Calmodulin and calmodulin-dependent protein kinase II inhibit hormone secretion in human parathyroid adenoma

Ming Lu^{1,2,3}, Erik Berglund¹, Catharina Larsson^{1,3}, Anders Höög⁴, Lars-Ove Farnebo¹ and Robert Bränström¹

¹Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital L1:03, SE-171 76 Stockholm, Sweden

²Department of Geriatric Endocrinology, First Affiliated Hospital of Guangxi Medical University, NanNing, People's Republic of China

³Center for Molecular Medicine (CMM), Karolinska University Hospital, SE-171 76 Stockholm, Sweden

⁴Department of Oncology–Pathology, Karolinska Institutet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden

(Correspondence should be addressed to M Lu at Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital; Email: ming.lu.1@ki.se)

Abstract

Intracellular calcium ($[Ca^{2+}]_i$) is the most relevant modulator of parathyroid hormone (PTH) secretion. Uniquely, an increase in $[Ca^{2+}]_i$ results in an inhibition of PTH secretion, and it probably exerts its function via calcium-binding protein pathways. The ubiquitous calcium-binding proteins, calmodulin and calmodulin-dependent protein kinase II (CaMKII), have well-established roles in regulated exocytosis in neurons and neuroendocrine cells. However, their roles in parathyroid cells and PTH secretion are still unclear. Using reverse transcription-PCR and western blot analysis, we have demonstrated the expression of calmodulin and CaMKII in human normal parathyroid and parathyroid chief cell adenomas. Blocking of calmodulin and CaMKII activity by the specific antagonists calmidazolium and KN-62 respectively caused a rise in PTH secretion from parathyroid

adenoma cells in spite of increased $[Ca^{2+}]_i$. The inhibitory effect of Ca^{2+} calmodulin on PTH secretion may be due to the absence of synaptotagmin 1 protein in parathyroid adenomas, as demonstrated by western blot analysis. An increased extracellular calcium level acutely lowered the amount of active phosphorylated CaMKII (pCaMKII) in adenoma cells *in vitro*, indicating the physiological importance of this pathway. Moreover, a negative correlation between the levels of pCaMKII in parathyroid adenomas and serum calcium was found in 20 patients with primary hyperparathyroidism. Taken together, these results show that calmodulin negatively contributes to the regulation of PTH secretion in parathyroid adenoma, at least partially via a CaMKII pathway.

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Introduction

The parathyroid glands are important organs for calcium regulation in the human body. Parathyroid cells sense small changes in serum calcium levels and adjust parathyroid hormone (PTH) secretion to keep serum calcium within a narrow range. In this sense, parathyroid cells are unique because intracellular Ca^{2+} ($[Ca^{2+}]_i$) inhibits PTH secretion, instead of stimulating secretion as in other endocrine cell types (Shoback *et al.* 1983). The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor located in the cell membrane. Upon binding of extracellular calcium ($[Ca^{2+}]_e$) to CaSR, a phospholipase C–inositol triphosphate pathway is activated, resulting in several fold increase in $[Ca^{2+}]_i$ and subsequent inhibition of PTH secretion. $[Ca^{2+}]_i$ has been established as the central second messenger for PTH secretion (Shoback *et al.* 1984), involving, e.g. the PLA2-AA and the MAP kinase pathways (Kifor *et al.* 2001, Almaden *et al.* 2002).

In addition to that, the mechanism behind $[Ca^{2+}]_i$ increase and PTH inhibition remains unclear.

Calmodulin is a calcium-binding protein involved in the sensing of increased $[Ca^{2+}]_i$ concentrations and subsequent signal transduction to a variety of cellular targets. Calmodulin-dependent protein kinase II (CaMKII) is a calmodulin-binding protein participating in functions such as exocytosis, $[Ca^{2+}]_i$ oscillation, and ion-channel activation (Wang 2008). Calmodulin is ubiquitously expressed and has been detected in both normal and pathological parathyroid (Brown *et al.* 1981). However, a correlation between levels of calmodulin expression, calcium sensitivity, and PTH secretion has not been demonstrated (Brown *et al.* 1981, Oldham *et al.* 1982). Expression of CaMKII has also been reported in hyperfunctioning human parathyroid cells where modulation of CaMKII activity was found to be calcium and calmodulin dependent (Kinder *et al.* 1987, Kato *et al.* 1991). However, the interaction between calcium,

calmodulin, and CaMKII in human parathyroid has not been clarified. In this study, we have investigated the relationship between calmodulin and CaMKII activity and PTH secretion.

Materials and Methods

Parathyroid tissue samples

Normal parathyroid tissue and parathyroid adenomas were collected with informed consent and ethical approval at the Karolinska University Hospital, Sweden. Histopathological diagnoses were according to the WHO classification (DeLellis *et al.* 2004). Histopathological examination of representative sections verified a high tumor cell content of the analyzed tissue samples.

Reverse transcription-PCR

The reverse transcription (RT)-PCR analysis included previously published samples of one normal parathyroid (Juhlin *et al.* 2010), and four or more parathyroid adenoma samples (Lu *et al.* 2010) for each gene analyzed. Total RNA was extracted using TRIzol reagent (Invitrogen), purified by DNase I (Amplification Grade, Invitrogen), and quantified by spectrophotometry. Total RNA (3 µg) from each sample was reverse transcribed into 40 µl cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Cat. no. 18080-051). In all, 2 µl of each cDNA were used for amplification of calmodulin 1, 2, and 3 genes (*CALM1*, *CALM2*, and *CALM3*) and of CaMKII α, β, γ, and δ genes (*CAMK2A*, *CAMK2B*, *CAMK2G*, and *CAMK2D*) with the Platinum Taq DNA polymerase high-fidelity kit (Invitrogen). Gene-specific forward and reverse PCR primers (Table 1) were designed in house according to published genomic data and PCR primer design guidelines. *CAMK2A*, *CAMK2B*, *CAMK2G*, and *CAMK2D* have

several transcript variants due to alternative splicing. Primers were designed based on the common sequence of each gene; therefore, it does not identify specific transcript variants. The PCR reactions were performed in 20 µl reactions with amplification conditions as follows: initial denaturation for 2 min at 94 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 50–55 °C, and 1 min at 68 °C. Human brain cDNA (Invitrogen, Cat. no. B1110033) was used as a positive control. The PCR products were subsequently size verified by agarose gel electrophoresis and were observed and photographed under u.v. light. To further verify the quality, all PCR products were purified using a PCR purification kit (Qiagen, Cat. no. Q28104) and were sequenced with the assistance of the KISeq core facility at Karolinska Institutet, Stockholm, Sweden.

Western blot analysis

Twenty previously published parathyroid chief cell adenomas and one normal parathyroid biopsy specimen (Lu *et al.* 2008) were used for western blot analysis (No. 1–20, Table 2), using previously described methodology (Lu *et al.* 2008). Total proteins were extracted using 1% NP-40 lysis buffer supplied with protease inhibitors, and then quantified with a Bio-Rad protein assay. Following separation by SDS-PAGE, proteins were blotted onto nitrocellulose membranes and incubated overnight at 4 °C with primary antibodies, followed by appropriate secondary antibodies. The following primary antibodies and dilutions were applied: monoclonal rabbit anti-calmodulin that targets the single calmodulin protein commonly encoded by the *CALM1*, *CALM2*, and *CALM3* genes (EP799Y, Abcam, Cambridge, UK, Cat. no. ab45689) at dilution 1:1000; polyclonal anti-endogenous CaMKII antibody that detects total CaMKII α and β subunits levels (Acris, Herford, Germany, Cat. No. AP02774PU-S) at 1:500; polyclonal anti-CaMKIIp-Thr286 antibody specific for the phosphorylated form of CaMKII α and β subunits (pCaMKII, Acris, Cat. no. AP2526PU-S) at 1:800; monoclonal anti-synaptotagmin 1 antibody (SYSY, Goettingen,

Table 1 Details and primers used for reverse transcription-PCR of the *CALM1–3* and *CAMK2A*, *CAMK2B*, *CAMK2G*, and *CAMK2D* genes

Gene symbol	Gene location	Protein product	Primer orientation	Primer sequence (5'–3')	Amplicon length (bp)	Annealing temperature (°C)
<i>CALM1</i>	14q24–q31	Calmodulin	Forward	CAGATATTGATGGAGACGGA	629	51
			Reverse	GAGCACACGAAGTACAAGAG		
<i>CALM2</i>	2p21	Calmodulin	Forward	GACAAAGATGGTGATGGAAC	394	51
			Reverse	GTCTTCACTTTGCTGTCATC		
<i>CALM3</i>	19q13.2	Calmodulin	Forward	ACAAGGATGGAGATGGCAC	313	57
			Reverse	ATCTCATCCACCTCCTCATC		
<i>CAMK2A</i>	5q32	CaMKII α subunit	Forward	ACCAGCTCTTCGAGGAATTG	241	56
			Reverse	GTGACCAGGTCTGAAGATCAG		
<i>CAMK2B</i>	7p14.3	CaMKII β subunit	Forward	GAGAGAGAGGCTCGGATCTG	395	56
			Reverse	TGTCCACAGGCTTGCCATAC		
<i>CAMK2G</i>	10q22	CaMKII γ subunit	Forward	GACTTCTGAAACATCCAAC	431	55
			Reverse	CACCAGGAGGATATACAGGA		
<i>CAMK2D</i>	4q26	CaMKII δ subunit	Forward	TAGCAAATCCAAGGGAGCAG	377	55
			Reverse	ATGGGTGCTTCAGTGCCTC		

Table 2 Clinical characteristics and protein expression levels

Case number	Age at operation	Sex (M/F)	Serum Ca ²⁺ total (mM)	Serum PTH (ng/l)	Weight (g)	Protein expression (ratio)	
						pCaMKII/ β -actin	Calmodulin/ β -actin
1	61	M	2.72	156	0.84	2.40	2.32
2	59	F	2.66	241	1.92	1.47	2.02
3	50	F	2.90	211	3.07	0.43	2.46
4	67	F	2.78	104	3.07	0.51	2.20
5	57	F	2.86	185	1.52	0.48	2.94
6	52	F	2.60	68	0.56	0.21	2.11
7	59	F	2.79	114	1.06	1.28	1.57
8	22	M	2.67	104	1.80	0.96	1.32
9	63	F	3.19	351	7.60	0.62	1.60
10	54	F	2.88		2.10	1.97	1.42
11	61	M		83	1.78	1.68	1.29
12	66	F	2.98	234	1.70	0.76	1.36
13	58	F	2.97	358	8.50	0.37	1.26
14	74	M	2.80	879	1.10	1.06	1.33
15	75	M	2.42	125	3.15	1.22	1.40
16	62	F	2.86	244	1.54	1.46	1.24
17	42	M	2.82	149	4.28	0.78	1.82
18	62	F	3.07	424	5.07	0.34	1.57
19	84	F	2.96	596	1.92	0.78	1.39
20	90	F	3.26	485	6.28	0.16	1.58

Reference values: serum Ca²⁺ (2.0–2.6 mM), PTH (10–65 ng/l). Clinical characteristics have been published in [Lu et al. \(2008\)](#).

Germany, Cat. no. 105011) at 1: 1000; and anti- β -actin (Sigma, Cat. no. A5441) used as a protein loading control. The results were visualized using enhanced chemiluminescence and by exposing to hyperfilm. The band density was measured by ImageJ software (NIH, Bethesda, Maryland, USA), and the levels of pCaMKII and calmodulin were determined by normalization against the density of β -actin.

Preparation of parathyroid adenoma cells

Fresh parathyroid adenoma tissues were collected at operation, quickly transferred to the laboratory in ice-cold MEM medium, and digested into cells with 1.5 mg/ml type II collagenase and 0.1 mg/ml DNase. The cells were cultured in DMEM/F-12 (GlutaMAX, Gibco) supplied with 10% FCS and 1% penicillin–streptomycin for either [Ca²⁺]_i measurement or PTH secretion studies. All experiments were performed within 72 h after isolation. Calmidazolium was purchased from Sigma, and KN-62 was obtained from Calbiochem (San Diego, CA, USA). Stock solutions were prepared in DMSO, and the final concentration of DMSO was <1%.

Measurement of [Ca²⁺]_i by Fura-2

After isolation, parathyroid adenoma cells were grown on glass cover slides overnight until they attached. Cells were loaded with 2.5 μ M Fura-2 AM (Invitrogen) at 37 °C for 30 min in extracellular solution (EC) containing (in mM): 125 NaCl, 4 KCl, 1 MgCl₂, 0.8 NaH₂PO₄, 20 HEPES, and 5.6 D-glucose with 1.0 CaCl₂. Slides were mounted into a 37 °C perfusion chamber and exposed to an inverted

fluorescence microscope (Axiovert 135 TV, Zeiss, Oberkochen, Germany) with a $\times 40$ oil objective. Fluorescence was provided by a SPEX fluorolog-2 CM1T111 spectrofluorimeter (SPEX Industries, Edison, NJ, USA) with the excitation wavelengths at 340 and 380 nm, and emission was monitored at 505 nm. Fluorescence imaging was detected by a cooled charge-coupled device camera (CCD, CH250 with KAF 1400, Photometrics, Tucson, AZ, USA) connected to an imaging system (Inovision, Durham, NC, USA). Fluorescence intensity was analyzed by ISEE software for UNIX (Inovision). The ratio of 340/380 nm emitted fluorescence was calculated to represent the [Ca²⁺]_i level.

Measurement of PTH secretion

Parathyroid adenoma cells were suspended in the medium overnight, allowing them to recover from collagenase digestion. Cell viability, assessed using Trypan blue, was >98%. Cells (1×10^4 – 1×10^5) were loaded into a column on the top of a 3/5 volume of P-4 gel. The column was carefully closed and kept in a cabinet with a constant temperature of 37 °C. A peristaltic pump at a speed of 500 μ l/3 min effectuated perfusion of the column. A 30 min preperfusion with EC containing 1.5 mM Ca²⁺ and basal amino acid, as previously described ([Conigrave et al. 2004](#)), was run before every experiment. Samples were collected every 3 min, quickly put on ice, and stored at –20 °C until use for PTH quantifications. Intact PTH was measured using an electrochemiluminescence immunoassay (Roche, Cat. no.11972219) at the routine clinical chemistry laboratory of Karolinska University Hospital. Each protocol was performed at least

Figure 1 Expression of calmodulin and CaMKII in human parathyroid. (A) RT-PCR analysis for agarose gel electrophoresis showing products of *CALM1–3*, *CAMK2A*, *CAMK2B*, *CAMK2G*, and *CAMK2D* genes in normal (N) and adenoma parathyroids, and human brain (P) used as control. Products were verified by sequencing expected for *CAMK2A* in parathyroid adenoma and normal. (B) Autoradiograms of western blots demonstrate protein expression of calmodulin and total CaMKII at expected product sizes in both normal and adenoma parathyroid.

three times from at least two different adenoma glands. To allow comparison, the initial PTH level in each experiment was set to the arbitrary value of 1, and the PTH levels were normalized correspondingly.

Short-term treatment of parathyroid adenoma cells with calcium

To investigate the effect of $[Ca^{2+}]_e$ on calmodulin and active pCaMKII, parathyroid adenoma cells were incubated with EC supplied with 0.5, 1.2, or 2.5 mM Ca^{2+} for 20 or 60 min respectively. After treatment, cells were washed twice with cold PBS, and then extracted for proteins. Untreated cells were used as negative control. The levels of calmodulin and pCaMKII were determined using western blot analysis.

Statistical analysis

All data are given as the mean $\pm$ S.D. If not indicated otherwise, experiments were repeated at least three times. Statistical significance was analyzed using paired and unpaired *t*-tests between the two groups or ANOVA and *post hoc* test for multiple groups. Nonparametric tests were used to compare protein expression levels with clinical parameters. A *P* value of <0.05 was considered significant.

Results

Expression of calmodulin and CaMKII in human parathyroid

Expression of the three calmodulin-encoding genes, *CALM1*, *CALM2*, and *CALM3*, and *CAMK2* genes was demonstrated by RT-PCR. As illustrated in Fig. 1A, single products of expected sizes were successfully amplified for *CALM1*, *CALM2*, *CALM3*, *CAMK2A*, *CAMK2B*, *CAMK2G*, and *CAMK2D* from human brain cDNA (positive control) and verified as correct by DNA sequencing. Products of the same sizes as the positive control were also amplified in parathyroid normal and adenoma samples. For all genes, except *CAMK2A*, the products were subsequently verified as correct by sequencing. Products amplified from parathyroid adenoma and normal tissue with *CAMK2A* primers were found to have the sequence of *CAMK2B*, suggesting that *CAMK2A* is not expressed in human parathyroid. Western blot analysis confirmed the expression of calmodulin and CaMKII on the protein level in normal and adenoma parathyroid samples (Fig. 1B).

Figure 2 Effect of calmidazolium and KN-62 on PTH secretion. (A–D) Addition of 1 and 10 μ M calmidazolium and 10 and 20 μ M KN-62 at 1.5 mM Ca^{2+} caused an increase in PTH secretion. As summarized in (E), calmidazolium and KN-62 increased PTH secretion in a dose-dependent manner (* $P<0.05$; ** $P<0.01$). The bars refer to time points of maximal observed effect.

Association of calmodulin and CaMKII with PTH secretion

The calmodulin antagonist calmidazolium was used to inhibit the calmodulin-regulated activity, and KN-62 was used to inhibit CaMKII activity. Parathyroid adenoma cells were loaded into a perfusion column and preperfused for 30 min with EC containing 1.5 mM Ca^{2+} before starting the experimental protocol. Thereafter, all experiments started with a 9 min perfusion of 1.5 mM Ca^{2+} , followed by an addition of drugs for 18 min. After washout, 0.5 mM Ca^{2+} was administered to verify parathyroid cell viability. Calmidazolium was tested at concentrations of 1 and 10 μM , and KN-62 was tested at 10 and 20 μM . Results are presented individually in Fig. 2A–D. In summary (Fig. 2E), 1 μM calmidazolium caused a $30 \pm 9.4\%$ increase in PTH secretion, whereas 10 μM calmidazolium led to a $260 \pm 130\%$ rise in PTH secretion. An addition of 10 μM KN-62 resulted in a $34 \pm 4\%$ increase in PTH secretion, and 20 μM KN-62 caused a $100 \pm 7.9\%$ increase in PTH secretion (Fig. 2E).

The effect of calmidazolium and KN-62 on $[\text{Ca}^{2+}]_i$

To investigate whether calmodulin and CaMKII are involved in the regulation of $[\text{Ca}^{2+}]_i$ signaling, we used the Ca^{2+} indicator Fura-2 to examine $[\text{Ca}^{2+}]_i$. Calmidazolium or KN-62 was added to parathyroid adenoma cells through a perfusion system after step stimulation by 0.5 and 1.5 mM Ca^{2+} . As shown in Fig. 3A, no significant change in $[\text{Ca}^{2+}]_i$ was observed after adding 1 μM calmidazolium. An elevation of $[\text{Ca}^{2+}]_i$ was observed after giving 10 μM calmidazolium. In the absence of Ca^{2+} , 10 μM calmidazolium only caused a small transient increase in $[\text{Ca}^{2+}]_i$, but the addition of 1.5 mM Ca^{2+} resulted in a higher rise of $[\text{Ca}^{2+}]_i$ than without the drug (Fig. 3B). Administration of 10 and 20 μM KN-62 did not show any effect on $[\text{Ca}^{2+}]_i$ (Fig. 3C).

Negative correlation between pCaMKII and serum calcium levels

To investigate the possible correlation between calmodulin and CaMKII levels and clinical pathological data, we analyzed the protein expression of calmodulin and the active form of CaMKII (pCaMKII) in 20 parathyroid chief cell adenomas. Calmodulin and pCaMKII were quantified by normalization to β -actin levels (Table 2). Clinical data for the 20 patients with primary hyperparathyroidism were: age at diagnosis 61.5 ± 14.7 years, total serum Ca^{2+} 2.85 ± 0.20 mM, serum PTH level 269 ± 208 ng/l, and tumor weight 2.8 ± 2.3 g (for details, see Table 2). Calmodulin was clearly expressed at comparable levels in all parathyroid adenomas, whereas the amount of pCaMKII showed a larger variation between the samples (Fig. 4A; Table 2). In our material, calmodulin protein levels were not found to correlate with serum Ca^{2+} , PTH, or tumor weight. In 8 out of 20 parathyroid adenomas, the pCaMKII signal was weak (Fig. 4A; Table 2). There was a negative correlation between the patients' total serum Ca^{2+} levels and the pCaMKII levels in the parathyroid adenomas

Figure 3 Measurement of $[\text{Ca}^{2+}]_i$ using Fura-2 and determination of ratios for 340/380 nm emitted fluorescence (F340/380).

(A) Application of 1 μM calmidazolium did not cause a significant change of $[\text{Ca}^{2+}]_i$, however, 10 μM calmidazolium induced an increase in $[\text{Ca}^{2+}]_i$. (B) In the absence of Ca^{2+} , 10 μM calmidazolium only induced a small transient increase in $[\text{Ca}^{2+}]_i$, however, addition of 1.5 mM Ca^{2+} caused a higher constant increase in $[\text{Ca}^{2+}]_i$; (C) 10 and 20 μM KN-62 had no effect on $[\text{Ca}^{2+}]_i$.

Figure 4 Demonstration of phosphorylated CaMKII (pCaMKII) expression in parathyroid adenoma. (A) Western blot analysis of calmodulin and pCaMKII (pT286) protein in 20 parathyroid adenomas (No. 1–20). β -Actin served as loading control. (B) There was a negative correlation between total serum Ca^{2+} and pCaMKII in the tumors. (C) The amount of pCaMKII in human parathyroid adenoma cells was reduced when extracellular Ca^{2+} was increased during 1 h incubation whereas no change on calmodulin was seen with the same treatment. The effect on pCaMKII level at different extracellular Ca^{2+} concentrations is summarized in (D) (* $P < 0.05$, NS $P > 0.05$). (E) The amount of pCaMKII at 2.5 mM Ca^{2+} was reduced at 20 min.

(Spearman's ρ , $r = -0.470$, $P < 0.05$; Fig. 4B). No correlation was observed between pCaMKII levels and serum PTH, tumor weight, or calmodulin protein levels. After treating parathyroid adenoma cells with EC containing 0.5, 1.2, and 2.5 mM Ca^{2+} for 60 min, no difference was observed in the expression of calmodulin protein, but a clear reduction in pCaMKII was detected at 2.5 mM Ca^{2+} (Fig. 4C and D). The effect could already be demonstrated after 20 min of incubation (Fig. 4E).

Absence of synaptotagmin 1 in human parathyroid adenomas

The expression of synaptotagmin 1 was investigated using western blot analysis. A product at the expected molecular size (~ 60 kDa) was clearly detected in the rat insulinoma INS-1 cell line (Lang *et al.* 1997) used as positive control. However, the same products were undetectable in all of the human parathyroid adenoma samples investigated (Fig. 5), suggesting that synaptotagmin 1 is rarely expressed in parathyroid adenoma.

Discussion

$[\text{Ca}^{2+}]_i$ is the central player for hormone secretion in endocrine cells. Parathyroid cells are not exclusive even though the parathyroid cell distinguishes itself because of the negative regulation by calcium. In the same way as in other endocrine cells, activation of receptors evokes a rise in $[\text{Ca}^{2+}]_i$ by mobilization of cellular $[\text{Ca}^{2+}]_i$ and calcium influx. $[\text{Ca}^{2+}]_i$ of 100–200 nM, which is close to the resting $[\text{Ca}^{2+}]_i$ in other cell types, results in a maximal release of PTH (Shoback *et al.* 1984). A typical rise of $[\text{Ca}^{2+}]_i$ induced by increased $[\text{Ca}^{2+}]_e$ in parathyroid cells is biphasic, including a transient increase followed by a steady-state increase probably via store-operated calcium channels (Lu *et al.* 2010). Some compounds, such as dopamine, potentiate PTH secretion without changing $[\text{Ca}^{2+}]_i$ (Nemeth *et al.* 1986). Therefore, it is likely that additional signaling pathways are involved in PTH secretion. $[\text{Ca}^{2+}]_i$ signaling involves many Ca^{2+} -binding proteins acting as calcium sensors or adaptors, which transduce signaling to cell processes via enzymatic reactions and protein–protein interaction.

Figure 5 Absence of synaptotagmin 1 expression in human parathyroid adenomas. Western blot analysis demonstrated a product of expected size for synaptotagmin 1 in INS-1 cells (P), but not in human parathyroid adenomas. Subsequent incubation with anti- β -actin demonstrated equal loading between the lanes.

Calmodulin, the most universal Ca^{2+} -binding protein in eukaryotic cells, has been demonstrated in human parathyroid (Brown *et al.* 1981, Oldham *et al.* 1982). These authors found that the amount of calmodulin does not differ between normal parathyroid and parathyroid adenomas (Oldham *et al.* 1982), but it is increased in secondary hyperparathyroidism (Brown *et al.* 1981). It is known that the single calmodulin protein is encoded by three genes *CALM1*, *CALM2*, and *CALM3* (Fischer *et al.* 1988). By using RT-PCR, we showed expression of all three calmodulin genes in human normal and adenomatous parathyroid tissue. Western blot confirmed its expression at the protein level. By analyzing 20 adenomas, we found that the calmodulin protein level did not correlate with serum Ca^{2+} , PTH, or tumor weight. Incubation for 60 min with 0.5, 1.2, and 2.5 mM Ca^{2+} did not alter the expression of calmodulin. These findings suggest that the amount of calmodulin is not associated with calcium-regulated PTH secretion, which is in accordance with previous studies (Brown *et al.* 1981, Oldham *et al.* 1982). Calmidazolium, a powerful and frequently used calmodulin blocker, was found to cause a significant dose-dependent increase in PTH secretion. Notably, the effect of 10 μM calmidazolium was 8.6-fold higher than 1 μM calmidazolium. The strong pharmacological action of 10 μM calmidazolium may cause emptying of the secretory vesicle pool and result in reduced response to the subsequent stimulation of 0.5 mM Ca^{2+} . These results imply that a calmodulin-regulated pathway is involved in PTH secretion.

Among numerous calmodulin-regulated enzymes, Ca^{2+} /CaMKII is the best-established calmodulin-regulated kinase participating in exocytosis. CaMKII has four subunits: α , β , γ , and δ . It regulates neurotransmitter release (Stefani *et al.* 1997), insulin secretion (Bhatt *et al.* 2000, Yamamoto *et al.* 2003), and catecholamine secretion (Schweitzer *et al.* 1995) via phosphorylation of synapsin I. By using calmodulin affinity chromatography, CaMKII (only α subunit) has been purified from human parathyroid cells (Kinder *et al.* 1987, Kato *et al.* 1991). The function of CaMKII in parathyroid cells has never been investigated. In this paper, we have demonstrated expression of the genes *CAMK2B*, *CAMK2G*, and *CAMK2D* encoding the CaMKII subunits β , γ and δ but not *CAMK2A*, which encodes the CaMKII subunit α in human parathyroid using RT-PCR. Inhibition of

CaMKII activity by the specific blocker KN-62 resulted in a dose-dependent increase in PTH secretion. This finding indicates that CaMKII is involved in the regulation of PTH release. The weaker effect of KN-62 compared with calmidazolium suggests that the CaMKII pathway is only partially involved in calmodulin-regulated PTH secretion.

In order to investigate the mechanism of calmodulin and CaMKII-regulated PTH secretion, $[\text{Ca}^{2+}]_i$ measurements were performed. Our results showed that 1 μM calmidazolium caused no significant change in $[\text{Ca}^{2+}]_i$, whereas, surprisingly, 10 μM calmidazolium induced an increase in $[\text{Ca}^{2+}]_i$. No obvious change in $[\text{Ca}^{2+}]_i$ by 10 or 20 μM KN-62 was demonstrated. Together with the PTH secretion data, this shows that the stimulation of PTH secretion by calmidazolium and KN-62 is dissociated from the $[\text{Ca}^{2+}]_i$ level. It is therefore possible that calmodulin directly influences the final step of hormone secretion, i.e. exocytosis. Calmodulin has been shown to interact with several exocytotic proteins, such as VAMP-2, Munc-13, and rab-3. Several of these exocytotic proteins are expressed in human parathyroid (Lu *et al.* 2008). Giovanni *et al.* showed that calmodulin itself has an inhibitory effect on exocytosis, because the binding of calmodulin to VAMP-2 impairs SNARE-mediated membrane fusion. However, when calmodulin is combined with synaptotagmin 1, the calmodulin-synaptotagmin 1 complex will overcome the inhibitory effect of calmodulin and accelerate the membrane fusion (Di Giovanni *et al.* 2010). These observations suggest that the existence of synaptotagmin 1 has an important role in Ca^{2+} -mediated membrane fusion. Interestingly, we could not detect synaptotagmin 1 in parathyroid adenomas. The absence of synaptotagmin 1 can at least partially give an explanation for the unusual Ca^{2+} /calmodulin-inhibited hormone secretion in human parathyroid adenoma cells.

Calmidazolium induced an increase in $[\text{Ca}^{2+}]_i$, which is in agreement with previous findings reported in other cell types and species, such as endothelial cells (Watanabe *et al.* 1999) and hepatoma cells (Schlatterer & Schaloske 1996, Liao *et al.* 2009). As shown in Fig. 3B, addition of calmidazolium caused a dramatic increase in $[\text{Ca}^{2+}]_i$ in the presence, but not in the absence, of $[\text{Ca}^{2+}]_e$. These findings suggest that the mechanism of increased $[\text{Ca}^{2+}]_i$ by calmidazolium could be due to calcium influx, i.e. induction of Ca^{2+} leakage from intracellular stores followed by calcium influx. This action has been argued to be unrelated to calmodulin. However, calmodulin was reported to inhibit thapsigargin-induced Ca^{2+} current (Vaca 1996), interrupt activation of calcium influx through TRPC1 channels (Vaca & Sampieri 2002), and inhibit Ca^{2+} release from the endoplasmic reticulum in skeletal muscle (Buratti *et al.* 1995). These observations suggest that an increased $[\text{Ca}^{2+}]_i$ induced by calmidazolium, as shown here in human parathyroid adenoma cells, may at least partially be calmodulin dependent.

The pCaMKII is active, whereas the dephosphorylated CaMKII is not. Autophosphorylation at threonine 286 residue increases the Ca/CaM affinity to CaMKII and

prolongs the activated state of CaMKII. Since antibodies against pCaMKII γ and δ are not available, we verified the level of T-286 pCaMKII α and β . By analyzing 20 chief cell adenomas, we found that the levels of pCaMKII in the tumors were negatively correlated with the patients' total serum Ca^{2+} levels. In cellular studies, a remarkable reduction in CaMKII activity was seen after incubation of cells with 2.5 mM Ca^{2+} , compared with 0.5 and 1.2 mM Ca^{2+} . Taken together, our results clearly show that high calcium suppresses the CaMKII activity in human parathyroid cells. The pathophysiological role of CaMKII in human parathyroid is still, however, unclear.

In conclusion, our results show that calmodulin and CaMKII are involved in the regulation of PTH secretion. In addition, we show that the amount of active CaMKII, namely pCaMKII, is reduced by increased calcium. Further studies are needed to understand the function of calmodulin/CaMKII in parathyroid cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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