The calcium sensing receptor: from understanding parathyroid calcium homeostasis to bone metastases

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ABSTRACT

The cloning of the calcium sensing receptor (CaR) confirmed that parathyroid cells monitor extracellular calcium concentration ($[Ca^{2+}]_{ext}$) via a receptor-type mechanism. This led to the hypothesis that abnormalities in the expression and/or function of the CaR could explain the biochemical abnormalities in primary hyperparathyroidism (PHPT).

Cultured cells from parathyroid adenomas of patients operated for PHPT were used to monitor real-time changes in intracellular calcium concentration ($[Ca^{2+}]_i$) as measured by fluorescent microscopy using the Fura-2/AM dye. We found that CaR agonists trigger release of intracellular calcium pools and such responses are amplified by increasing the affinity of IP_3 receptors. Using confocal microscopy to monitor membrane trafficking in living parathyroid cells labelled with the fluorescent dye FM1-43, we found that a decrease in $[Ca^{2+}]_i$ rather than an absolute change in $[Ca^{2+}]_{ext}$ is the main stimulus for exocytosis from human parathyroid cells. These data suggest that, in PHPT, a defective signalling mechanism from the CaR allows cells from parathyroid adenomas to maintain low $[Ca^{2+}]_i$ with uninhibited PTH secretion in the face of hypercalcaemia.

Over longer periods of time, CaR controls parathyroid proliferation via changes in tyrosine phosphorylation. We found that multiple proteins of molecular weight 20–65 kDa are phosphorylated within 10–60 min in response to CaR agonists.

Further work demonstrated that high $[Ca^{2+}]_i$ stimulates the expression of *bcl-2* oncoprotein in cultured human parathyroid cells and that, in parathyroid adenomas, predominant expression of *bcl-2* rather than *bax* oncoprotein might prevent apoptosis and explain the slow growth rate of these tumours.

More recently, it became apparent that CaR stimulates cell proliferation in several cell types not involved in calcium homeostasis. Using archived histological material from 65 patients who died with metastatic breast cancer, we identified CaR expression predominantly in tumours from patients who developed bone rather than visceral metastases (35 of 49 versus 7 of 16; $P < 0.01$, chi-squared test).

These data suggest that CaR expression has the potential to become a new biological marker predicting the risk of bone metastases in patients with breast cancer. A prospective study should investigate if patients with CaR-positive tumours are more likely to develop bone metastases and whether they could benefit more from prophylactic treatment with bisphosphonates or the newly developed CaR antagonists.

KEYWORDS

Calcium sensing receptor – Primary hyperparathyroidism – Cell proliferation – Breast cancer – Bone metastasis

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The parathyroid glands were first described at autopsy in 1862 in an Indian rhinoceros by Sir Richard Owen from the Hunterian Museum of the Royal College of Surgeons of England,¹ but their role in calcium homeostasis was only recognised decades later. Calcium is an essential element throughout the phylogenetic tree and the free extracellular calcium concentration ($[Ca^{2+}]_{ext}$) is maintained within a narrow range (1.0–1.3 mM). A central role in the rapid control of calcium homeostasis is played by the parathyroid hormone (PTH), whose co-ordinated actions on

bone, kidney and intestine increase the flow of calcium into the extracellular fluid.²

There is an inverse sigmoidal relationship between PTH secretion and $[Ca^{2+}]_{ext}$. The initial changes in the secretory rate of PTH in response to low $[Ca^{2+}]_{ext}$ take place within seconds through the release of preformed hormones from storage granules. Within 15–30 min, there is also an increase in the net rate of PTH synthesis. Prolonged hypocalcaemia promotes parathyroid cellular hypertrophy and proliferation within days to weeks.

Cloning of the calcium sensing receptor (CaR)

By the late 1980s, a body of indirect evidence suggested that $[Ca^{2+}]_{ext}$ acts on parathyroid cells through a cell surface, receptor-like mechanism which signals the cells to alter the

intracellular calcium concentration ($[Ca^{2+}]_i$), as reviewed by Brown³.

A major breakthrough in the understanding of parathyroid calcium homeostasis came in 1993 when Brown *et al.*⁴ reported the cloning of the bovine parathyroid calcium-ion

sensing receptor (CaR) followed by isolation of cDNAs for human parathyroid CaR from a parathyroid adenoma.⁵ The human CaR consists of 1078 aminoacids with a highly conserved structure compared with the rat and bovine CaRs. The CaR is the first example of a cell-surface receptor coupled with G proteins that recognises an inorganic ion rather than a molecule as principal ligand.

Mutations that inactivate the CaR have been identified in kindreds with familial hypocalciuric hypercalcaemia – an autosomal dominant disorder characterised by life-long hypercalcaemia with relative hypocalciuria and normal or mildly elevated PTH concentrations. Individuals homozygous for such mutations present with neonatal severe hyperparathyroidism – a life-threatening condition with marked hypercalcaemia and parathyroid hyperplasia. The impact of different CaR mutations on the severity of the clinical syndrome has recently been reviewed.⁶

CaR is also expressed in non-parathyroid tissues but its functional significance in tissues not involved in calcium homeostasis is yet to be fully understood. Examples of such CaR-positive tissues include calcitonin-secreting C cells from medullary thyroid carcinomas, kidney cells, osteoblasts and osteocytes, nervous system, lung (alveolar type II epithelial cells secreting pulmonary surfactant express), mononuclear bone marrow cells and cells of several haematopoietic lineages, keratinocytes.

Intracellular signalling from the CaR in primary hyperparathyroidism

Primary hyperparathyroidism (PHPT) is a common endocrine disorder characterised by hypercalcaemia in the presence of

high PTH levels. The lack of correlation between the weight of parathyroid adenomas and the maximum calcium levels and PTH concentration (Fig. 1) suggests that a functional defect rather than the total mass of parathyroid cells represents the cause of the excessive PTH secretion in PHPT. We, therefore, explored whether different steps of the intracellular signalling cascade are defective in cells from parathyroid adenomas of patients operated for PHPT.

Fluorescent microscopy using the Fura-2/AM dye allowed real-time monitoring of changes in $[Ca^{2+}]_i$ in individual cells. Our results, summarised in Figure 2, have shown that in cultured human parathyroid cells:

1. CaR agonists trigger release of Ca^{2+} intracellular calcium pools.
2. Depletion of intracellular Ca^{2+} stores using thapsigargin (an inhibitor of intracellular Ca^{2+} pumps) causes a sustained rise in $[Ca^{2+}]_i$ and blunts further responses to CaR agonists.⁷
3. Modulation of IP_3 receptor affinity by thimerosal increases the responsiveness of parathyroid cells to CaR agonists.⁸
4. Ceramides (C2 *N*-acetyl-D-erythro-sphingosine) induce rapid increases in $[Ca^{2+}]_i$ through discharge of Ca^{2+} from the IP_3 -sensitive intracellular pools necessary for CaR responsiveness.⁹

Abnormalities of these mechanisms could be significant for the pathogenesis of PHPT because it would induce a shift of the response curve between $[Ca^{2+}]_{ext}$ and $[Ca^{2+}]_i$ and, therefore, it could explain the main functional abnormality described in PHPT. Drugs that modulate the responsiveness

Figure 1 Pre-operative plasma calcium levels and PTH concentration in patients with primary hyperparathyroidism do not correlate with the weight of the parathyroid adenomas. Data derived from a cohort of 300 consecutive unselected patients with PHPT. Patients presented with hypercalcaemia (normal range, 2.20–2.65 mmol/l) and uninhibited PTH levels (normal range, 0.8–8.5 pmol/l).

of this signalling cascade could, therefore, have the potential to control excessive PTH secretion in PHPT.

CaR controls exocytosis in human parathyroid cells

A very challenging unsolved paradox is that parathyroid cells seem to be the only endocrine model in which a raise in intracellular calcium leads to inhibition rather than stimulation of hormone secretion (such as in other endocrine cells and neurons). Exocytosis of cytoplasmic granules containing preformed hormones is known to involve numerous proteins that interact in the process of docking of granules and their fusion with the plasma membrane. All such interactions are triggered or accelerated by a raise in $[Ca^{2+}]_i$ in all endocrine cells apart of parathyroid cells. Despite extensive investigations, it remains unknown what is the mechanism unique to parathyroid cells.

In an attempt to characterise further the coupling between CaR agonists and secretion/exocytosis in human

parathyroid cells, we have used confocal microscopy to monitor membrane trafficking in living parathyroid cells labelled with the fluorescent dye FM1-43. Once exocytosis takes place, the slight increase in the total cell surface leads to an increased uptake of the fluorescent dye on the surface of the cell and this process can be visualised and quantified in real time on confocal microscopy (Plate 1). Experiments showed that exocytosis could be triggered by a drop in $[Ca^{2+}]_i$ during incubation in high $[Ca^{2+}]_{ext}$, which should have inhibited secretion. In contrast, maintaining a high $[Ca^{2+}]_i$ prevented the uptake of FM1-43 during incubation in low $[Ca^{2+}]_{ext}$, which should have stimulated secretion.¹⁰

We also found that after long incubation (5-30 min) in the presence of FM1-43, the dye accumulates in the cytoplasm with a granular distribution suggesting targeting of the secretory compartment. This led to a cell model in which the granule movements could be visualised and exocytosis monitored and quantified based on the destaining kinetics.¹⁰ These data

were subsequently confirmed using parathyroid cells transfected with a hybrid plasmid made of green fluorescent protein (GFP) and phogrin – a protein constituent of secretory granule membranes.¹¹ In such transfected cells, secretory granules appear fluorescent on confocal microscopy and their intracytoplasmic movement and their fusion with the plasma membrane can be visualised in real time.¹¹

This group of experiments have demonstrated that a decrease in $[Ca^{2+}]_i$, rather than an absolute change in $[Ca^{2+}]_{ext}$ is the main stimulus for secretion from human parathyroid cells. Such data reinforce the hypothesis that, in PHPT, a defective signalling mechanism from the CaR allows cells from parathyroid adenomas to maintain low $[Ca^{2+}]_i$ with uninhibited PTH secretion in the face of hypercalcaemia.

Tyrosine phosphorylation modulates the responsiveness of the CaR

Over longer periods of time, CaR controls parathyroid proliferation via changes in tyrosine phosphorylation. We found that multiple proteins of molecular weight 20–65 kDa are phosphorylated within 10–60 min in response to CaR agonists (Fig. 3).¹² Subsequently, it was confirmed by others that tyrosine phosphorylation is an alternative intracellular signalling for CaR in several cell models¹⁵ and such mechanism could be important for the control of parathyroid cell proliferation.

Hypercalcaemia-induced expression of bcl-2 in parathyroid tumours

Over 90% of patients with PHPT have a single parathyroid adenoma. Such tumours are monoclonal¹⁴ and can reach between 100 mg and 10 g in weight (*i.e.* between 2–200-fold increase in weight compared with normal glands). At the time of surgical treatment, parathyroid adenomas have a very slow growth rate and their rate of DNA synthesis and values of proliferative markers are much too low to account for the size attained,¹⁵ suggesting that the rates of cell division and tumour growth decline as the condition progresses.

One hypothesis is that parathyroid adenomas may occur due to a defect in the calcium-sensing mechanism, which leads to the accumulation of cells with a higher calcium set point (*i.e.* the plasma calcium concentration needed for half-maximal inhibition of PTH secretion). These cells would have a rapid initial growth but slow down as tumour size reaches an asymptotic value corresponding to the total rate of parathyroid hormone secretion needed to raise the $[Ca^{2+}]_{ext}$ according to the new set-point. The relationship between calcium-sensing mechanisms and proliferation rates is, however, not well understood.

We explored the possibility that parathyroid cell apoptosis is inhibited in PHPT and investigated the role of bcl-2 protein. The *bcl-2* proto-oncogene was originally identified in B-cell lymphomas and subsequently found in a wide range of normal and tumour tissues.¹⁶ In humans, bcl-2 protein is associated with activated and undifferentiated cells which are undergoing terminal differentiation and need protection from apoptosis, such as hormone- or growth factor-dependent glandular epithelium (breast, prostate), differentiating epithelium with stem or proliferating cells (skin, intestine) and long-lived postmitotic cells (*e.g.* neurones).

We demonstrated that the expression of bcl-2 oncoprotein in cultured human parathyroid cells is stimulated during 12–72 h incubation with drugs known to raise $[Ca^{2+}]_i$ (thapsigargin and the Ca^{2+} -ionophore A23187).¹⁷ Subsequently, we evaluated *bcl-2* and *bax* expression by immunohistochemistry in 48 parathyroid adenomas and 8 biopsies of normal glands from patients with PHPT. Homogeneous, intense, cytoplasmic, bcl-2-immunostaining was demonstrated in 45 of 48 parathyroid tumours while bax immunostaining was less intense (21 of 48 tumours were negative or weak). Normal glands and the normal rim at the edge of parathyroid adenomas showed more intense bcl-2 immunostaining and less bax immunostaining compared with the corresponding adenomas. It appears, therefore, that in parathyroid adenomas predominant expression of bcl-2 rather than bax oncoprotein might prevent apoptosis and explain the slow growth rate of these tumours.¹⁸

These results suggest that the predominant expression of *bcl-2* versus *bax* in parathyroid adenomas could be

Table 1 Relationship between CaR-expression and development of bone metastases in patients with breast cancer

	CaR-positive tumours	CaR-negative tumours	P-value
Number of patients	49	16	
Skeletal metastases	35 of 49 (71%)	7 of 16 (43%)	< 0.01
Bone metastases developed in first 12 months	6 of 13 with intense CaR expression 6 of 21 with weak CaR expression	1 of 7	< 0.05
Median metastases-free intervals	13 months for those with intense CaR expression 22 months for those with weak CaR expression	24 months	< 0.05

important for parathyroid adenoma growth. Furthermore, stimulation of *bcl-2* expression by high $[Ca^{2+}]_i$ could act as a survival mechanism for normal parathyroid cells exposed to hypercalcaemia.

Expression of CaR in advanced breast cancers

In 1889, Stephen Paget first described the preferential dissemination of breast cancer to bone and he proposed the 'seed and soil' theory that the micro-environment of the organs to which cancer cells spread serves as fertile soil for their growth. The predilection of breast cancer to metastasise to bone in about 70% of women with advanced disease was subsequently confirmed by large autopsy studies and recognised in multiple clinical studies. Currently, there are no criteria to identify at the time of the initial diagnosis those patients likely to develop bone metastases.

We explored the hypothesis that CaR expression acts as a selection mechanism for tumour cells having a survival/functional advantage in the presence of high concentrations of calcium, such as those present in the bone micro-environment. Tumours expressing the CaR would be, therefore, more likely to initiate the development of clinically significant bone metastases. This hypothesis was encouraged by the observation that activation of the CaR in human breast cancer cell lines MDA-MB-231 and MCF-7 triggers PTHrP secretion and transactivation of the epidermal growth factor receptor.¹⁹

With Ethical Committee approval, we analysed archived histological sections of primary tumours from patients who died with advanced breast cancer. Over a 5-year period, 108 patients with breast cancer were found to have positive bone scans, of whom 42 patients died before the study began. Of the patients with negative bone scans, 23 died when known to have extensive liver or lung metastases. Immunohistochemistry was performed on histological slides from 65 patients with primary breast cancer using a commercially available antiCaR antibody (AffinityBioReagents, Cambridge, UK). CaR expression was identified in both ductal and lobular breast carcinomas, with a predominant cytoplasmic

distribution (Plate 2). The risk of developing skeletal metastasis was significantly higher in patients with CaR-positive tumours (Table 1). The median metastases-free interval was shortest in patients with intense CaR expression (Table 1).²⁰

Our study was the first to report the incidence of expression of CaR in patients with advanced breast cancer. These data complement our previous report of CaR expression in 9 of 14 specimens of bone metastases from patients previously diagnosed with breast cancer.²¹ A subsequent preliminary report from another group confirmed our findings using quantitative real-time polymerase chain reaction.²²

It appears that breast cancers that produce bone metastases are more likely to have a very intense CaR expression. This situation is opposite to that found in colonic carcinomas, which are known to have visceral metastases and only very rarely to metastasise to the bone. CaR has been identified in human colon epithelium, where it is restricted to normal colon mucosa and to differentiated areas of colonic adenocarcinomas, but is absent in poorly or undifferentiated tumours.²⁵ These data suggest that a loss of CaR expression in the transition from normal colonic epithelial cells to malignant adenocarcinoma cells is associated with a very low potential of colonic carcinomas to generate bone metastases.

These data suggest that CaR expression has the potential to become a new biological marker predicting the risk of bone metastases in patients with breast cancer. The lack of any recent further publications addressing this hypothesis might suggest that others failed to demonstrate a convincing statistical correlation between CaR expression and development of bone metastases. So far, therefore, the hypothesis is based on data derived from a very small cohort of patients. A prospective study should investigate if patients with CaR-positive tumours are more likely to develop bone metastases and whether they could benefit more from prophylactic treatment with bisphosphonates.

Conclusions

The cloning of the calcium sensing receptor (CaR) from parathyroid cells by Brown *et al.*⁴ confirmed the unexpected existence of a plasma-membrane receptor recognising an ion

as its principal ligand. The wide expression of CaR raises ongoing questions about its role in tissues not involved in calcium homeostasis.

Our studies using human parathyroid cells from patients with primary hyperparathyroidism explored the intracellular signalling cascade previously described in bovine parathyroid cells and provided new data on the timing and modulation of secretion-coupled membrane turnover in response to changes in $[Ca^{2+}]_{ext}$.

With more direct clinical significance, we reported for the first time that CaR expression in human breast cancers correlates with the ability to metastasise to the bone. Future studies might confirm the use of CaR expression as a prognostic marker for the risk for skeletal metastases in patients diagnosed with breast cancer. This could lead to the use of the newly developed CaR-antagonists as potential treatment for patients with high risk of developing bone metastases.

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