EXPERIMENTAL STUDY

Calcium stimulates parathyroid hormone-related protein production in Leydig tumor cells through a putative cation-sensing mechanism

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Abstract

The production of parathyroid hormone-related protein (PTHrP) is regulated by a variety of hormones and growth factors. Previous research has shown that several PTHrP-producing cells are influenced by extracellular calcium (Ca_o^{2+}) concentration, with elevated levels increasing PTH-like activity released by cultured H500 rat Leydig tumor cells through a post-transcriptional mechanism. We have investigated the hypothesis that calcium stimulates PTHrP production in H500 cells by interacting with a cell membrane-associated cation-sensing receptor. Besides increased Ca_o^{2+} concentration, magnesium and the polycationic antibiotic neomycin also increased PTHrP production in a concentration-dependent manner. In the presence of the calcium ionophore, ionomycin, which markedly elevated cytosolic free calcium, the stimulation by Ca_o^{2+} of PTHrP could still be detected. These results indicate that increasing Ca_o^{2+} stimulates PTHrP production, possibly through a putative cell membrane-associated calcium-sensing mechanism. RT-PCR revealed the presence of a very small amount of calcium-sensing receptor coding mRNA.

European Journal of Endocrinology 142 500–505

Introduction

Parathyroid hormone-related protein (PTHrP) is recognized as a major mediator of hypercalcemia of malignancy. Apart from its effect on the renal tubular reabsorption of calcium, it increases bone resorption in the vicinity of the cancer cells (for review see 1). More recently, its crucial role in normal bone development and growth has been described (2). PTHrP is expressed in numerous tissues and its production is controlled by a large variety of hormones, growth factors, or cytokines, in a tissue-specific manner (3–5). In certain cell types, elevated extracellular calcium concentrations (Ca_o^{2+}) have been reported to positively influence PTHrP production (6–9). It is increasingly recognized that calcium regulates cell proliferation and function in many cell systems (10–12). Various extracellular cation-sensing mechanisms are located in the cell membrane (13–16), which could be involved in these calcium-triggered processes. In a previous study (6), we reported that in rat Leydig tumor cells, an experimental model of humoral hypercalcemia of malignancy, an increase in Ca_o^{2+} concentration stimulated PTHrP production in a *de novo* protein synthesis-dependent manner, apparently through a post-transcriptional process. In the present study, we extended this observation and provide evidence that the effects of Ca_o^{2+} on PTHrP production

involve a putative cell membrane-associated calcium-sensing mechanism.

Materials and methods

Cell culture

Mice H-500 Leydig tumor cells were propagated in RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Confluent cells were split 1/10 twice a week. On day 3 in culture, media were replaced with fresh 10% FCS RPMI-1640 medium. On day 4 in culture, the monolayers were rinsed with phosphate-buffered saline and supplemented with calcium-free Dulbecco's modified Eagle's medium containing 1% FCS, adjusted to the different cation concentrations by the addition of an appropriate amount of calcium or magnesium chloride. Incubation in the same medium was continued for 16 h, unless otherwise indicated. Conditioned media (CM) were collected, centrifuged at 1000 *g* for 10 min to remove cell debris and stored at -80°C .

Radioimmunoassay

Immunoreactive PTHrP was measured in 1% FCS-containing CM by competitive RIA performed under

non-equilibrium conditions as follows: 100 μ l CM or 100 μ l synthetic human PTHrP(1–34) standard (Auspep Inc., Melbourne, Australia) in 1% FCS culture medium, and 100 μ l barbitol-acetate buffer, pH 8.6, containing 0.1% bovine serum albumin (RIA grade; Sigma Co., St Louis, MO, USA) were incubated with 100 μ l rabbit antihuman PTHrP(1–34) antibodies (Peninsula, Belmont, CA, USA) in duplicate at 4 °C for 24 h. Synthetic PTHrP(1–34) was radiolabeled by a chloramine-T procedure and purified on C18 Sep-Pak cartridge columns (Waters Associates, Milford, MA, USA). Approximately 10 000 c.p.m. of the radiolabeled tracer were then added for an additional 24 h at 4 °C. Sheep antirabbit IgG antiserum was used for the separation of the bound and free fractions. Under these conditions the detection limit was 2 pg PTHrP/tube, non-specific binding was less than 2.0%, and half-maximal displacement occurred with about 20 pg/tube (3). One per cent of FCS-containing unconditioned medium did not displace the bound tracer.

Measurement of cytosolic free calcium

Cells were grown on glass coverslips and loaded with a fluorescent calcium-sensing indicator by incubation with 2 mM fura-2 acetoxymethyl ester (Calbiochem, Lucerne, Switzerland) for 30 min at 37 °C in RPMI medium with 5% FCS. Subsequently, cells were washed with the same medium. Experiments were carried out in 3 ml of a medium containing 140 mM NaCl, 5 mM KCl, 1 g/l dextrose, 1 mM Na₂HPO₄·2H₂O, 0.4 mM CaCl₂, 20 mM HEPES, 1 mM MgSO₄·7H₂O, pH 7.55, in which two to four coverslips were suspended. Fluorescence emission was recorded at 505 nm using an excitation wavelength of 350 nm. Calibration of fluorescence signals (F) was performed by using 1 μ M ionomycin (Calbiochem) to saturate the dye with Ca²⁺ (F_{max}), and then quenched by the addition of 1 mM MnCl₂ (F_{min}) (17). Calculations of cytosolic free calcium (Ca_i²⁺) were carried out according to the following equation: $224 \times ((F - F_{\min}) / (F_{\max} - F))$ (18).

RNA extraction and Northern blot analysis

Total RNA was extracted from confluent rat H500 Leydig cells and from human parathyroid gland by the guanidine thiocyanate method as previously described (19). Subsequently, mRNA was purified using Gibco polydT columns (Gibco-BRL, Basel, Switzerland) according to the manufacturer's recommendations. Total RNA used in RT-PCR experiments was isolated from confluent rat H500 Leydig cells using Qiagen Rneasy total RNA kit (Qiagen, Basel, Switzerland). The PTHrP antisense RNA probe was labeled with [α -³²P]UTP by *in vitro* transcription of the PTHrP cDNA using T7 polymerase (Boehringer-Mannheim, Lucerne, Switzerland). Northern blot analysis was performed with 1 μ g mRNA denatured in glyoxal, electrophoresed in 1.5%

agarose gel and transferred overnight onto nylon filters (Hybond-N; Amersham International plc, Amersham, Bucks, UK). The filters were baked at 80 °C for 2 h, and hybridized with the RNA probe. Prehybridization (3 h) and hybridization (overnight) were carried out at 65 °C in 50% formamide, 1×Denhardt's solution, 50 mM PIPES, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, and 100 μ g/ml denatured herring sperm DNA. The blots were washed twice for 20 min at 65 °C in 3×SSC, 2×Denhardt's solution, and then twice for 20 min at 70 °C in 0.2×SSC, 0.1 SDS, and 0.1% NaPPi. After immersion in 1% SDS at 95 °C, the same filters were rehybridized with *Bam*HI fragment of the human cyclophilin probe as control (20).

RT-PCR and PCR

RT-PCR was performed using the Titan One Tube RT-PCR kit from Boehringer-Mannheim following the supplier's recommendations. RNA template (1 μ g) was extracted from the rat H500 Leydig cells, and from human parathyroid gland, as a positive control, for the detection of the calcium-sensing receptor (CaR). Primers used for RT-PCR were N1F: GCTTTGAGTGTGTGGAG-TGTGTCC, and N2R: ATCTGCATGAAGGTGCAGAGG. Taking into account the homology between human and rat CaR cDNAs, these primers were designed to detect both human and rat cDNA (human CaR mRNA, accession number, U20760; *Rattus norvegicus* kidney CaR mRNA, accession number, U10354). This set of primers span one intron of the CaR gene (21). For the purpose of a second round of PCR, we used the nested primer pairs N3F: CTGCTCCAATGAGAACCACAC, and N4R: CAGCAGGAAGTGCAGGTTGAG. PCR reaction was carried out in 50 μ l using Qiagen Taq DNA polymerase (Qiagen). Temperature cycling protocol was 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, for 30 cycles. The reaction was concluded with a 10-min elongation at 72 °C. Ten microliters of the RT-PCR and PCR products were fractionated on a 1.2% agarose gel. An automated DNA sequencer (Perkin Elmer 310) was used for sequence analysis.

Other methods

PTHrP production was corrected for total cell protein, using the method of Lowry (22). Ca_i²⁺ levels were modulated by using either the calcium ionophore ionomycin, or chelators such as BAPTA/AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester) (23), or Quin-2/AM (23, 24). All these reagents were from Calbiochem. As phorbol myristate acetate (PMA), they were dissolved in dimethyl-sulfoxide, the latter at a final concentration of 0.1%.

Statistical analysis

Results are means $\pm$ S.E.M. of separate experiments, all performed in at least triplicate. Significance of differences

was evaluated using an analysis of variance, with Bonferroni's criterion for multiple comparison.

Results

Effects of cations on PTHrP production

Increasing concentrations of Ca_o^{2+} or magnesium were associated with an increment of immunoreactive PTHrP released into the culture medium (Fig. 1). To evaluate whether the cation-sensitive PTHrP-stimulating mechanism was also influenced by polycations, we examined the effects of the antibiotic neomycin (25). Neomycin mimicked the effects of calcium on PTHrP release and it appeared to potentiate the stimulation caused by Ca_o^{2+} (Fig. 2).

Ca_i^{2+} and PTHrP production

We investigated changes in Ca_i^{2+} in response to an increase in the concentration of Ca_o^{2+} under conditions where PTHrP was consistently stimulated. The acute increment from 0.4 to 3.0 mM was associated with a transient increase of Ca_i^{2+} of 30% (from 140 ± 17 to 212 ± 34 nM, $P < 0.05$, $n = 12$ separate experiments). To evaluate whether high magnitude modifications of Ca_i^{2+} could alter PTHrP release, Leydig tumor cells were incubated for varying periods of time with $1 \mu\text{M}$

Figure 1 Effects of increasing concentrations of calcium or magnesium on PTHrP production in cultured Leydig tumor cells. On day 3 after seeding, cells were incubated with varying concentrations of the cations for 16 h. The medium was assayed for PTHrP released. The results are expressed as pg PTHrP(1–34)/mg cell protein and are the means $\pm$ S.E.M. of four replicates.

Figure 2 Effects of neomycin on PTHrP production. On day 3 after seeding, cells were incubated with increasing concentrations of neomycin, in the presence of 0.4 or 3.0 mM calcium, for 16 h. The medium was assayed for PTHrP released. The results are expressed as pg PTHrP(1–34)/mg cell protein and are the means $\pm$ S.E.M. of four replicates. Effect of neomycin: $P = 0.0007$ and $P = 0.0001$ for 0.4 and 3.0 mM calcium respectively. ^a $P < 0.05$ as compared with no addition of neomycin; ^b $P < 0.05$ as compared with 0.01 mM neomycin. All the values in the 3.0 mM calcium curve significantly differed from the 0.4 mM one.

ionomycin, which dissipates the extra/intracellular calcium gradient (26, 27). Under these conditions, increasing concentrations of Ca_o^{2+} still caused a concentration-dependent increment of PTHrP production (Fig. 3). These experiments suggested that changes in Ca_o^{2+} concentration may influence PTHrP production, independently of Ca_i^{2+} concentration. Conversely, in an attempt to trap the Ca_i^{2+} we used the calcium chelators BAPTA/AM and Quin-2/AM, but no modification of the calcium-stimulated PTHrP production was observed (Table 1).

Effects of protein kinase C (PKC) stimulation by PMA on the calcium-dependent change of PTHrP production

To investigate whether the PKC signal transduction pathway was involved in calcium-stimulated PTHrP production, we tested the effect of PMA. Leydig tumor cells were incubated for 16 h with 0.1 mM of the phorbol ester PMA. The effects of 3.0 mM calcium as compared with 0.4 mM on PTHrP release were not modified by PMA ($+172 \pm 7\%$ in controls and $+174 \pm 15\%$ in PMA-treated cells), but PMA was associated with higher levels of PTHrP mRNA (Fig. 4). Similarly, the response to

Figure 3 Effects of the calcium ionophore ionomycin on PTHrP production. On day 3 after seeding, cells were incubated in the presence of 0.4 or 3.0 mM calcium, with or without 1 μ M ionomycin, for various periods of time. The medium was assayed for PTHrP. The results are expressed as pg PTHrP(1–34)/mg cell protein and are the means $\pm$ S.E.M. of triplicates.

calcium was not altered in the presence of the PKC inhibitor staurosporine (data not shown). In contrast, as previously suggested, Ca_o^{2+} increased PTHrP production without changes in mRNA, irrespective of PMA treatment.

Search for a CaR in cultured Leydig tumor cells

Using RT-PCR analysis, we searched for the presence of the extracellular CaR, which has been characterized in

Table 1 Effects of extracellular calcium on PTHrP production in the presence of modulators of cytosolic free calcium concentration. Culture medium was conditioned with Leydig tumor cells for 16 h in the presence of 0.4 mM or 3.0 mM extracellular calcium, and the various modulators. Number of experiments (each experiment included triplicates) in parentheses.

	PTHrP production (%)	
	0.4 mM Ca_o^{2+}	3.0 mM Ca_o^{2+}
Controls (22)	100	172 $\pm$ 7*
Ionomycin (1 μ M) (4)	100	203 $\pm$ 23*
BAPTA/AM (25 μ M) (2)	100	189 $\pm$ 22*
BAPTA/AM (50 μ M) (2)	100	189 $\pm$ 47*
Quin-2/AM (10 μ M) (2)	100	152 $\pm$ 12*

* $P < 0.05$.

Figure 4 Northern blot analysis of polyA⁺ RNA (1 μ g/lane) isolated from Leydig tumor cells incubated for 16 h with 0.4 or 3.0 mM calcium in the presence or absence of PMA (see cell culture in Materials and methods).

parathyroid cells (13). No amplified product was detected from 1 μ g Leydig tumor cell total RNA unlike that from human parathyroid total RNA (Fig. 5). A second round of PCR with nested primer-amplified products which were found to be 100% homologous to the CaR was carried out (Fig. 5, lane 4). These results indicate the presence of a very small amount of CaR mRNA in the Leydig tumor cells.

Discussion

We previously reported that the production of PTHrP in rat Leydig tumor cells was augmented by increased Ca_o^{2+} (6). In the present work, we have confirmed that calcium stimulation of PTHrP production in Leydig tumor cells acts at a post-transcriptional level. Furthermore, we have provided evidence that the Ca_o^{2+} effect on PTHrP production is poorly correlated with changes in the Ca_i^{2+} concentration and seems to implicate a cell membrane-sensitive mechanism. Indeed, the response was not specific to calcium, since other divalent cations such as magnesium and the polycationic antibiotic neomycin also stimulated PTHrP production. The response to magnesium was similar to the calcium

Figure 5 RT-PCR analysis of CaR RNA from rat H500 Leydig cells. Amplification and analysis were carried out as specified in the Materials and methods section. Lane 1, positive control, RT-PCR carried out on parathyroid RNA with the primer pair N1F-N2R; lanes 2 and 3, RT-PCR carried out on Leydig tumor cells RNA with the primer pairs N1F-N2R and N3F-N4R respectively; lane 4, amplification of the RT-PCR shown in lane 2 with the nested primer pair N3F-N4R.

response, with neomycin mimicking the calcium effect but to a lesser extent. The effect was markedly enhanced in the presence of a high concentration of calcium.

We then evaluated the modulation of Ca_i^{2+} response to changes in Ca_o^{2+} concentrations. The alteration in Ca_i^{2+} was minimal and transient. Furthermore, to evaluate if a change in the Ca_i^{2+} concentration modified the PTHrP release, we treated the cells with the ionophore ionomycin, which dissipates the extra/intracellular calcium gradient. Under these conditions, the production of PTHrP was still stimulated by the increment of calcium in the medium. This finding suggests that the increase of Ca_i^{2+} concentration was insufficient to account for the response to Ca_o^{2+} changes in terms of PTHrP release. Moreover, we showed that chelators of Ca_i^{2+} , such as BAPTA/AM or Quin-2/AM, did not modify the response to 3 mM Ca_o^{2+} .

All these results suggest that the effect of calcium could be mediated by a membrane calcium-sensing mechanism. The most extensively studied example of calcium-sensing receptor is that present in parathyroid cells (CaR) and which regulates PTH secretion in response to changes in Ca_o^{2+} concentration (28). CaR has also been identified in kidney (29), brain (30), keratinocytes (31), and other tissues including thyroid C cells (28, 32). CaR is a transmembrane calcium-sensing receptor coupled to G protein, the stimulation of which results in an inhibition of cAMP synthesis, a stimulation of phospholipase C and of inositol phosphate turnover, and increments in Ca_i^{2+} . The CaR effect is presumed to use the PKC pathway (13). Using RT-PCR, we searched for the presence of CaR mRNA, but the messenger was detected only after a second round of PCR. In addition, we found that stimulation of the PKC by PMA did not significantly change either the PTHrP release in the medium or the level of PTHrP transcription in response to increased Ca_o^{2+} concentration. These results indicate the presence of a putative calcium-sensing mechanism in Leydig tumor cells. However, the small amount of CaR mRNA detected and the apparent independence of the PKC pathway suggests the involvement of a calcium-sensing mechanism distinct from the extensively studied CaR present in the parathyroid gland (13, 28).

Acknowledgements

Mrs C Decrind and L Ackermann are acknowledged for their expert technical assistance. We are indebted to Dr M Aubert for the labeling of the PTHrP tracer, and to Dr JF Manen for PCR product sequencing. We thank Mrs M Perez for typing the manuscript. This work was supported by the Swiss National Science Research Fund (grant no. 32-49839.96).

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Received 17 August 1999

Accepted 5 January 2000