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Co-purification of Calcium Transport-stimulating and DNA Synthesis-stimulating Agents with Parathormone-like Activity Isolated from the Hypercalcaemic Strain of the Walker 256 Tumour

F. de Miguel, R. García-Cañero and P. Esbrit

One of the strains of the Walker 256 carcinosarcoma induces in the rat a humoral hypercalcaemia of malignancy (HHM) syndrome which is similar to that reported in human patients. We have isolated from this tumour a chromatographic fraction which displays an adenylate cyclase stimulating activity in dog kidney cortical membranes, similar to that of a parathormone (PTH) related protein isolated from various HHM related tumours. In addition, this fraction stimulated initial calcium (Ca) uptake in confluent Madin-Darby canine kidney (MDCK) cell monolayers in a dose-dependent manner. Maximal stimulation of Ca uptake was associated with an enhanced Ca efflux from MDCK cells preloaded with the cation, and with an increased DNA synthesis in these cells. These activities might be involved in development of increased tubular calcium reabsorption in Walker 256 tumour-bearing rats.
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INTRODUCTION

HUMORAL HYPERCALCAEMIA of malignancy (HHM) is a syndrome which is now known to result from the elaboration by tumours of humoral factors that stimulate bone resorption and/or tubular calcium (Ca) reabsorption [1]. A parathormone (PTH) related protein (PTHrp), capable of interacting with PTH receptors in bone and kidney, has been suggested as a likely mediator for the HHM syndrome associated with human and animal tumours [2, 3]. However, tumour-derived growth factors have also been proposed as mediators for this syndrome [4]. The hypercalcaemic strain of the rat Walker 256 carcinosarcoma is one of the few known animal models for HHM [5]. The hypercalcaemia associated with this tumour appears to involve

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an increase of both bone resorption and renal Ca reabsorption [6, 7]. This tumour secretes a factor(s) which interacts with adenylate cyclase coupled PTH receptors in bone and kidney cells [6, 8, 9]. However, the relationship of this adenylate cyclase stimulating activity (ACSA) to the increased bone resorption and renal Ca reabsorption associated with the Walker 256 tumour is unclear at present.

In the present studies, we have partially purified from the Walker 256 tumour a chromatographic fraction which displays ACSA. In addition, this fraction also exhibited Ca transport and DNA synthesis-stimulating activities in cultured Madin-Darby canine kidney (MDCK) cells. The latter activities might be involved in the pathogenesis of increased renal Ca reabsorption in Walker 256 tumour-host rats.

MATERIALS AND METHODS

Female Wistar rats (200 g), were used for continuous transplantation of the hypercalcaemic variant of the Walker 256 tumour (kindly supplied by A.J.S. Davies, Institute of Cancer Research, London), as described [7]. Tumours (20 g) were harvested at 12–14 days after transplantation and homogenised on ice in a glass homogeniser in 0.1 mol/l Tris-HCl, pH 7.4. Homogenised tissue was then extracted by 8 mol/l urea/0.2 mol/l HCl/0.1 mol/l cysteine followed by 20% ethanol/10 mmol/l acetic acid/0.1 mol/l NaCl, as described by Stewart *et al.* [10, 11]. The lyophilised ethanol–NaCl extract, resuspended in 0.15 mol/l ammonium acetate, pH 4.5, was applied to a 2.5 × 90 cm Ultrogel AcA-54 (IBF, Villeneuve-la-Garenne, France) column, run in the same buffer. Fractions of 5 ml were collected, pooled and kept lyophilised at −20°C. ACSA-containing fraction pool was rechromatographed on Ultrogel AcA-54, as described above. The resulting peak of ACSA was used for Ca transport and DNA synthesis studies.

ACSA in tumour extracts was assayed by using partially purified canine renal cortical membranes, according to Stewart *et al.* [11]. Briefly, lyophilised samples suspended in 5 mmol/l acetic acid containing 0.5 mg/ml bovine serum albumin (BSA) (Sigma), were incubated for 30 min at 30°C with 50 mmol/l Tris-HCl, pH 7.4, 5 mmol/l $MgCl_2$, 10 mmol/l KCl, 1 mmol/l EGTA, 40 U/ml creatine phosphokinase (Sigma), 9 mmol/l creatine phosphate (Sigma), 0.13 mmol/l ATP (Sigma), 1.85 kBq (α ^{32}P)ATP (370 GBq to 1.85 TBq/mmol; Amersham, UK), 1 mmol/l cAMP (Sigma), 2 mmol/l dithiothreitol (Sigma), 100 μmol/l 5′-guanylyl imidodiphosphate (Sigma) and 60 μg membrane protein. The reaction was stopped by diluting with a solution containing 2 mmol/l ATP, 0.5 mmol/l cAMP, and 1.11 kBq (^{3}H)cAMP (1.11 TBq/mmol; Amersham) to test recovery, and boiling the mixture for 3 min. (^{32}P)cAMP was separated from (α ^{32}P)ATP by sequential chromatography on Dowex AG 50W-X4 (Bio-Rad, Richmond, California) and neutral alumina (Sigma) columns, and counted by liquid scintillation. Bovine (b)PTH(1-34) (Peninsula, Belmont, California), or alternatively human (h)PTHrp(1-34) (Bachem, Bubendorf, Switzerland), was used as standard. In some experiments, ACSA was assayed in the presence of the competitive PTH analogue, [Nle^{8,18},Tyr^{34}]bPTH(3-34) amide [bPTH(3-34)] (Peninsula). In our hands, cAMP recovery in the assay was usually more than 70%.

MDCK cells from the American Type Culture Collection (CCL 34) were used. This cell line has been extensively characterised, and shown to display various morphological and functional features of thick limbs and distal tubules when grown as a monolayer [12–14]. Cells were plated at 1.5 × 10^4/cm^2 in 24-well culture dishes in Dulbecco's modified Eagle's medium (DME): Ham's F12 (Flow, UK), 1:1 (v/v), containing 10% fetal bovine serum (Flow), and allowed to grow to confluence in a 5% CO_2 incubator at 37°C. Serum-containing medium was replaced by the same medium containing 0.2% BSA instead of serum, 24 h before the experiments.

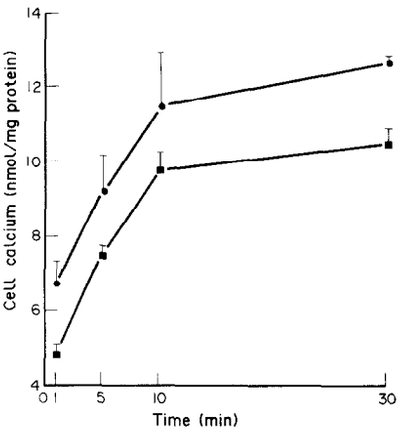

Fig. 1. Time course of Ca uptake from the luminal side of MDCK cells grown on Millipore filters. Ca uptake was stopped with (■) or without (●) La^{3+} in the washing solution. Points represent means (S.E.) of 2–9 determinations.

Confluent MDCK monolayers were washed with Hanks' balance salts solution (1.2 mmol/l $CaCl_2$), pH 7.4, before addition of 37 kBq $^{45}CaCl_2$ (370 GBq to 1.48 TBq/g Ca; Amersham) and the test samples in 0.5 ml of Hanks' solution. At the end of incubation, Ca uptake was stopped by adding ice-cold 250 mmol/l sucrose, with or without 0.5 mmol/l $LaCl_3$, and then cells were washed twice with the same solution. Cell Ca was extracted overnight with 2 ml 0.1 mol/l HNO_3, and radioactivity measured in the resulting supernatants.

To characterise Ca fluxes in MDCK cell monolayers, a minimum of 1.7 × 10^5 cells/cm^2 were seeded on Millicell-HA filters (0.45 μm, Millipore, Bedford, Massachusetts) placed in 24-well dishes, and incubated as described above. At different times after seeding, the cell monolayers were tested for their permeability to inulin. Culture medium (0.5 ml) containing 10^{-7} mol/l (^{3}H)inulin (18.5 kBq, 37–185 GBq/mmol; Amersham) was added to the apical side of the monolayer, and the same volume of medium without inulin was added to the other side. After various incubation periods, 10 μl aliquots were removed from the basolateral side compartment and their radioactivity was counted.

Transepithelial resistance of MDCK cell monolayers on Millipore filters was measured with a Millicell-ERS voltohmmeter (Millipore).

Confluent monolayers of serum-depleted cells on filters were washed and then incubated as described above, with $^{45}CaCl_2$ (37 kBq) and the test samples added to either the apical or the basolateral side of the monolayer. Ca uptake was stopped and cell Ca was extracted, as above.

For measurement of Ca efflux, confluent MDCK cells on Millipore filters were serum-depleted and then preincubated with $^{45}CaCl_2$ only in the apical bathing solution for 45 min at 37°C, as described for uptake measurements. Ca uptake in these cells had reached equilibrium at this time (Fig. 1). Then, the $^{45}CaCl_2$-containing medium was replaced by 0.5 ml Hanks' medium, and samples in Hanks' solution were added to the basolateral side. Ca efflux was stopped and cell Ca was extracted

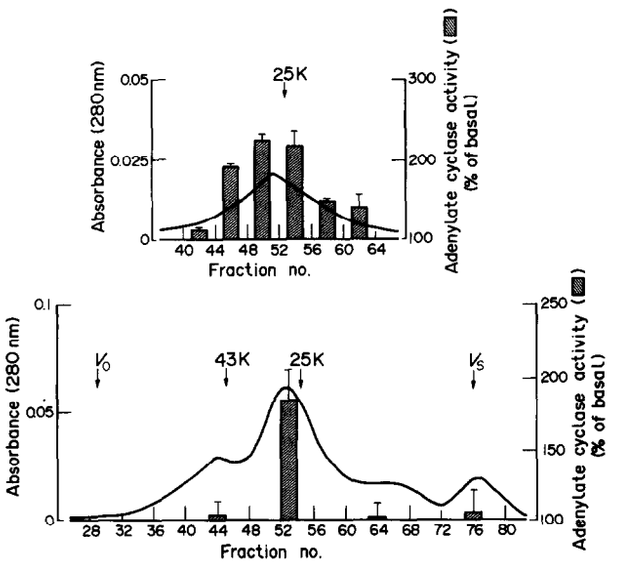

Fig. 2. Gel filtration on an Ultrogel AcA 54 column of an ethanol-NaCl extract from Walker tumour homogenates. Column markers were IgG (V_o), ovalbumin (43 K), chymotrypsinogen (25 K) and vitamin B_{12} (V_s). Inset: Pooled ACSA-containing fractions were rechromatographed in the same column. Values are means (S.E.) of 2–6 determinations.

as described above. Ca efflux towards the basolateral side was calculated as the percentage of cell Ca over that found both intracellularly and in the basolateral side at each time.

DNA synthesis was measured in serum-depleted MDCK cells grown to confluence on Millipore filters. The cells were incubated for 18 h in culture medium with 18.5 kBq (^{3}H)thymidine (740 GBq/mmol; New England Nuclear, Boston) or the sample at the apical or at the basolateral side of the monolayer, respectively. DNA was extracted by heating with 7% perchloric acid, and incorporated radioactivity in supernatants counted, as described previously [15].

Cells were lysed in 0.5% sodium dodecyl sulphate, 0.5 mol/l NaOH. Then, protein was precipitated by 5% trichloroacetic acid and resolubilised in 0.5 mol/l NaOH. Proteins were measured by Bradford's method [16].

Kinetics analysis was performed by using a non-linear regression program (Enzfitter, Elsevier-Biosoft), fitting the values to a triple exponential decay curve. Statistical differences were evaluated by Student's t test.

RESULTS AND DISCUSSION

The gel filtration profile of ethanol/NaCl extract of the Walker tumour homogenates is shown in Fig. 2. ACSA eluted in the region of the chromatogram corresponding to an apparent molecular weight of 20–30 kD (F II). Repurification of F II on the same column yielded a well defined peak of ACSA (Fig. 2, inset).

The pooled ACSA-containing fractions (FR II, numbers 49–55; Fig. 2, inset) displayed dose-dependency parallel to those of bPTH(1-34) and hPTHrp(1-34), so that maximal activity was obtained at about 1 mg protein/ml, equivalent to that of 1 nmol/l bPTH(1-34) or 5 nmol/l hPTHrp(1-34) (Fig. 3a), and had a specific activity of 2.2 ng eq. of bPTH(1-34)/mg protein, or 9.3 ng eq. of hPTHrp(1-34)/mg protein, since the latter was less sensitive than bPTH(1-34) in the ACSA assay (Fig. 3a), as previously reported [17]. This represents a purification of about 20-fold over the activity of the first urea extract. This activity of FR II and that of either bPTH(1-34) or hPTHrp(1-34) were inhibitable by the PTH analogue, bPTH(3-34) (Fig. 3b). These properties of FR II are similar to those of the PTHrp isolated from various HHM-associated tumours [18].

On the other hand, FR II induced a dose-dependent stimulation of the initial rate (1 min) of Ca uptake by MDCK cells grown on plastic dishes (Fig. 4). Maximal uptake in stimulated cells, which was 20% over that seen with saline medium (control), occurred with FR II at 200 ng/ml, decreasing at higher concentrations.

Since maximal Ca uptake induced by FR II was low, the next experiments were carried out with MDCK grown on Millipore filters. This approach allows exposure of putative receptors on either the apical or the basolateral cell side to the agonist, and also analyse the sidedness of the effects of this agonist on Ca uptake by MDCK monolayers. Therefore, we first determined if the initial seeding density of MDCK cells on filters was satisfactory to achieve a confluent monolayer, impermeable to small molecules and electrically asymmetric. As shown in Fig. 5, MDCK monolayers grown for 5–8 days after seeding on Milli-

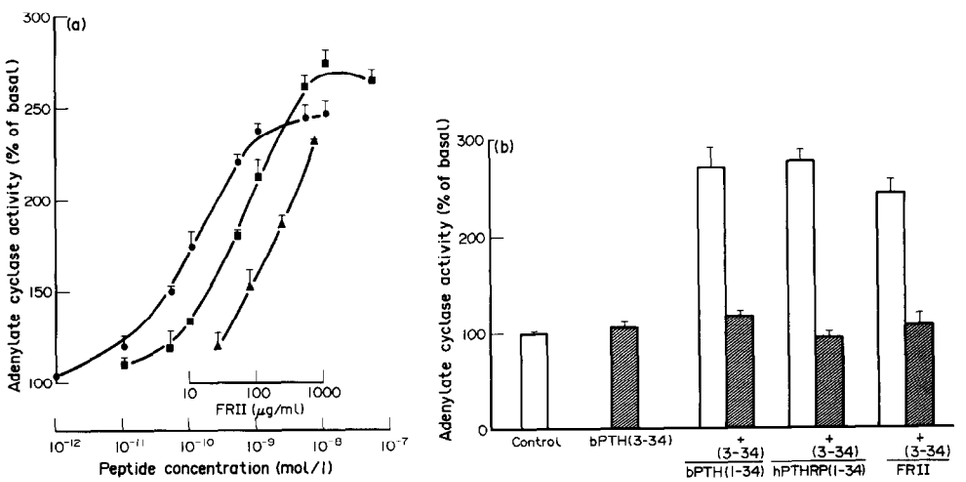

Fig. 3. (a) Dose-response curves of bPTH(1-34) (●), hPTHrp(1-34) (■) and FR II (▲) in the canine renal cortical adenylate cyclase assay. Each point represents the means (S.E.) of two measurements. (b) Inhibition of ACSA by [Nle^{8,18},Tyr^{34}]bPTH(3-34) amide. The latter was used at a concentration 1000-fold higher than that of the agonists tested. Values are means (S.E.) of four determinations.

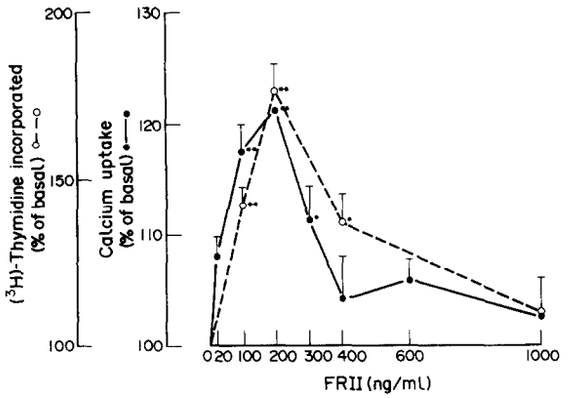

Fig. 4. Dose dependency of FR II on Ca uptake (●) or DNA synthesis (○) in MDCK cells. 100% activity corresponds to 5.0 (S.E. 0.7) nmol min per mg protein (●) or 84 (4) dpm/μg protein (○), respectively. Points represent means (S.E.) of 3–4 determinations. $P < *0.05$, and $**P < 0.01$, compared with basal activity.

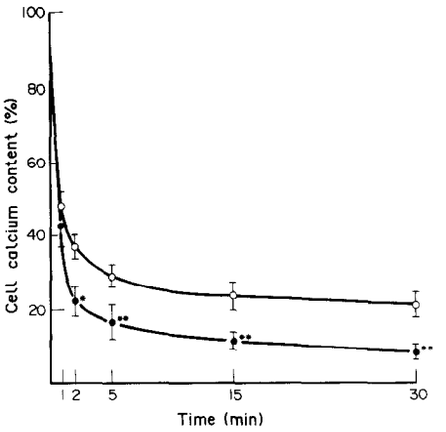

Fig. 6. Time-course of Ca efflux towards the basolateral side from MDCK cells preloaded with $^{45}CaCl_2$, grown on Millipore filters. (○), control solution; (●), FR II-containing solution. Points represent means (S.E.) of 3 determinations. 100% cell Ca content represents 9111 (S.E. 813) dpm/mg protein. $*P < 0.05$, and $**P < 0.01$, compared with control.

pore filters, were impermeable to inulin compared to filters with no cells. At this time, MDCK monolayers developed a transepithelial resistance, corrected for both fluid and filter resistance, of 71.1 (S.E. 5.5) ohm cm^2. These data are consistent with previous findings by other investigators [14, 19].

FR II (200 ng/ml) at the basolateral cell surface was found to induce a stimulation of Ca uptake from the apical side into the cell, which was 152 (S.D. 6)% ($P < 0.01$, $n = 3$) compared to control (6.7 (0.6) nmol/min per mg protein). No effect on Ca uptake was observed when FR II was at the apical cell surface. About 20% of Ca uptake represents binding to the outside of the cell membrane since La^{3+}, which displaces Ca from the binding sites [20], in the stopping solution, inhibited 20% of Ca uptake (Fig. 1).

This stimulation of Ca uptake in MDCK cells by FR II, was associated with a dose-dependent DNA synthesis in these cells (Fig. 4). This finding is interesting since various growth factors displaying bone-resorbing activity, synthesised by some HHM-related tumours including the Walker tumour, might contribute to the mechanism of hypercalcaemia in the tumour host [4, 21]. Whether the mitogenic activity for MDCK cells reported herein has also bone-resorbing activity awaits further studies.

In addition, FR II at the dose which maximally increased Ca uptake, significantly increased cell Ca loss from MDCK cells during the slower phase of Ca efflux to the basolateral side (Fig. 6). Cell Ca loss mainly represents transmembrane movement of intracellular Ca since addition of 0.5 mmol/La^{3+} to the solution used to initiate efflux inhibited cell Ca loss by more than 70%. The efflux rate constant between 5 and 30 min was about 2.5-fold higher in the presence of FR II compared to that of the control (82 (9) dpm/min per mg protein). Doses of FR II higher than 200 ng/ml were found to inhibit its effect on Ca efflux, in a similar manner to that observed for Ca uptake- and DNA synthesis-stimulating activities. This suggests the presence of inhibitory contaminants in the rather crude FR II fraction, as recently reported for another HHM-related tumour [22]. Further purification is obviously necessary before any definite conclusions on this subject can be drawn.

This is the first time to our knowledge that the same chromatographic fraction which stimulates the unidirectional Ca transport, from the apical to the basolateral cell side, and DNA synthesis in distal tubule-like cells, has been shown to co-elute with ACSA isolated from an HHM-related tumour. These effects of FR II on Ca transport and DNA synthesis in MDCK cells reported herein might be related to the mechanism of the increased renal Ca reabsorption in Walker tumour-host rats. Whether different molecular entities are responsible for these activities requires further studies. However, recent data in our laboratory, obtained with HPLC-further purified tumour extracts, suggest that the stimulatory effect of FR II on DNA synthesis in MDCK cells might be unrelated to ACSA in this fraction.

Our findings described in the present report also demonstrate that MDCK cells can be a valuable model for studying the mechanism of action of tumour-derived factors in the distal tubule.

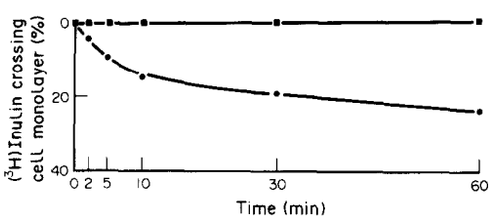

Fig. 5. Permeability to (^{3}H)inulin of Millipore filters seeded with MDCK cells grown for 5–8 days (■), or no cells (●). Points represent mean (S.E.) of two single experiments on three different filters. Symbols are bigger than S.E. bars.

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Encapsulation of Doxorubicin in Thermosensitive Small Unilamellar Vesicle Liposomes

Jean-Louis Merlin

The optimisation of the formulation of thermosensitive, doxorubicin-containing small unilamellar liposomes is described. The liposomes were first strictly defined in terms of size distribution and size stability and a quality level was defined. The suspension contained more than 95% vesicles with a maximal diameter of 50 nm and kept this level for a minimum of 24 hours. Several lipid mixtures were tested in defined thermal conditions usable for *in vitro* experiments: 43°C in fetal calf serum-containing medium. The mixture yielding the best differential thermal stability (DTS) defined as the difference of release between 37°C and 43°C exposures was found to be a dipalmitoylphosphatidylcholine/distearoylphosphatidyl–choline/cholesterol mixture in 5:4:2 molar ratio yielding 72% DTS. These thermosensitive liposomes were evaluated betwen pH 6.00 and 8.00 since hyperthermia-induced lethality was reported to be enhanced by pH variations. Their release capacity was not altered by any pH variations. Incorporation of doxorubicin within these liposomes was then performed. The release kinetics at 37° and 43°C were determined. It is proposed to use this formulation in *in vitro* experiments on tumour cells, although a decrease of DTS was evident.
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INTRODUCTION

SELECTIVE LOCALISATION of antitumour drugs is a goal that may result in better control of cancers. Most antitumour agents interact with non-malignant tissues reducing the therapeutic effectiveness when given systemically. Liposome-encapsulated drugs appear to represent an interesting alternative opportunity [1, 2]. The combination of liposome-mediated drug delivery and hyperthermic treatment is an original approach and synergistic applications have been demonstrated on tumour models with encapsulated methotrexate [3, 5], cisplatin [6] or bleomycin [7, 8], hyperthermia playing the double role of treating the tumour and triggering the local efflux of the drug from the liposomes. To our knowledge, no experimentation has been reported with doxorubicin encapsulated in thermosensitive liposomes.

Our experiments were designed to evaluate a series of liposomes prepared with dipalmitolyphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and cholesterol in order to optimise the thermosensitivity. The molar ratios of these