Solubilization of functional calcitonin receptors

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Calcitonin (CT) binding activity has been extracted from a membrane fraction of human placenta using the zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (Chaps). Approximately two-thirds of the available binding sites were extracted using 5 mM-Chaps. The binding characteristics of ^{125}I -labelled salmon CT (^{125}I -sCT) to the solubilized extract were similar to those obtained previously with placental membranes and other targets such as osteoclasts, renal cells and certain human cancer cell lines. ^{125}I -sCT binding was saturable (B_{max} , 75 ± 6 fmol/mg of protein, $n = 3$) and Scatchard analysis revealed a single class of high-affinity binding sites (K_d 165 ± 28 pM, $n = 3$). In competitive-binding studies, various species-specific CTs and CT analogues showed the same rank order of potencies as seen in CT bioassays and several unrelated peptides did not compete at high doses. A biologically active CT analogue, [Arg^{11,18},Lys¹⁴]sCT, derivatized with the photoreactive phenylazide cross-linking agent, *N*-hydroxysuccinimidyl-4-azidobenzoate, was used to identify receptor components of $M_r \sim 88\,000$ and $\sim 71\,000$ in both particulate placental membranes and the solubilized extract. Receptor components of M_r 85–90 000 have been identified in other CT target cells previously using chemical- and photoaffinity-labelling techniques. These results demonstrate the first successful solubilization of the CT receptor in a form which purification.

Introduction

In mammals, calcitonin (CT) acts to inhibit bone resorption [1,2] by interaction with specific receptors on the bone-resorbing cell, the osteoclast [3,4]. This has been the basis of the clinical use of CT in conditions associated with excessive bone resorption such as Paget's disease and osteoporosis.

Solubilization of receptors in a functional state is a basic requirement for their purification. Attempts to solubilize the CT receptor using a variety of detergents including Lubrol-PX [5], *n*-octyl β -D-glucopyranoside and Triton X-100 (J. M. Moseley, unpublished work) have resulted in alteration of the binding characteristics so that little progress has been made with its purification. However, some success has been achieved with rat kidney membranes solubilized with Triton X-100 [6].

Recently, we have identified high-affinity CT receptors in a human placental membrane fraction [7]. In this report, the solubilization of CT receptors from these membranes using the zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (Chaps) [8] is described. The solubilized receptors retained all the characteristics of the membrane-bound receptor, including affinity, specificity and apparent M_r on SDS/polyacrylamide-gel electrophoresis (PAGE).

MATERIALS AND METHODS

Chemical and hormones

Synthetic human CT (hCT) was provided by Dr. W. Rittel, Ciba-Geigy, Basle, Switzerland. Synthetic salmon CT (sCT), porcine CT (pCT), des-Leu¹⁶-sCT, des-Phe¹⁶-

hCT and [Gly⁸]hCT were provided by Dr. R. C. Orłowski, Armour Pharmaceutical Co., Kanakee, IL, U.S.A. Aminosuberate eel CT ([ASu¹⁻⁷]eCT) and synthetic human parathyroid hormone [PTH-(1–34) (hPTH)] were provided by Dr. J. Murase, Toyo Jozo Co., Shizuoka, Japan. Synthetic adrenocorticotropin [ACTH-(1–24)] was obtained from Ciba-Geigy. Purified human growth hormone (hGH) and human prolactin (hPRL) were gifts from Professor H. G. Burger, Prince Henry's Hospital, Melbourne, Australia. Human epidermal growth factor (hEGF) was a gift of Dr. E. Nice, Ludwig Cancer Institute, Melbourne, Australia. The sCT analogue [Arg^{11,18},Lys¹⁴]sCT was synthesized on an Applied Biosystems 430A Automatic Peptide Synthesizer, cleaved from the resin with anhydrous HF and purified by reversed-phase h.p.l.c. The [Arg^{11,18},Lys¹⁴]sCT chromatographed as a single peak on reversed-phase h.p.l.c. and had the expected amino acid composition following quantitative amino acid analysis. The derivative, [Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT was prepared from *N*-hydroxysuccinimidyl-4-azidobenzoate (Pierce Chemical Co., Rockford, IL, U.S.A.) and purified by reversed-phase h.p.l.c. Both [Arg^{11,18},Lys¹⁴]sCT and [Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT are equipotent with native sCT (C. S. D'Santos, unpublished work). Na¹²⁵I was obtained from the Radiochemical Centre, Amersham, U.K. Chaps and Extracti-Gel D were obtained from Pierce Chemical Co. All other chemicals were reagent grade.

Solubilization of placental membrane fraction

The membrane fraction was prepared from full-term human placentae as previously described [9]. Frozen

Abbreviations used: CT, calcitonin; PTH, parathyroid hormone; ACTH, adrenocorticotropin; GH, growth hormone; PRL, prolactin; EGF, epidermal growth factor; the prefixes h, s, p, e and b refer to human, salmon, pig, eel and bovine hormones, respectively; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

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membranes were thawed, resuspended (with 10 strokes of a loose-fitting Dounce homogenizer) in 10 mM-Tris/HCl, pH 7.8, containing Chaps and then stirred for 1 h at 4 °C. After centrifugation at 105 000 *g* for 1 h at 4 °C, the supernatant was collected for binding assay or stored frozen at -80 °C for later use. Protein concentrations were assayed by the method of Lowry *et al.* [10] or the Amido-Black method [11], using bovine serum albumin (Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex, U.K.) as the standard. In certain experiments, Extracti Gel-D was used, according to the manufacturers' recommendations, to remove detergent from the extract.

¹²⁵I-sCT binding to solubilized placental membranes

¹²⁵I-Labeling of the peptides was carried out using low-concentration chloramine-T oxidation and QUSO G32 purification as previously described [12,13]. Specific activities for all tracers were 150–200 μ Ci/ μ g. Several experiments were performed to determine the optimum conditions to separate receptor-bound ¹²⁵I-sCT from free ¹²⁵I-sCT. Precipitation of hormone-receptor complexes with polyethyleneglycol 6000 at a final concentration of 12 % (w/v) and bovine γ -globulin at a final concentration of 1 mg/ml produced satisfactory results. The routine binding assay was performed as follows: placental membrane extract was incubated overnight at 4 °C with 0.1 nM-¹²⁵I-sCT alone or together with unlabelled peptides in 50 mM-Tris/HCl, 1 mM-EDTA, bovine serum albumin (0.2 mg/ml) and 4 mM-Chaps. Non-specific binding was assessed in the presence of excess unlabelled sCT (300 nM). Bound and free tracer were separated by the addition of bovine γ -globulin and polyethyleneglycol 6000 in 100 mM-potassium phosphate buffer, pH 7.4,

Fig. 1. Effect of Chaps concentration on solubilization of placental CT receptors

Placental membranes (10 mg of protein/ml) were solubilized with increasing concentrations of Chaps as indicated and centrifuged at 105 000 *g* for 1 h. The extracts were incubated with 0.1 nM-¹²⁵I-sCT with and without excess unlabelled sCT for 18 h at 4 °C to determine specific binding (□). The addition of tracer and unlabelled sCT reduced the Chaps concentration of these incubations to 80 % of that used for solubilization. The percentage of the membrane protein solubilized at each concentration of Chaps is shown (Δ).

Fig. 2. Effect of Chaps concentration on ¹²⁵I-sCT binding to solubilized CT receptors

Placental membranes (10 mg of protein/ml) were solubilized in 5 mM-Chaps as described in the Materials and methods section. The Chaps was then removed from 20 ml of the extract by chromatography on a column of Extracti-Gel D (0.8 cm × 5 cm). Chaps powder was then added to aliquots of the extract to final concentrations as indicated and specific binding assessed by incubation with 0.1 nM-¹²⁵I-sCT in the absence and presence of excess unlabelled sCT.

followed by incubation on ice for 20 min and centrifugation in a Beckman microfuge. Bound radioactivity in the pellets was counted in a Packard Autogamma γ -counter with 70 % efficiency. The data are expressed as means of triplicate determinations. S.E.M. values are not shown, but in all cases were less than 5 % of the means.

Photoaffinity labelling of the CT receptor with ¹²⁵I-[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT

Photoaffinity labelling was carried out on both particulate placental membranes and on the extract of membranes solubilized in Chaps. All procedures, up to the irradiation step, were carried out under safe-light conditions. Placental membranes were incubated overnight at 4 °C with 0.2 nM-¹²⁵I-[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT alone or together with unlabelled sCT in 100 mM-potassium phosphate buffer, 1 mM-EDTA and bovine serum albumin (1 mg/ml), pH 7.0. The membranes were centrifuged in a Beckman microfuge, washed and resuspended in the phosphate buffer, placed in a Petri dish on ice and irradiated for 15 min with a 100 W long-wave (peak emission of 366 nm) u.v. lamp at a distance of 10 cm. They were recentrifuged and the pellets solubilized in 50 mM-Tris/HCl, 1 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) mercaptoethanol, pH 6.8 (sample buffer). Extracts were heated at 95 °C for 3 min, centrifuged at 105 000 *g* for 1 h and then analysed by SDS/PAGE by the method of Laemmli [14].

Solubilized placental membrane extracts were treated in a similar way. After irradiation, 80 μ l aliquots of the extract (containing ~ 250 μ g of protein) were added to 80 μ l of double-strength sample buffer, boiled and subjected to SDS/PAGE.

Fig. 3. Effect of pH on ^{125}I -sCT binding to solubilized CT receptors

Placental membranes (10 mg of protein/ml) were solubilized in 5 mM-Chaps as described in the Materials and methods section. The indicated pH values were obtained by the addition of Tris/HCl (pH 6.0–9.0) ($\square$, $\blacksquare$) or Tris/maleate (pH 5.5–7.0) ($\diamond$, $\blacklozenge$) to final concentrations of 50 mM. Binding was assessed by incubation with 0.1 nM- ^{125}I -sCT in the absence (closed symbols) and presence (open symbols) of excess unlabelled sCT.

Fig. 4. Time course of ^{125}I -sCT binding to solubilized CT receptor

Three 5 ml aliquots of the placental membrane extract were incubated with 0.1 nM- ^{125}I -sCT in the absence ($\blacksquare$) or presence ($\square$) of excess (300 nM) unlabelled sCT at 4 °C. At the indicated times, 100 μl aliquots were assayed for binding as described in the Materials and methods section. After 1 h, as indicated by the arrow, 300 nM-unlabelled sCT was added to one of the incubations previously incubated with tracer alone ($\triangle$) to assess the reversibility of binding.

RESULTS

Detergent solubilization

The effect of increasing concentrations of Chaps on the solubilization of functional CT receptors from placental membranes is shown in Fig. 1. Solubilization with Chaps at concentrations of 4–6 mM resulted in the highest specific binding of ^{125}I -sCT. The percentage of protein solubilized increased from 34% with 2 mM-Chaps to 44% with 10 mM-Chaps and thereafter did not increase. We investigated the possibility that the detergent might interfere with binding by removing Chaps from the extract with Extracti-Gel D, and then replacing it at varying final concentrations by the addition of Chaps powder before performing the ^{125}I -sCT binding assay. As shown in Fig. 2, ^{125}I -sCT binding was optimum at Chaps concentrations of 2–3 mM, but remained at 80% of maximum at 5 mM-Chaps, which was the concentration used for solubilization. Therefore, we routinely employed 5 mM-Chaps for both the solubilization and binding steps.

Specific binding was not dependent on divalent cations, but was actually slightly reduced in the presence of 10 mM- Ca^{2+} or Mg^{2+} (84% and 86%, respectively). EDTA (1 mM and 5 mM) had no significant effect. Nevertheless, 1 mM-EDTA was included as a precaution to inhibit Ca^{2+} -dependent protease activity. As shown in Fig. 3, specific binding was maximal at pH 6.5 in either 50 mM-Tris/HCl or 50 mM-Tris/maleate buffer, although there was little variation in the range pH 6–7.

Under the conditions employed, non-specific binding was consistently less than 20% of total binding and the separation method was rapid, simple and reproducible. The specific-binding capacity of the solubilized extracts

was not significantly affected by storage at 4 °C for 7 days or at –80 °C for 3 months.

Time course of binding and dissociation

^{125}I -sCT binding was maximal by 6 h at 4 °C and thereafter remained constant to 18 h (Fig. 4). Addition of excess unlabelled sCT at 1 h prevented further binding, but did not produce dissociation even after 18 h (Fig. 4). Maximum binding was achieved more rapidly (by 90 min) at 22 °C, but was only 75% that achieved at 4 °C (results not shown).

Saturation analysis

When solubilized extracts of placental membranes were incubated with increasing concentrations of ^{125}I -sCT, the binding was saturable (Fig. 5a) and Scatchard analysis [15] of the specific binding data (Fig. 5b) was linear, suggesting a single class of binding sites. Results from three separate experiments produced a mean ($\pm$ S.E.M.) K_d of 165 ± 28 pM and a mean B_{max} of 75 ± 6 fmol/mg of protein.

Specificity

Competitive-binding studies (Fig. 6) showed that binding to the solubilized CT receptor retained the same high degree of specificity for CT as seen in the particulate membranes [7]. The relative potencies of species-specific CTs and analogues as observed in the hypocalcaemic bioassays [16–18] and binding assays [13,19,20] was preserved. The unrelated peptide hormones bPTH-(1–34), EGF, hPRL, hGH and ACTH-(1–24) did not compete at high concentrations.

Fig. 5. Saturation analysis of ^{125}I -sCT binding to solubilized placental membranes

(a) Aliquots of Chaps-solubilized placental membrane extract (final protein concentration 2.5 mg/ml) were incubated with increasing concentrations of ^{125}I -sCT with and without excess unlabelled sCT for 18 h at 4 °C. Points represent specific binding. (b) Scatchard analysis of the specific binding data from (a). K_d 200 pM; B_{max} 82 fmol/mg of protein.

Photoaffinity labelling

Analysis by SDS/PAGE of particulate placental membranes photolabelled with ^{125}I -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT resulted in labelled bands at $M_r \sim 88000$ and ~ 71000 (Fig. 7a). These bands were not seen in the absence of photolysis nor in the presence of excess (300 nM) unlabelled sCT and they were reduced in the presence of 0.1 or 0.3 nM-sCT. The results were essentially the same with solubilized extract (Fig. 7b). However, rather than single bands at ~ 88000 and ~ 71000 , there were duplex bands at $\sim 86\text{--}92000$ and $\sim 71\text{--}75000$. Also, a non-specific (present in the presence of excess unlabelled sCT) band at ~ 38000 was evident. The experiments were repeated three times with identical results.

Specifically labelled material seen at the top of the

Fig. 6. Specificity of ^{125}I -sCT binding to solubilized CT receptor

Aliquots of placental membrane extract were incubated with 0.1 nM- ^{125}I -sCT alone or together with increasing concentrations of unlabelled sCT (■), [ASu¹⁻⁷]eCT (□), des-Leu¹⁶-sCT (◇), pCT (◆), hCT (▲), [Gly⁸]hCT (△), PRL (+), EGF (○), bPTH-(1-34) (●), hCGRP (×), or hGH (◐) for 18 h at 4 °C.

11% (w/v) gels (Fig. 7) was not resolved using gradient gels of 7.5–15% (w/v) acrylamide. Furthermore, bands of higher M_r were not seen when SDS/PAGE was performed under non-reducing conditions (results not shown).

DISCUSSION

The CT receptor has been difficult to obtain in significant amounts in solubilized form for several reasons. Although it is expressed in several cancer cell lines [12,13,19–21], it is a minor component of the cell protein in these cases. It is most abundant in osteoclasts [4], but unfortunately, it is not practical to prepare these cells in sufficient numbers to undertake large-scale work. Finally, previous attempts to solubilize CT receptors have resulted in alterations in their kinetic properties, indicating that significant physical changes have occurred in the receptor molecule [5,6, J. M. Moseley, unpublished work]. In no case have studies been carried out with several CT analogues to demonstrate preservation of the solubilized receptors' ability to recognize functional changes in CT structure. These problems have been overcome in the present work by using the zwitterionic detergent Chaps, which was specifically designed to combine the useful properties of the sulphobetaine-type and the bile salt anionic detergents [8].

Solubilization of the placental CT receptor using 5 mM-Chaps resulted in complete preservation of receptor binding characteristics. The time course of binding was similar to that observed in particulate membranes [7]. Furthermore, as has been noted in various membrane preparations [21,22], rat osteoclasts [4] and human cancer cells [13,19,20], as well as particulate placental membranes [7], the binding was essentially irreversible. Accordingly, calculations of kinetic data using Scatchard analysis are approximations, as we have frequently

Fig. 7. Photoaffinity labelling of the placental CT receptor

(a) Placental membranes were incubated with 0.2 nM- ^{125}I -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT, washed, irradiated, solubilized and analysed on SDS/PAGE as described in the Materials and methods section. Lane 1: no photolysis. Lanes 3–5: 300, 0.1 and 0.3 nM-unlabelled sCT, respectively. (b) Solubilized placental membrane extract was incubated with 0.2 nM- ^{125}I -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT, irradiated and analysed on SDS/PAGE. Lane 1: no photolysis. Lanes 3–6: 300, 0.1, 0.3 and 1 nM-unlabelled sCT, respectively. (a) and (b) Labelled bands are shown on the left. The band at M_r 38000 is non-specific. Protein standards ($M_r \times 10^{-3}$) are shown on the right with arrowheads. Standards used were (M_r): myosin, 200000; galactosidase, 116000; phosphorylase *b*, 97400; bovine serum albumin, 66200; ovalbumin, 42700 and carbonic anhydrase, 31000. The distal end of the gel, containing free tracer was excised before autoradiography.

stressed [4,7,20]. However, comparisons can be made with results obtained from CT binding to receptors from other sources. The affinity of the solubilized receptor for sCT is only slightly lower than that observed in

particulate membranes (K_d 168 ± 28 pM for solubilized compared with 88 ± 15 pM for particulate receptor). The competitive-binding studies demonstrate that the rank order of binding potencies of other CTs to the solubilized receptor is maintained and that binding is highly specific for CT, with other non-related peptides not binding.

We have previously reported that the $B_{\max}$ for ^{125}I -sCT binding to particulate placental membranes was 58 ± 11 fmol/mg of protein [7]. In this study we have shown that after extraction with 5 mM-Chaps, the $B_{\max}$ is 75 ± 6 fmol/mg of protein with solubilization of approximately one-third of the membrane protein. Thus, as well as resulting in retention of desirable affinity and specificity characteristics, solubilization with Chaps is efficient, resulting in extraction of approximately 50% of the available binding sites.

A structural analogue of sCT, [Arg^{11,18},Lys¹⁴]sCT, which retains full biological activity after derivatization at the Lys¹⁴ position, has been used to produce the photoactive, derivatized analogue [Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT. With this agent we have been able to photoaffinity label specific receptor components of $M_r \sim 88000$ and ~ 71000 in particulate placental membranes and in the solubilized extract. In the extract, each of these components consisted of duplex bands, either reflecting greater resolution or a detergent effect. Recently, we have used this sCT analogue derivatized with another photoactive cross-linking agent, 4-fluoro-3-nitrophenylazide, to demonstrate labelling of a single receptor component of $M_r \sim 85000$ in placental membranes [7]. This was similar to our earlier results from chemical cross-linking and photoaffinity-labelling experiments using ^{125}I -labelled native sCT, which identified single CT receptor components of $M_r \sim 80000$ – 90000 on a human lung cancer cell line (BEN), human breast cancer cell lines (T47D and MCF 7) [23,24] and rat osteoclasts [4]. Thus, the labelling of the $M_r \sim 71000$ component is a new finding which could be a feature of the use of ^{125}I -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT as the photoaffinity agent in the placental CT receptor studies.

The results presented here show that Chaps can be used to solubilize CT receptors from placental membranes in a non-denatured form which retains all the binding characteristics of the membrane-bound receptor. This, together with identification of the abundant source of CT receptors in the placenta, provides new opportunities for purification of the CT receptor.

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