Reversible calcitonin binding to solubilized sheep brain binding sites

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In this study we have solubilized and characterized binding sites for calcitonin (CT) from sheep brainstem. Autoradiography of ^{125}I -labelled salmon CT (^{125}I -sCT) binding to sheep diencephalon revealed a similar pattern of binding to that seen in other species, although the extent of distribution was greater in the sheep. CT binding activity could be extracted from membranes with either CHAPS or digitonin, but not with β -octyl glucoside. ^{125}I -sCT binding was saturable, with a dissociation constant for CHAPS-solubilized membranes of $2.8 \pm 0.5 \text{ nM}$ and a maximum binding site concentration of $6.2 \pm 1.6 \text{ pmol/mg}$ of protein. In competition binding studies, various CTs and their analogues demonstrated a similar rank order of potency to that seen in other CT receptor systems. Optimal binding occurred in the pH range 6.5–7.5, and was decreased in the presence of NaCl concentrations greater than 200 mM. In contrast with most other CT receptor binding systems, in which binding is poorly reversible, the binding of ^{125}I -sCT to sheep brain binding sites underwent substantial dissociation upon addition of excess unlabelled sCT, with 40% and 46% dissociation after 2 h at 4 °C in particulate and solubilized membranes respectively. Photoaffinity labelling of the binding site with the biologically active analogue ^{125}I -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT and analysis on SDS/PAGE under reducing conditions revealed a specific protein band of $M_r \sim 72000$ in both solubilized and particulate brain membranes. This is in accordance with the molecular size of CT receptors in other tissues where two species of receptor have been identified, one of $M_r \sim 71000$ and another of $M_r \sim 88000$. These results demonstrate the presence of high concentrations of CT binding sites in sheep brain which display different kinetic properties to those of CT receptors found in other tissues.

INTRODUCTION

Calcitonin (CT), a 32-amino-acid peptide hormone, is an important calciotropic hormone (Martin *et al.*, 1988). Accordingly, high densities of CT receptors are present in bone cells (Nicholson *et al.*, 1986) but are also present in kidney (Sexton *et al.*, 1987) and brain (Fischer *et al.*, 1981a,b; Henke *et al.*, 1983; Guidobono *et al.*, 1987). Although the physiological role of CT in the brain is unknown, central injection of salmon CT (sCT) induces potent effects, including analgesia (Pecile *et al.*, 1975; Fabbri *et al.*, 1985; Morton *et al.*, 1986), decreased appetite (Twery *et al.*, 1982; Freed & de Beaupaire, 1984; Tannenbaum & Goltzman, 1985) and gastric acid secretion (Morley *et al.*, 1981; Maeda-Hagiwara & Watanabe, 1985; Lenz *et al.*, 1986), as well as modulation of secretion of prolactin (Chihara *et al.*, 1982; Clementi *et al.*, 1983), growth hormone (Tannenbaum & Goltzman, 1985) and adrenocorticotropin (Rapisarda *et al.*, 1984). In addition, central administration of CT may also affect the extrapyramidal motor system (Nicolletti *et al.*, 1982; Twery *et al.*, 1986) as well as directly altering membrane excitability of individual neurons (Gerber *et al.*, 1985; Twery & Moss, 1985; Shimizu & Oomura, 1986). These actions correlate well with the anatomical location of CT receptors in the central nervous system (Olgiati *et al.*, 1983; Henke *et al.*, 1983; Fabbri *et al.*, 1985; Guidobono *et al.*, 1986, 1987).

CT-receptor interactions have been studied in a number of tissues and cancer cell model systems. In most systems studied to date, the interaction of CT with its receptor results in virtually irreversible binding (Findlay *et al.*, 1980; Lamp *et al.*, 1981;

Fischer *et al.*, 1981b; Chao & Forte, 1983; Nicholson *et al.*, 1986, 1988). This kinetic behaviour is believed to be related to the persistent activation of adenylate cyclase in the CT-responsive cell line T47D (Lamp *et al.*, 1981) and the prolonged action of CT in its major physiological target, the osteoclast (Nicholson *et al.*, 1987).

More recently, functional CT receptors have been solubilized from human placental membranes using the zwitterionic detergent CHAPS (Nicholson *et al.*, 1988). The present paper describes the identification and solubilization of a CT binding site from sheep brain which displays reversible CT binding kinetics while retaining the ability to discriminate among CT analogues which would be expected for a CT receptor.

A preliminary report of this work was presented at the 10th International Conference on Calcium Regulating Hormones.

MATERIALS AND METHODS

Materials

Synthetic human CT (hCT) was provided by Dr. W. Rittel, Ciba-Geigy, Basel, Switzerland. Synthetic sCT, pig CT (pCT), des-Leu¹⁶-sCT and [Gly⁸]hCT and bovine parathyroid hormone-(1–34) were provided by Dr. R. C. Orlowski, Armour Pharmaceutical Co., Kanakee, IL, U.S.A. Epidermal growth factor was a gift from Dr. Ed Nice, Ludwig Institute, Melbourne, Australia. Synthetic human parathyroid hormone-(1–34) was obtained from Auspep, Parkville, Victoria, Australia. Rat α -calcitonin-gene-related peptide (CGRP) and atrial natriuretic peptide were from Peninsula, Belmont, CA, U.S.A. ATP-agarose (linked through

Abbreviations used: CT, calcitonin (the prefixes s, h and p refer to salmon, human and pig respectively); CGRP, calcitonin-gene-related peptide; PMSF, phenylmethanesulphonyl fluoride; PEG, poly(ethylene glycol).

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C-8) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Digitonin was from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. CHAPS and β -octyl glucoside were from Pierce Chemical Co., Rockford, IL, U.S.A. The sCT analogue [Arg^{11,18},Lys¹⁴]sCT and its photoactive derivative [Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT were synthesized and purified as described previously and both peptides were equipotent with native sCT in biological assay (D'Santos *et al.*, 1988). ¹²⁵I-labelled sCT was prepared as described (Findlay *et al.*, 1980). Specific radioactivities ranged from 150 to 200 μ Ci/ μ g.

Tissue preparation

Sheep brains were collected fresh from the abattoir and transported on ice before dissection (~30 min). The hypothalamus, midbrain and medulla oblongata were dissected free of surrounding tissue and either snap-frozen (for autoradiography) in isopentane at -40 °C and stored at -70 °C until use, or used to prepare membranes.

Membrane preparation

Dissected brainstem tissue was blended for 30 s in 4–5 vol. of lysis buffer [25 mM-Tris/HCl (pH 7.3)/1 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride (PMSF)] then homogenized in an Ultra-Turrax (13000 rev./min) for a further 2 min. The homogenate was centrifuged for 15 min at 1500 *g* and the supernatant was retained. The pellet was rehomogenized in an equal volume of lysis buffer and recentrifuged at 1500 *g* and the supernatant was again retained. The low-speed supernatants were then centrifuged for 20 min at 40000 *g* and the pellets were retained. The pellets were resuspended in lysis buffer with 3 strokes of a motorized glass-Teflon homogenizer then centrifuged again for 20 min at 40000 *g*. Pellets were resuspended in lysis buffer (2–3 mg/ml) and stored at -70 °C until use. All procedures were carried out at 4 °C. Proteins were assayed by the Amido Black method (Schaffner & Weissman, 1973).

Particulate membrane binding

Membranes were thawed and centrifuged at 48000 *g* for 20 min at 4 °C and rehomogenized in 50 mM-Tris/HCl buffer, pH 7.3, containing 1 mM-PMSF, 1 mM-EDTA, 1 mg of bacitracin/ml and 0.2% BSA (buffer A). Portions of membrane (~0.1 mg of protein/ml) were incubated as specified with ¹²⁵I-sCT (0.1 nM) in the presence or absence of unlabelled sCT. Reactions were terminated by centrifugation in a Beckman Microfuge for 3 min at 4 °C. The pellets were overlaid with 1 ml of ice-cold phosphate-buffered saline (100 mM-phosphate) and Microfuged again for 90 s. The pellets were counted for radioactivity in a Packard γ -radiation counter.

Autoradiography

Coronal sections (10–20 μ m) of hypothalamus were cut and processed for autoradiography as previously described (Sexton *et al.*, 1986). Sections were preincubated for 15 min in 50 mM-Tris/HCl buffer, pH 7.3, containing 100 mM-NaCl, 0.2% BSA and 0.4 mM-bacitracin at room temperature, and then incubated for 90 min in 5 ml of the same buffer containing ¹²⁵I-sCT (~82 pM) in the presence or absence of 0.1 μ M unlabelled sCT. The slide-mounted sections were then rapidly washed four times with 1 min transfers through 50 mM-Tris/HCl, pH 7.3, containing 100 mM-NaCl at 0 °C, and dried in a stream of cold air. The dried sections were exposed to Agfascopix CR3B (Agfa, Gevaert, Belgium) X-ray film for 7–21 days in X-ray cassettes.

Membrane solubilization

Membranes were thawed, centrifuged at 4 °C for 20 min at 40000 *g* and resuspended with two strokes of a glass-Teflon homogenizer in an equivalent volume of solubilization buffer (50 mM-Tris/HCl, pH 7.3, containing 1 mM-EDTA, 1 mM-PMSF, 10 mM-benzamidine, 10 μ g of pepstatin/ml, 10 μ g of antipain/ml, 20 μ g of leupeptin/ml and 1 mg of bacitracin/ml) and solubilized using 5 mM-CHAPS unless otherwise specified, for 1 h at 4 °C. After centrifugation at 100000 *g* for 1 h at 4 °C, supernatants were retained for use in binding characterization studies.

Binding of ¹²⁵I-sCT to solubilized membranes (~0.03 mg of protein) was performed, and separation of bound and free CT was achieved by poly(ethylene glycol 6000) (PEG) precipitation as previously described (Nicholson *et al.*, 1988). Briefly, ¹²⁵I-sCT (0.1 nM unless otherwise specified) was incubated in solubilization buffer containing 0.2% BSA. After incubation, bound and free tracer were separated by the addition of bovine γ -globulin (1 mg/ml) and PEG (10%, w/v, final concentration) in 100 mM-potassium phosphate buffer, pH 7.3, followed by incubation on ice for either 10 min or 2 min (kinetic experiments) and centrifugation in a Beckman Microfuge for 5 min. No change in precipitated radioactivity was observed between the two incubation times. Pellets were counted for radioactivity in a Packard γ -radiation counter with 70% efficiency. Data are expressed as means of triplicate determinations. Binding data were analysed by the iterative curve-fitting program (LIGAND) of Munson & Rodbard (1980). Values shown are means $\pm$ S.E.M.; S.E.M. values for binding studies are not shown but were less than 5% of the means in all experiments.

Cross-linking studies

Photoaffinity labelling was carried out on both particulate and CHAPS-solubilized membranes. All procedures before irradiation were implemented under safelight conditions. After centrifugation and rehomogenization in buffer A, sheep brain membranes (~2 mg of protein) were incubated for 18 h at 4 °C with 0.3 nM-¹²⁵I-[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT in the presence or absence of unlabelled sCT. After incubation, the membranes were centrifuged in a Beckman Microfuge, washed and resuspended in 100 mM-phosphate buffer, pH 7.0, placed in a Petri dish on ice and irradiated for 15 min with a 100 W long-wave (peak emission 366 nm) u.v. lamp at a distance of 10 cm. They were then recentrifuged and the pellets were solubilized in 50 mM-Tris/HCl/1% (w/v) SDS/10% (v/v) glycerol/5% (v/v) β -mercaptoethanol, pH 6.8 (sample buffer). Extracts were boiled for 3 min, centrifuged at 100000 *g* for 1 h and then analysed by SDS/PAGE by the method of Laemmli (1970). Solubilized brain extracts were treated in a similar manner. After incubation and irradiation, 80 μ l portions (~250 μ g of protein) were added to 80 μ l of 2 $\times$ concentrated sample buffer, boiled and analysed by SDS/PAGE. The resulting gels were subjected to autoradiography with Kodak X-AR 5 X-ray film in X-ray cassettes for ~7 days.

ATP chromatography

Solubilized brain extract (~40 mg total protein) was supplemented with 3 mM-MgCl₂ and applied to a 1 ml ATP-agarose affinity column. Binding of ¹²⁵I-sCT to solubilized material was assessed before chromatography and in column flow-through fractions. The column was then washed with 50 ml of buffer B [50 mM-Tris/HCl, pH 7.4, 5 mM-CHAPS, 10% (v/v) glycerol, 1 mM-EDTA and 3 mM-MgCl₂] containing 0.5 M-NaCl, followed by 30 ml of buffer B. The column was developed with buffer B containing 4 mM-ATP.

RESULTS

Autoradiography

Analysis of ^{125}I -sCT binding to sections through sheep brain diencephalon revealed discrete patterns of binding. Moderate- to high-density binding occurred in most of the hypothalamus, with highest densities in the paraventricular and arcuate nuclei and median eminence (Figs. 1a, 1c and 1d). High-density binding was also observed in parts of the hippocampus and amygdala (Figs. 1c and 1d). Moderate-density binding was present in discrete nuclei of the thalamus, including paraventricular thalamic nucleus and part of the lateral ventroposterior thalamic nucleus (Figs. 1a, 1c and 1d). Moderate-to-low levels of binding were observed in other thalamic nuclei, and in the ventrolateral putamen, medial septum (Fig. 1a) and cortex (Figs. 1c and 1d). Binding was absent if sections were incubated in the presence of 300 nM unlabelled sCT (Fig. 1b).

Detergent solubilization

Functional CT binding sites were solubilized with either the zwitterionic detergent CHAPS or the non-ionic detergent digitonin, but not with another non-ionic detergent, β -octyl glucoside (Fig. 2). Maximal binding activity was extracted with 6–8 mM-CHAPS and 0.4% digitonin respectively. As CHAPS and digitonin were similarly effective in solubilizing binding sites, CHAPS was chosen for subsequent experiments due to greater ease of handling. A concentration of 5 mM was used, since more than 75% of maximal specific binding was solubilized at this concentration with only 7% of total protein, compared with 17–20% of total protein at the higher CHAPS concentrations.

Binding specificity

Fig. 3 illustrates the specificity of binding of ^{125}I -sCT to CHAPS-solubilized sheep brain membranes, with competition by unlabelled CTs and CT analogues. The unrelated peptides parathyroid hormone ($2\text{ }\mu\text{M}$), epidermal growth factor ($10\text{ }\mu\text{M}$), atrial natriuretic peptide ($3\text{ }\mu\text{M}$) and α -rat CGRP ($3\text{ }\mu\text{M}$) did not compete for binding (results not shown). Analysis of sCT competition binding isotherms revealed an average K_d of $2.8 \pm 0.5\text{ nM}$, with a maximal binding site concentration (B_{max}) of $6.2 \pm 1.6\text{ pmol/mg}$ of protein. K_d and B_{max} values for the particulate membranes were $0.98 \pm 0.24\text{ nM}$ and $0.9 \pm 0.2\text{ pmol/mg}$ of protein respectively. Binding was optimal in the pH range 6.5–7.5 (Fig. 4), and NaCl decreased binding at concentrations greater than 200 mM (results not shown).

Solubilization of binding sites in the presence of 10% glycerol increased recovered binding by 40–60%. This was in part due to an increased efficiency in the extraction of total protein, but there was also an increase in specific activity of binding sites of 20–25%. In a representative experiment, analysis of binding isotherms demonstrated that the addition of 10% glycerol to the solubilization buffer increased recovery of extractable binding sites from 7.1 ± 1.0 (– glycerol) to 9.3 ± 1.4 (+ glycerol) pmol/mg of protein, with a slight increase in affinity. K_d values in the absence and presence of glycerol were $3.9 \pm 0.8\text{ nM}$ and $2.6 \pm 0.6\text{ nM}$ respectively. Accordingly, subsequent experiments were performed using membranes solubilized in the presence of glycerol.

Time course of binding

Association of ^{125}I -sCT to both particulate and solubilized membranes was time- and temperature-dependent, with $\sim 80\%$

Fig. 1. Autoradiography of ^{125}I -sCT binding to sheep brain diencephalon

Coronal sections of sheep brain diencephalon were incubated with ^{125}I -sCT (0.1 nM) in the absence (a, c, d) or presence (b) of 300 nM-sCT. Panel (b), depicting non-specific binding, corresponds to the level of the section in panel (a). Abbreviations are: Amg, amygdala; Arc, arcuate nucleus; C, caudate nucleus; CM, central medial thalamic nucleus; Cx, cortex; DH, dorsal hypothalamus; DM, dorsomedial hypothalamus; Hi, hippocampus; ME, median eminence; MS, medial septum; Pa, paraventricular nucleus, hypothalamus; PVA, paraventricular thalamic nucleus; Pu, putamen; VPL, lateral ventroposterior thalamic nucleus; ZI, zona incerta.

Fig. 2. Effect of detergent concentration on solubilization of sheep brain CT binding sites

Sheep brain membranes (~ 3 mg/ml) were solubilized as described with increasing concentrations of (a) digitonin (■, total binding; □, non-specific binding), (b) β -octyl glucoside (■, total binding; □, non-specific binding), or (c) CHAPS (●, specific binding; ○, protein level), in solubilization buffer. The extracts were incubated for 18 h at 4°C with 2 nM- (a, b) or 0.1 nM- (c) ${}^{125}\text{I}$ -sCT with or without 300 nM unlabelled sCT to determine specific binding. The addition of tracer and unlabelled hormone decreased the detergent concentration during the assay to 80% of that used for solubilization.

of maximal binding occurring by 4 h at 4°C (Fig. 5). Association at 22°C was more rapid, reaching equilibrium by 60 min (results not shown). The presence or absence of glycerol in the solubilization buffer had no apparent effect on the rates of association and dissociation of binding.

In most other CT receptor systems studied, ligand binding is resistant to displacement by addition of excess unlabelled hormone. In contrast, addition of 300 nM unlabelled sCT to particulate or solubilized sheep brain membranes displaced 40% and 46% respectively of prebound ${}^{125}\text{I}$ -sCT after a 2 h incubation at 4°C (Fig. 5), with 70% displaced after 24 h (results not shown). Similar results were seen with solubilized sites prebound at 22°C . That this was specific for CT was shown by the failure

Fig. 3. Specificity of ${}^{125}\text{I}$ -sCT binding to CHAPS-solubilized sheep brain membranes

Solubilized extracts were incubated with 0.1 nM- ${}^{125}\text{I}$ -sCT in the presence of the unlabelled peptides at the concentrations indicated. Peptides used were: sCT (●), pCT (○), des-Leu¹⁶-sCT (■), hCT (□) and [Gly⁸]hCT (▲).

Fig. 4. Effect of pH on binding of ${}^{125}\text{I}$ -sCT to solubilized membranes

Membranes were solubilized as described in either Mes (○, ●) or Tris/HCl (□, ■) buffer, and the pH was adjusted as indicated. Samples were incubated with 0.1 nM- ${}^{125}\text{I}$ -sCT in the presence (○, □) or absence (●, ■) of 300 nM-sCT. Maintenance of pH was confirmed at the conclusion of the incubation.

of human parathyroid hormone-(1-34) ($1 \mu\text{M}$) to displace binding, whereas α -rat CGRP ($1 \mu\text{M}$) induced only minor displacement of binding after 2 h at 4°C and had no effect at 22°C (results not shown).

Photoactive labelling

Photoaffinity labelling of the receptor with the biologically active sCT analogue ${}^{125}\text{I}$ -[Arg^{11,18},4-azidobenzoyl-Lys¹⁴]sCT, and analysis on SDS/PAGE and autoradiography, revealed a specific receptor band of $M_r \sim 72000$ in both solubilized and particulate brain membranes (Fig. 6). These bands were not detected in the absence of photolysis or in the presence of excess unlabelled sCT (800 nM). A number of non-specific proteins of lower M_r were also observed.

ATP-agarose chromatography

Recently, Dana *et al.* (1990) indicated that members of the 70000- M_r family of heat-shock proteins can bind sCT with nanomolar affinity, and suggested that some binding sites studied in tissues such as placenta may be heat-shock proteins. These

Fig. 5. Time course of association of 4 °C of ^{125}I -sCT binding to particulate (a) and solubilized (b) brain membranes

Particulate membranes or membranes solubilized in the presence of glycerol (10%) were incubated at 4 °C with 0.1 nM ^{125}I -sCT in the presence (○) or absence (●) of 300 nM-sCT. At the times indicated, samples were removed and assayed for bound ligand as described. Reversibility of binding was assessed by the addition of 300 nM-sCT (■) to the incubation at the time indicated by the arrow.

Fig. 6. Photoaffinity labelling of CT binding sites from sheep brain

Particulate (a) and solubilized (b) membranes were cross-linked with 0.2 nM of the photoactive derivative ^{125}I -[Arg 11,18 ,4-azidobenzoyl-Lys 14]sCT as described. Membranes were irradiated with u.v. light in the absence (0) or presence of 300 nM-sCT (sCT) or not irradiated (-u.v.). Samples were run on a 7.5–15% polyacrylamide gel in the presence of SDS. Protein standards ($M_r \times 10^{-3}$) are shown on the left. Standards used were (M_r): phosphorylase b, 97000; BSA, 66200; ovalbumin, 42700; carbonic anhydrase, 30000.

proteins, however, are unlikely to correspond to the binding site identified in the current study, as the 70000- M_r heat-shock proteins avidly bind ATP and are retained on ATP-agarose (Welch & Feramisco, 1985; Dana *et al.*, 1990), whereas binding of ^{125}I -sCT to the solubilized sheep brain CT binding site was not altered by passage through ATP-agarose (results not shown).

DISCUSSION

Autoradiography of ^{125}I -sCT binding in the sheep diencephalon illustrated high binding densities in parts of the hypothalamus, the amygdala and the hippocampus. Comparison of the patterns of binding across species demonstrates that high-density binding to regions such as the hypothalamus, amygdala and caudoventral putamen is conserved in sheep, rat (Olgati *et al.*, 1983; Henke *et al.*, 1983), cat (Guidobono *et al.*, 1987) and human (Sagar *et al.*, 1984; P. M. Sexton, unpublished work). However, the distribution of binding in cat and sheep is wider than that in rat and human, with binding present in the hippocampus and much of the thalamus, while the sheep appears to have an even broader distribution than does the cat, with binding also present in the cortex. Thus central CT receptors are likely to subserve a subset of similar functions in all species, but may also have additional actions in species such as sheep and cat.

Earlier attempts to solubilize CT receptors using the non-ionic detergents Lubrol PX (Queener *et al.*, 1975) or β -octyl glucoside and Triton X-100 (J. M. Moseley, unpublished work) resulted in alterations in binding properties from those seen in either cells or membranes. Similarly, β -octyl glucoside was ineffective in solubilizing CT binding sites in the current study. This may result from the inability of the long alkyl chains of β -octyl glucoside and the polyoxyethylene derivatives, which associate more readily with hydrophobic regions of the proteins, to disrupt the ionic interactions between the hydrophilic residues of the proteins (Helenius *et al.*, 1979). In this study, sheep brain CT binding sites were successfully solubilized with the zwitterionic and non-ionic steroid derivatives CHAPS and digitonin respectively. We have previously reported solubilization of functional CT receptors from human placental membranes using CHAPS (Nicholson *et al.*, 1988). The differences in CHAPS concentration required for optimal solubilization of receptor in the brain (6–8 mM) and placenta (4–6 mM) probably reflect differences in the protein and lipid content of the two tissues. The success of both CHAPS and digitonin in solubilizing CT receptors may be related to the ability of these two steroid derivatives to substitute for cholesterol, a lipid which may stabilize the structures of certain membrane proteins (Rooney *et al.*, 1984).

Association of ^{125}I -sCT to both particulate and CHAPS-solubilized CT binding sites was time- and temperature-dependent, with optimal binding in the physiological ranges of pH and NaCl concentration. Furthermore, competition binding studies demonstrate that the relative binding potencies of various CTs in sheep brain is similar to that seen in other CT receptor systems (Findlay *et al.*, 1980, 1983, 1985; Fischer *et al.*, 1981a,b; Nicholson *et al.*, 1986, 1988; Sexton *et al.*, 1987). However, despite this similarity to other CT receptors, the sheep brain CT hormone-binding site complex was readily dissociable upon addition of excess unlabelled sCT. This is in contrast with CT receptors studied in osteoclasts (Nicholson *et al.*, 1986) and in various CT-receptor-bearing cell lines (Findlay *et al.*, 1980; Lamp *et al.*, 1981), where binding is virtually irreversible, or in kidney, where binding is slowly or incompletely reversible (Goltzman, 1980; Chao & Forte, 1983). Furthermore, in rat (Fischer *et al.*, 1981a; P. M. Sexton, unpublished work) and human (Fischer *et al.*, 1981b) particulate brain membranes, binding is also poorly reversible, suggesting that the observations

in sheep brain reflect a species variation in the behaviour of the receptor rather than tissue differences. High concentrations of CGRP induced a minor displacement of radiolabelled CT in solubilized membranes, consistent with the low cross-reactivity of CGRP with CT binding sites in rat brain (Goltzman & Mitchell, 1985; Henke *et al.*, 1985), whereas the unrelated parathyroid hormone had no effect, illustrating the specificity of the effect for CT.

According to autoradiographic studies, both osteoclasts (D'Santos *et al.*, 1988) and placenta (Nicholson *et al.*, 1988) contain two forms of the receptor of $M_r \sim 88000$ and $M_r \sim 71000$, whereas in the cell lines UMR 106-06 (D'Santos *et al.*, 1988), BEN and T47D (Moseley *et al.*, 1986) only the higher- M_r form has been resolved. In the current study, a single protein of $M_r \sim 72000$ was identified, corresponding to the lower- M_r form of the receptor seen in osteoclasts and placenta. No evidence for a higher- M_r form of the protein was seen in sheep brain. Studies on the granulocyte/macrophage-colony-stimulating factor and interleukin 2 receptors demonstrated two M_r forms of these receptors which correspond to high- and low-affinity binding states (Gearing *et al.*, 1989; Hatakeyama *et al.*, 1989). These studies revealed that low- M_r /low-affinity forms require the association of another membrane protein(s) to acquire high affinity for the hormone. Indeed, only the high-affinity receptor is associated with ligand internalization and signal transduction. Evidence from renal cell binding studies, in which reversibility was observed at 4 °C but not at 20 °C, suggests that the occurrence of high-affinity binding may be an energy-dependent process (Chao & Forte, 1983). Given the apparently lower affinity of the sheep brain binding site (as indicated by its more rapid dissociation), it is possible that low-affinity binding is a function of a lower- M_r form of the receptor, with high affinity conferred in the higher- M_r form of the receptor after coupling of a secondary protein, e.g. a guanine-nucleotide-binding protein.

The current results highlight the potential for heterogeneity in different CT receptor populations. Comparison of the biochemical characteristics and kinetics of CT binding sites in other sheep tissues (e.g. bone or kidney) and the brain of other species should help to elucidate the nature of the differences in CT binding observed between sheep brain and other systems.

This work was supported by grants from the National Health and Medical Research Council of Australia. P.M.S. is an Australian Research Council Post-Doctoral Research Fellow. H.-G.S. was supported by the Deutsche Forschungsgemeinschaft.

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