obtained from Sigma (Deisenhofen, Germany), PKC pseudosubstrate PKC-(19–36) was from Saxon Biochemicals (Hannover, Germany) and calphostine from Calbiochem (Bad Soden, Germany). Radiochemicals were purchased from Amersham-Buchler (Braunschweig, Germany). All other chemicals were of analytical grade.

RESULTS

Stimulation of PKC

In order to evaluate the effect of PTH on PKC, the ability of PTH-(28–48) to increase PKC specific activity in the membrane fraction of adult cardiomyocytes was investigated. For comparison, time-course experiments were carried out in the presence of either PTH-(28–48) (100 nM) or PMA (100 nM), a receptor-independent activator of PKC (Figure 1). Within 10 min of incubation, PTH-(28–48) and PMA stimulated PKC to an activity of 262±34% and 266±22% respectively (not significantly different). After 15 min, however, the stimulatory effect of PTH-(28–48) had started to decline, in contrast with the effect of PMA. The stimulation of PKC by PTH-(28–48) was dose-dependent (Figure 2). Over the PTH-(28–48) range 1–100 nM PKC activity was significantly enhanced.

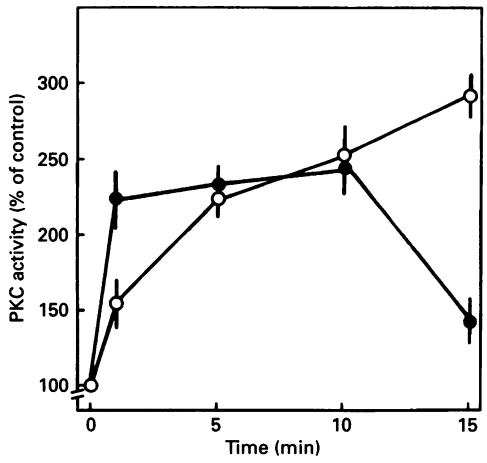

Figure 1 Time course of PKC stimulation in the membrane fraction of cardiomyocytes in the presence of 100 nM PMA (○) or 100 nM PTH (28–48) (●)

Data are given as means±S.D. of three cell preparations. At all times, the increase in PKC activity was significantly different from controls ($P < 0.01$). Basal activity was 112±16 pmol of phosphate incorporated/10 min per 10 μg of membrane protein.

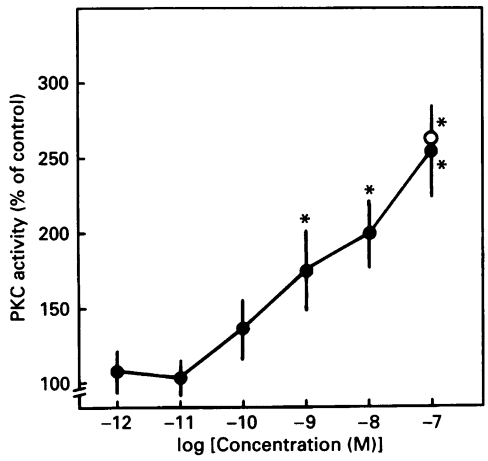

Figure 2 Stimulation of PKC in the membrane fraction of cardiomyocytes by 10 min exposure of the cells to 100 nM PMA (○) or various concentrations of PTH-(28–48) (●)

Data are given as means±S.D. of three cell preparations; *$P < 0.05$ compared with control. Basal values were the same as in Figure 1.

Table 1 Inhibition of membranous PKC activity by addition of PKC-(19–36)

PKC activity was measured in the membrane fraction of cardiomyocytes under control conditions and after a 10 min exposure to 1 μM PMA or 100 nM PTH-(28–48) in the absence or presence of the pseudosubstrate PKC-(19–36), which blocks Ca^{2+}-dependent isoforms of the enzyme. Data are given as means±S.D. of three cell preparations. * $P < 0.05$, compared with inhibition by 1 μM PMA alone; † $P < 0.05$, compared with 100 nM PTH-(28–48) alone.

	PKC activity (pmol/10 min per 10 μg)	Inhibition (%)
Control	118±11	0±9
Control + PKC-(19–36) (1.0 μM)	98±12	17±9
PMA	295±50	0±17
PMA + PKC-(19–36) (0.1 μM)	240±10	31±4*
PMA + PKC-(19–36) (1.0 μM)	211±11	47±5*
PMA + PKC-(19–36) (10.0 μM)	165±15	73±9*
PTH-(28–48)	299±48	0±17
PTH-(28–48) + PKC-(19–36) (1.0 μM)	203±22	68±11†

Table 2 Effect of Ca^{2+} concentration and PMA on basal PKC activity of a membrane preparation

PKC activity was measured in the membrane fraction of cardiomyocytes under control conditions as described in the Materials and methods section, and either without the addition of Ca^{2+} ions or with the addition of PMA to the incubation tubes. Data are given as means±S.D. of three cell preparations. *$P < 0.05$, compared with activity in the presence of 1 μM $CaCl_2$.

	PKC activity	
	(pmol/10 min per 10 μg)	(%)
$CaCl_2$ (1 μM)	123±14	100±11
$CaCl_2$ (0 μM)	11±5	9±4*
$CaCl_2$ (1 μM) + PMA (1 μM)	140±22	114±18

To distinguish between Ca^{2+}-dependent and -independent isoforms of PKC, a specific PKC inhibitor peptide, PKC-(19–39), was used. This inhibitor peptide contains a sequence that is homologous to the regulatory domain of the Ca^{2+}-dependent isoforms of PKC and can therefore be used as a tool to identify the presence of these isoforms [22]. The specific activity of PKC in membrane preparations from PMA-stimulated cardiomyocytes was dose-dependently inhibited by PKC-(19–39) (Table 1). At a concentration of 1 μM, this peptide also inhibited PKC in membrane preparations from PTH-(28–48)-stimulated cardiomyocytes, but not in membrane preparations from control cultures. PKC activity fell to about 10% in the absence of Ca^{2+}, and membrane preparations from control cultures could not be activated by subsequent addition of PMA (Table 2).

Table 3 Effect of PTH-(28–48) and PMA on PKC activity measured with a fraction of nuclear proteins

Phosphorylation of nuclear proteins by the membrane fraction of cardiomyocytes was measured under control conditions and after 10 min exposure to 100 nM PTH-(28–48) or 1 μM PMA. To identify the PKC-dependent part of total phosphorylation, 10 μM calphostine, a PKC inhibitor, was used. Values are given as means ± S.D. of three cell preparations. *$P < 0.05$, compared with the control.

	PKC activity (pmol/10 min per 10 μg)	Phosphorylation (%)
Control	241 ± 27	100 ± 11
Control + calphostine	253 ± 21	105 ± 9
PTH-(28–48)	415 ± 31	172 ± 13*
PTH-(28–48) + calphostine	224 ± 9	93 ± 4
PMA	434 ± 35	180 ± 15*
PMA + calphostine	238 ± 19	99 ± 7

The relevance of this PKC assay was further tested by replacing the specific, but artificial, substrate peptide by nuclear proteins isolated from cardiomyocytes. Membrane fractions from cardiomyocytes stimulated by PTH-(28–48) (100 nM) or PMA (100 nM) increased phosphorylation of nuclear proteins from cardiomyocytes by 72 and 80% respectively compared with membrane preparations from control cultures (Table 3). The augmented rate of phosphorylation of nuclear proteins was abolished in the presence of calphostine, a potent and highly specific inhibitor of PKC, indicating that the increase in phosphorylation was indeed dependent on PKC. Phosphorylation of nuclear proteins by membrane preparations from control cultures, however, could not be inhibited by calphostine. In these experiments the same pattern of phosphorylated nuclear proteins was identified by SDS/PAGE in membranes from control cultures and those treated with PTH-(28–48) or PMA.

Direct effects on cellular cyclic AMP content and functional correlates

As an indirect assay of adenylate cyclase activity in intact cardiomyocytes, cyclic AMP accumulation in cultures was determined in the presence of the phosphodiesterase inhibitor IBMX. IBMX was added to cultures 15 min before the experiments were performed. The β-adrenoceptor agonist isoprenaline (10 μM) increased cyclic AMP content from 1.4 ± 0.6 pmol/mg of protein (control) to 41.5 ± 7.4 pmol/mg of protein within 5 min. Exposure of cardiomyocytes to isoprenaline did not increase cyclic AMP levels further (Figure 3). Direct activation of adenylate cyclase by the addition of forskolin (1 μM) increased cyclic AMP content to 71.2 ± 9.8 pmol/mg of protein within 5 min and 150.3 ± 12.4 pmol/mg of protein within 10 min (Figure 3). In contrast, PTH-(1–34) at a concentration of 100 nM had no effect on cellular accumulation of cyclic AMP (Table 4).

The effect of increased levels of cyclic AMP on the functional properties of cardiomyocytes was tested in two assays. First, the maximal cell shortening of electrically stimulated freshly isolated cardiomyocytes was analysed. Cell shortening depends on cellular cyclic AMP content as described previously [23]. Isoprenaline (10 μM) increased maximal cell shortening by 67% on average (Table 5). In contrast, the N-terminal PTH fragment PTH-(1–34) had no effect at concentrations up to 1 μM. For the second

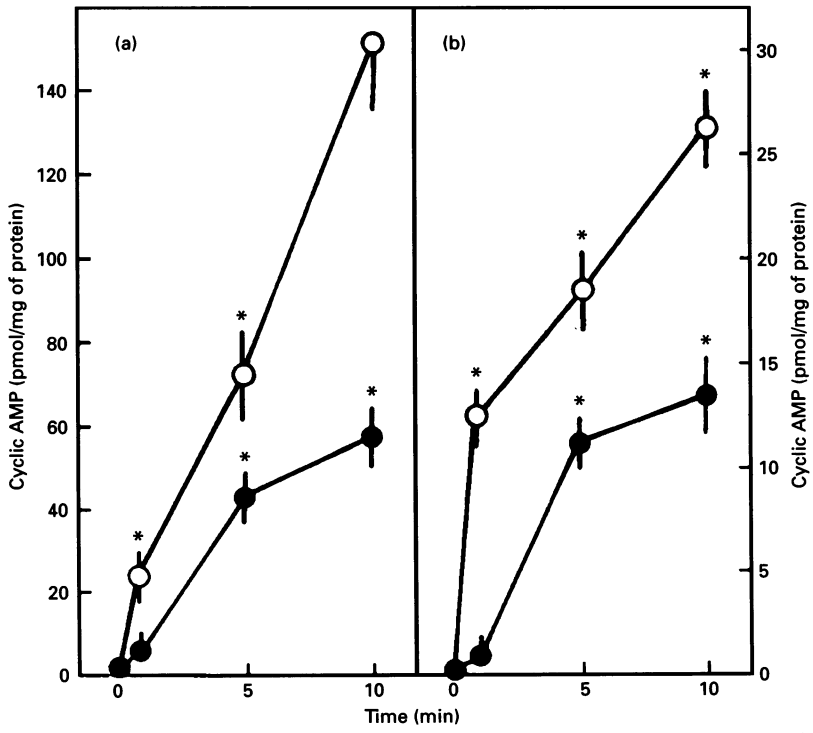

Figure 3 Time course of cyclic AMP accumulation in cardiomyocytes after the addition of isoprenaline (●) or forskolin (○) in the presence (a) or absence (b) of the phosphodiesterase inhibitor IBMX

Basal values are the same as those given in Tables 4 and 7. Data are means ± S.D. of three cell preparations; *$P < 0.05$, compared with control.

Table 4 Accumulation of cyclic AMP in cardiomyocytes after 5 min exposure to isoprenaline or PTH-(1–34) in the presence of IBMX

Stimulation of cyclic AMP accumulation in cultures of cardiomyocytes was measured after 5 min exposure to 10 μM isoprenaline or 100 nM PTH-(1–34) in the presence of the phosphodiesterase inhibitor IBMX. Data are given as means ± S.D. of three cell preparations. *$P < 0.05$, compared with control.

	Cyclic AMP (pmol/mg of protein)
Control	1.41 ± 0.55
Isoprenaline	41.53 ± 7.41*
PTH-(1–34)	2.12 ± 0.89

Table 5 Effect of PTH on maximal cell shortening of electrically stimulated cardiomyocytes

Cardiomyocytes were electrically paced (0.5 Hz) and maximal cell shortening was determined under control conditions and after 5 min exposure to PTH-(1–34), isoprenaline or PTH-(28–48). Data are means ± S.D. *$P < 0.05$, compared with control; †$P < 0.05$, compared with cell shortening in the presence of isoprenaline alone; $n = 20$.

	Maximal cell shortening ($dL/L \times 100$)
Control	6.9 ± 0.7
PTH-(1–34) (0.01 μM)	7.4 ± 0.9
PTH-(1–34) (0.10 μM)	7.3 ± 0.5
PTH-(1–34) (1.00 μM)	6.1 ± 0.5
Isoprenaline (10 μM)	11.5 ± 0.5*
Isoprenaline (10 μM) + PTH-(1–34) (1 μM)	8.2 ± 0.6†
Isoprenaline (10 μM) + PTH-(28–48) (1 μM)	9.6 ± 0.6†

functional test, 3-day serum-free cultures were used. At this stage of culture, an increase in cellular cyclic AMP content provokes spontaneous contractile activity of the cardiomyocytes, as shown previously [13]. Isoprenaline (10 μM) and the cell-permeable cyclic AMP analogue dibutyryl cyclic AMP (1 mM) induced contractile activity, but PTH-(1–34) had no effect at 0.1 and 1 μM (Table 6).

Table 6 Effect of PTH on spontaneous contractile activity of 3-day cultures

Cardiomyocytes were cultured for 3 days under serum-free conditions. Subsequently, beating frequencies were determined under control conditions, and 30 min after the addition of 10 μM isoprenaline, 1 mM dibutyryl cyclic AMP (dbcAMP), PTH-(1–34), PTH-(28–48) or 10 μM calphostine. Data are means ± S.D. *$P < 0.05$, compared with control; †$P < 0.05$ compared with frequency in the presence of isoprenaline (10 μM) alone; $n = 20$.

	Frequency (beats per min)
Control	3 ± 2
PTH-(1–34) (0.1 μM)	9 ± 3
PTH-(1–34) (1.0 μM)	12 ± 4
Isoprenaline	141 ± 14*
dbcAMP	121 ± 16*
Isoprenaline + PTH-(1–34) (0.01 μM)	135 ± 12*
Isoprenaline + PTH-(1–34) (0.10 μM)	128 ± 21*
Isoprenaline + PTH-(1–34) (1.00 μM)	94 ± 13*†
Isoprenaline + PTH-(1–34) (1.0 μM) + calphostine	143 ± 22*
Isoprenaline + PTH-(28–48) (0.01 μM)	142 ± 19*
Isoprenaline + PTH-(28–48) (0.10 μM)	140 ± 18*
Isoprenaline + PTH-(28–48) (1.00 μM)	82 ± 17*†
Isoprenaline + PTH-(28–48) (1.00 μM) + calphostine	151 ± 30*

Table 7 Accumulation of cyclic AMP in cardiomyocytes after 5 min exposure to isoprenaline in the absence of IBMX

Accumulation of cyclic AMP in the absence of IBMX under control conditions and after 5 min exposure to isoprenaline (10 μM) and simultaneously 100 nM PTH-(28–48), 1 μM PMA and 10 μM calphostine. Data are means ± S.D. from three different cell preparations. *$P < 0.05$, compared with control; †$P < 0.05$, compared with accumulation in the presence of isoprenaline (10 μM) alone.

	Cyclic AMP (pmol/mg of protein)
Control	0.11 ± 0.08
Isoprenaline	11.81 ± 0.83*
Isoprenaline + PTH-(28–48)	3.80 ± 0.51†
Isoprenaline + PMA	4.12 ± 0.19†
Isoprenaline + PTH-(28–48) + calphostine	11.73 ± 0.69
Isoprenaline + PMA + calphostine	13.12 ± 1.82

Indirect effects on cellular cyclic AMP content

It was investigated whether the co-presence of PTH with isoprenaline (1 μM) could alter the increase in cellular cyclic AMP content caused by the β-adrenoceptor agonist. In the presence of a non-selective phosphodiesterase inhibitor (IBMX), the isoprenaline-induced accumulation of cyclic AMP was not affected by the co-presence of PTH-(28–48). In the absence of this inhibitor, however, PTH-(28–48) (100 nM) reduced the isoprenaline-induced accumulation of cyclic AMP by 42% on average (Table 7). This effect of PTH-(28–48) could be mimicked by PMA. In addition, the PTH-(28–48) and PMA effects on isoprenaline-induced accumulation of cyclic AMP could be reversed by the addition of calphostine.

In two functional assays related to cellular cyclic AMP content, i.e. electrical stimulation of maximal cell shortening and spontaneous contractions in 3-day cultures, an antagonistic effect of PTH to isoprenaline was also evident (Tables 5 and 6). This antagonistic effect of PTH could be abolished in the presence of calphostine (Table 6).

DISCUSSION

Stimulation of PKC

With the use of the synthetic PTH peptide PTH-(28–48) the present study has demonstrated that PTH stimulates PKC in adult cardiomyocytes. This peptide has been shown to activate PKC in osteoblasts [11] and stimulates the proliferation of cartilage- and bone-derived cells, i.e. classic target cells for PTH, both *in vitro* and *in vivo* [10,24,25]. The same fragment was recently found to stimulate protein synthesis and induce creatine kinase activity in adult cardiomyocytes [4]. A comparison of these results suggests that activation of PKC-dependent signal-transduction pathways by PTH obeys a similar structure–function relationship in cardiomyocytes and classic PTH-target cells.

The stimulation of PKC activity by the addition of PTH-(28–48) was transient, with a maximum observed after 10 min incubation. Such a transient stimulation was also found for PTH activation of PKC in bone-derived cells [11]. In contrast, the presence of PMA resulted in long-term activation of this enzyme.

The phorbol ester mimics the action of diacylglycerol, the post-receptor activator of PKC. Diacylglycerol is rapidly metabolized to the corresponding phosphatidic acid [26] but PMA is only poorly metabolized; its effect therefore persists for longer periods of time.

In osteoblasts, the PTH-dependent stimulation of PKC is blocked completely by the addition of PKC-(19–39), a specific inhibitor of Ca^{2+}-dependent isoforms of PKC [27]. In membrane preparations of cardiomyocytes neither this inhibitory peptide nor calphostine influenced basal phosphorylation of the PKC-specific substrate peptide. Basal activity, however, required Ca^{2+} ions for activity. It is likely therefore that other Ca^{2+}-dependent kinases are able to use the substrate peptide used in this study. However, we were interested in PMA- and PTH-(28–48)-induced PKC activity in cardiomyocytes. This induced activity was completely sensitive to PKC-(19–39), indicating that activation of PKC in cardiomyocytes mainly involves the Ca^{2+}-dependent isoforms. This activation must involve translocation of PKC from the cytosol to membranes, because in membrane preparations from unstimulated cardiomyocytes addition of PMA did not activate PKC.

Direct and indirect effects of PTH fragments on cellular cyclic AMP content

PTH activates adenylate cyclase in various classic targets (for a review see ref. [5]) but not in all cells [27,28]. The N-terminal part of the peptide is responsible for this effect. We found that the synthetic N-terminal PTH fragment PTH-(1–34) does not stimulate the adenylate cyclase of ventricular cardiomyocytes from adult rats. These results are in agreement with the finding that the cyclic AMP content of papillary muscles incubated with PTH-(1–34) also remains unaltered [9]. Interestingly, the adenylate cyclase of neonatal cardiomyocytes has been reported to respond to PTH-(1–34) [1,8], suggesting that coupling of PTH receptors to adenylate cyclase is lost during cardiomyocyte maturation. A differentiation-mediated decrease in coupling of PTH to adenylate cyclase in classic target cells, e.g. osteoblasts, has also been reported [29]. In osteoblasts, loss of functional coupling was found at the level of the catalytic component of the adenylate cyclase. This cannot be the reason for the loss of responsiveness of adenylate cyclase to PTH in cardiomyocytes, as it was strongly activated by β-adrenoceptor stimulation and forskolin.

The inability of PTH-(1–34) to stimulate adenylate cyclase in ventricular cardiomyocytes from adult rats is also reflected in its lack of effect on cyclic AMP-dependent functional correlates such as contractile shortening under electrical stimulation and spontaneous beating in 3-day cultures. These cellular functions were stimulated by isoprenaline but not by PTH-(1–34). The functional data underline the physiological significance of the biochemical data.

Even though PTH-(1–34) was ineffective in stimulating adenylate cyclase in cardiomyocytes from adult rats, PTH may nevertheless modulate cellular cyclic AMP levels indirectly via its PKC-stimulatory capacity. The co-presence of PTH-(28–48) with β-adrenoceptor stimulation reduced cellular cyclic AMP accumulation and its functional correlates, but only in the absence of phosphodiesterase inhibition. This finding indicates that PTH exerts a functional antagonism to adenylate cyclase stimulation via activation of phosphodiesterase activity. This effect of PTH could be mimicked by PMA and abolished by calphostine, indicating that PKC activation triggers this inhibitory effect. The present study does not allow us to decide whether stimulation of phosphodiesterase activation is a direct or indirect effect of PKC. A similar example of indirect modulation of cyclic AMP-dependent signal transduction by a PKC-dependent pathway, however, has been described for the 'cross talk' between α_1- and β-adrenoceptor stimulation in cardiomyocytes [30].

Conclusions

In adult cardiomyocytes, PTH stimulates PKC but not adenylate cyclase. The mechanism of PKC activation involves translocation of Ca^{2+}-dependent isoforms to the membrane. PKC activation is also the cause of the inhibitory effect of PTH on cyclic AMP accumulation. The effects of PTH on the haemodynamic behaviour of the adult heart as well as growth-regulatory effects on cardiomyocytes seem to be mediated exclusively by activation of the PKC-dependent pathway.

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