Covalent Cross-linking of a Photoactive Derivative of Calcitonin to Human Breast Cancer Cell Receptors*

Jane M. Moseley, David M. Findlay, and T. John Martin
From the University of Melbourne, Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, 3077 Australia

Jeffrey J. Gorman
From the Howard Florey Institute for Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria, 3052 Australia

A photoaffinity derivative of salmon calcitonin has been produced by transglutaminase-mediated incorporation of N-(β-aminoethyl)-4-azido-2-nitroaniline into the hormone. The derivative, purified by high pressure liquid chromatography, retained the abilities to bind to cultured T47D breast cancer cells and to stimulate adenylate cyclase in these cells. In both these respects it was equipotent with synthetic salmon calcitonin.

Photolysis of the 125I-labeled photoactive salmon calcitonin derivative bound to T47D cells was accompanied by specific labeling to only one component (Mₐ ≈ 85,000) as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Labeling was observed only upon photolysis and was inhibited by unlabeled synthetic salmon calcitonin but not by the inactive calcitonin analogue, 8-glycine human calcitonin. Reduction did not alter the apparent molecular weight of the calcitonin receptor complex. No macromolecular forms of calcitonin were produced by photolysis in the absence of T47D cells.

Specific, high affinity receptors for calcitonin have been demonstrated in human lymphoid cell lines (1, 2), in a human lung cancer cell line (3, 4), and in two human breast cancer cell lines MCF7 and T47D (5-7). In the cancer cells, calcitonin stimulates adenylate cyclase (3-7), and the ability of various calcitonins and analogues to compete for binding to the cells correlates closely with their relative efficacies in stimulating adenylate cyclase in homogenates of the same cells (4, 6). Furthermore, the potencies of the various peptides in these cells resemble their potencies in lowering plasma calcium in the rat bioassay (5, 6, 8, 9), an effect resulting from inhibition of bone resorption by calcitonin (10, 11), probably through a direct action of the hormone upon osteoclasts (9, 12). Thus, the calcitonin receptor in the cancer cells has certain major properties which would be expected of a "physiological" calcitonin receptor. It has not yet been possible to culture significant numbers of osteoclasts in reasonably pure form for biochemical study. For that reason we have used the human breast cancer cell line T47D in experiments aimed at further characterization of the properties of the calcitonin receptor.

The present report describes preparation of a biologically active photoactive derivative of salmon calcitonin and its application to the photoaffinity labeling of the calcitonin receptor on T47D cells. Photoaffinity labeling involves linkage of an aryl azido group to a peptide hormone and subsequent photoactivated cross-linkage of the hormone derivative to a membrane receptor of a responsive target cell (for review, see Ref. 13). The specific method used in this communication for linkage of an aryl azido group to salmon calcitonin exploits the ability of the transglutaminase enzymes to catalyze substitution of the γ-carboxamide side chain of peptide-bound glutamine residues with a variety of compounds containing primary amines (for review, see Ref. 14). A transglutaminase amine substrate containing the 4-azido-2-nitrophenyl group (15) was linked to salmon calcitonin and the photoactive hormone derivative was used to specifically label the calcitonin receptor of T47D cells.

**EXPERIMENTAL PROCEDURES**

Monolayer cultures of T47D cells were grown as previously described (6), using routine maintenance medium of RPMI 1640 with 10% fetal calf serum containing 10 ng/ml of insulin (NOVO Laboratories) and 1 mg/liter of hydrocortisone hemisuccinate. UMR 106, a clonal line of osteoblastic phenotype, established from a rat osteogenic sarcoma, was grown in monolayer culture as reported (16). Synthetic salmon calcitonin (lot K 715081, 4700 units/mg) and 8-glycine human calcitonin were gifts from the Armour Pharmaceutical Co., Kankakee, IL. Synthetic human calcitonin was provided by Ciba Geigy Ltd., Basel, Switzerland, and synthetic (ammonium bicarbinate) eel calcitonin was from the Toyoo Joyco Company, Japan. Phenylmethanesulfonyl fluoride and N-ethylmaleimide were obtained from BDH, Australia.

Transglutaminase was purified from guinea pig liver homogenates (17). The transglutaminase amine substrate, N-(β-aminoethyl)-4-azido-2-nitroaniline, was prepared as previously described (18), concentrations of this compound were determined based on a molar extinction coefficient of 4800 at 460 nm determined for N-(4-azido-2-nitrophenyl)glycine (18). 4-γ-(γ-Glutamyl)N-ethyl-4-azido-2-nitroanilino salmon calcitonin was prepared as follows. Salmon calcitonin (26.6 mg) was incubated with guinea pig liver transglutaminase (13.2 μM) and N-(β-aminoethyl)-4-azido-2-nitroaniline (8.5 mM) in 0.1 M Tris-chloride buffer containing 30 mM NaCl, 1 mM EDTA, and 50 mM CaCl₂ at pH 7.4 and 25 °C. The transglutaminase concentration was increased to 26 μM following incubation for 2 h at 25 °C. After a 4-h incubation, the suspension was made 5% (v/v) with respect to formic acid and calcitonin was freed of salts and excess N-(β-aminoethyl)-4-azido-2-nitroanilino by chromatography on Sephadex G-25 in 0.1 M formic acid. The reddish fractions eluting at the void volume were collected and lyophilized. The photoactive derivative of sCT was purified by reverse phase high pressure liquid chromatography using...

*The abbreviations used are: sCT, synthetic salmon calcitonin; SDS, sodium dodecyl sulfate.*
a Waters Associates system comprised of two model 600A pumps, a model 660 solvent programmer, and a model 440 detector set at 284 nm. Elution was at 2.5 ml/min from a µBondapak C18 column (3.9 mm x 30 cm; Waters Associates), using an isocratic mobile phase of either 38% acetonitrile in 0.1% trifluoroacetic acid (mobile phase 1) or 35% acetonitrile in 0.1 M sodium phosphate buffer at pH 2.1 (mobile phase 2). The purified derivative was stored at -20 °C after evaporation of acetonitrile under a stream of nitrogen. The 4-azido-2-nitroaniline group was protected from photolytic decomposition by performing the above procedures in dimmed light and wrapping all vessels in aluminum foil.

Amino acid analyses were performed with a Beckman 121 MB analyzer. Peptide concentrations were determined by amino acid analysis. Absorption spectra were recorded with a Varian Superscan spectrophotometer.

Iodination of synthetic salmon calcitonin and of the photoactive derivative was carried out to specific activities of 150 to 200 μCi/μg as previously described (4-7). Binding of labeled peptides to intact cell monolayers was studied as previously reported (19), by incubating replicate monolayer cultures of T47D cells in 4.5-cm² multiwell dishes performing the above procedures in dimmed light and wrapping vessels in aluminum foil.

Spectra of the purified sCT derivative (47 μg) were recorded before (solid line) and after (broken line) photolysis. Photolysis was performed for 20 min as described under “Experimental Procedures” with the sample contained in quartz spectrophotometer cuvettes.

Comparison of binding of 125I-labeled photoactive salmon calcitonin to T47D, UMR 106, and UMR 108 cell lines was carried out as described for the photoaffinity labeling except that the cells were grown as replicate cultures in 4.5-cm² multiwell dishes. Cell numbers were comparable for all lines, and culture dishes were divided for total binding and nonspecific binding (carried out in the presence of 3 x 10⁻⁷ M salmon calcitonin). Duplicate culture dishes allowed experiments to be carried out with or without the photolysis step.

Solubilization of the cells from each flask was carried out at 4 °C for 40 min in 300 μl of 0.05 M Tris (w/v), 0.1% Triton (v/v), 0.15 M NaCl, 10 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 0.06% N-ethylmaleimide (w/v), at pH 8.0. Following removal of cell debris by centrifugation for 30 s at 10,000 x g in a Beckman model B microfuge, the remaining extract was analyzed immediately. In all experiments, 80–90% of the total counts bound were recovered in the extract.

Polyacrylamide gel electrophoresis was carried out in the presence of 0.1% SDS (w/v), according to the method of Laemmli (20) on 10% gels (10% acrylamide and 0.25% bisacrylamide) in conjunction with a

\[
\begin{array}{c|c|c}
\text{Amino acid} & \text{Found} & \text{Expected} \\
\hline
\text{Asp} & 1.98 & 2 \\
\text{Thr}^* & 4.45 & 5 \\
\text{Ser}^* & 3.54 & 4 \\
\text{Glu} & 3.25 & 3 \\
\text{Pro} & 1.89 & 2 \\
\text{Gly} & 2.87 & 3 \\
\text{Cys} & 0.51 & 2 \\
\text{Val} & 1.0 & 1 \\
\text{Leu} & 5.18 & 6 \\
\text{Tyr}^* & 0.97 & 1 \\
\text{Lys} & 2.15 & 2 \\
\text{His} & 1.06 & 1 \\
\text{Arg} & 1.01 & 1 \\
\end{array}
\]

\* No corrections have been made for hydrolytic decomposition.

**TABLE 1**

Amino acid composition of photoactive salmon calcitonin derivative

Samples were hydrolyzed in duplicate for 24 h at 110 °C in sealed evacuated tubes containing 6 M HCl.

**FIG. 2.** Absorption spectra of photoactive salmon calcitonin. Spectra of the purified sCT derivative (47 μg) were recorded before (solid line) and after (broken line) photolysis. Photolysis was performed for 20 min as described under “Experimental Procedures” with the sample contained in quartz spectrophotometer cuvettes.
Photoaffinity Labeling of Calcitonin Receptor

3% stacking gel. Samples were diluted in sample buffer with or without 5% mercaptoethanol (v/v). In all cases, 150 µl (representing ~2.5 × 10⁶ cells) of the original extract were applied to each of the gels unless otherwise stated. Disc gels were sliced into 2-mm fractions and ¹²⁵I radioactivity measured in a Packard automatic γ spectrometer (model 5110) with an efficiency of 70%.

Slab gels (180 × 140 × 2.5 mm) were fixed in acetic acid: methanol:water (1:5:4) overnight, dried under vacuum, and exposed to Fuji Rx Safety x-ray film at -70 °C in the presence of a Dupont Cronex Lightning Plus intensifying screen.

Molecular weight standards comprising phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase (Pharmacia, South Seas) were run with each experiment and their mobilities are indicated where applicable.

---

**FIG. 3.** Biological activity of photoactive salmon calcitonin. Upper, effect of sCT () or photoactive salmon calcitonin (○) on adenylate cyclase activity in homogenates of T47D cells. Activity is expressed as picomoles of cAMP produced during a 30-min incubation. Lower, competition for binding of ¹²⁵I-sCT to intact T47D cells by sCT () or photoactive sCT (○) using described method (19).

**FIG. 4.** Binding of ¹²⁵I-labeled native and photoactive sCT to intact T47D cells. Labeled native (●) or photoactive sCT (○) were incubated for 1 h at 21 °C in the presence of increasing concentrations of unlabeled sCT and bound radioactivity measured (19).

**FIG. 5.** Identification of a high molecular weight complex of photoactive salmon calcitonin and a component from T47D cells. Upper, SDS-polyacrylamide disc gel electrophoresis of solubilized T47D cells which had been incubated with photoactive ¹²⁵I-sCT for 1 h at 21 °C in the absence () and presence (○) of 1 µg/ml of sCT followed by incubation for 30 min at 4 °C without photolysis. Lower, SDS-polyacrylamide disc gel electrophoresis of solubilized T47D cells which had been incubated with photoactive ¹²⁵I-sCT for 1 h at 21 °C in the absence () and presence (○) of 1 µg/ml of sCT followed by photolysis for 20 min at 4 °C. The arrows indicate the position of molecular weight standards resolved at the same time as the profiles illustrated.
Photoaffinity Labeling of Calcitonin Receptor

5849

Mr

- 94 K
- 67 K
- 43 K
- 20 K

sCT

FIG. 6. The effects of competition by synthetic salmon calcitonin and time on formation of the high molecular weight complex of photoactive salmon calcitonin and a component from T47D cells. SDS-polyacrylamide slab gel electrophoresis of solubilized T47D cells which had been incubated with photoactive $^{125}$I-sCT for 1 h at 21 °C (B), for 1 h at 21 °C in the presence of 1 μg/ml of sCT (C), for 30 min at 21 °C (D); E, F, and G as for B, C, and D except that the samples were treated at 100 °C for 3 min with sample buffer containing 5% mercaptoethanol prior to electrophoresis. A, medium derived from incubation B after photolysis. H, nonphotolysed photoactive $^{125}$I-sCT. The molecular weight markers indicated were run simultaneously on the same gel.

FIG. 7. The effect of competition by 8-glycine human calcitonin on formation of a high molecular weight complex of photoactive salmon calcitonin and a T47D cell component. SDS-polyacrylamide slab gel electrophoresis of solubilized T47D cells which had been incubated with photoactive $^{125}$I-sCT for 1 h at 21 °C plus: A, no addition; B, 1.0 pg/ml of 8-glycine synthetic human calcitonin; C, 0.5 pg/ml of 8-glycine synthetic human calcitonin; D, 0.5 pg/ml of 8-glycine synthetic human calcitonin (50 μl loaded). E shows nonphotolysed photoactive $^{125}$I-sCT. The molecular weight markers indicated were run simultaneously on the same gel.

RESULTS

High pressure liquid chromatography of sCT after incubation with guinea pig liver transglutaminase and N-(β-aminoethyl)-4-azido-2-nitroaniline revealed only a trace of the original native sCT, elution time 4.5 min, and appearance of a derivative peak eluting at 7.5 min (Fig. 1A). This peak could not have been due to excess photosensitive amine substrate, since the latter had been removed by gel chromatography on Sephadex G-25 prior to high pressure liquid chromatography. Furthermore, the elution time of the amino substrate was determined to be 2 min when chromatographed under identical conditions. The efficacy of the chromatographic system for separation of native sCT from the derivative was demonstrated by chromatography of a portion of the incubation mixture to which native peptide was added (Fig. 1B). Similar elution patterns were observed when mobile phase 2 was used, containing 0.1 M sodium phosphate buffer (pH 2.1), in place of trifluoroacetic acid. The spectral characteristics ($\lambda_{max} = 465$ nm) of the purified derivative (Fig. 2) indicated presence of the 4-azido-2-nitroaniline chromophore (18). In addition, the photosensitive nature of the purified derivative was evidenced by spectral changes associated with photolysis. The amino acid composition of the purified derivative (Table I) was in close agreement with the known structure of the native hormone (21). The ratio of concentration of derivative determined by absorbance at 465 nm to concentration determined by amino acid analysis was 0.85. This figure is consistent with a substitution level of 1 mol of 4-azido-2-nitroaniline/mol of photoactive salmon calcitonin derivative.

The isolated photoactive salmon calcitonin derivative retained full biological activity when compared with unmodified peptide for its ability to stimulate adenylate cyclase activity in T47D cell homogenates (Fig. 3). Similarly, the two peptides competed to a similar extent with $^{125}$I-labeled salmon calcitonin for binding to intact T47D cells (Fig. 3). Furthermore, when $^{125}$I-labeled native and photoactive sCT were incubated with T47D cells in culture, the amount of binding and sensitivity to competition by added unlabeled calcitonin were similar (Fig. 4). Kinetics of binding of the two labeled peptides...
Photoaffinity Labeling of Calcitonin Receptor

Fig. 8. Effect of photolysis upon binding of 125I-labeled photoactive sCT to T47D and UMR 106 cells. Upper, SDS-polyacrylamide disc gel electrophoresis of solubilized T47D cells which had been incubated in the absence (●) and presence (○) of 1 μg/ml of sCT. Conditions as for Fig. 5. Lower, SDS-polyacrylamide disc gel electrophoresis of solubilized UMR 106 cells which had been incubated in the absence (●) and presence (○) of 1 μg/ml of sCT. Experiment carried out at same time and same conditions as in upper panel. Arrows indicate position of molecular weight markers.

to T47D cells are not distinguishable. Whichever solvent system was used for high pressure liquid chromatography purification of sCT derivative, the biological activity was the same.

When the 125I-labeled photoactive calcitonin was incubated with T47D cells, photolysis yielded a single peak of radioactivity on disc gels, corresponding to a molecular weight (ligand plus bound component) of approximately 85,000 (Fig. 5). In the absence of photolysis only a small peak of radioactivity was observed but in both cases competition for this band occurred by addition of excess synthetic salmon calcitonin (Fig. 5). Autoradiography of slab gels also revealed formation of this specific binding corresponding to a molecular weight of approximately 70,000 was present in both extracts (Fig. 8).

TABLE II

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Without photolysis</th>
<th>With photolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total binding</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>T47D</td>
<td>6989 ± 96</td>
<td>1997 ± 82</td>
</tr>
<tr>
<td>UMR 106</td>
<td>1445 ± 168</td>
<td>1733 ± 241</td>
</tr>
<tr>
<td>UMR 108</td>
<td>1037 ± 44</td>
<td>1297 ± 157</td>
</tr>
</tbody>
</table>

* ± S.E., n = 4.

apparently reduced. A low molecular weight band was also observed (Fig. 6, A–G) which probably represents a noncross-linked derivative since its mobility coincided with that of the 125I-labeled salmon calcitonin derivative (Fig. 6H). Synthetic human (amino~uberic~) eel calcitonin, or the nonradioactive photoactive derivative prevented formation of the 85,000 molecular weight band (results not shown). However, 8-glycine human calcitonin, a biologically inactive analogue (4) did not do so, as shown on the autoradiograph in Fig. 7. All the slab gels revealed a minor band of radioactivity in the region of apparent M, = 65,000–75,000, the intensity of which varied between experiments. The nature of this has not yet been determined, but its formation is not influenced by inclusion of biologically active calcitonin peptides. Media from incubations which were subjected to photolysis produced only a single band of radioactivity in the position of the 125I-labeled salmon calcitonin (Fig. 7A) derivative indicating that neither copolymerization nor crosslinking to bovine serum albumin in the incubation medium had occurred.

Control experiments were carried out with cells known not to possess receptors or responses to calcitonin. 125I-labeled photoactive salmon calcitonin was incubated with replicate cultures of T47D, UMR 106, and UMR 108 cells (16). Total and non-specific binding was assessed with and without photolysis, and specific binding of 125I-labeled photoactive sCT to UMR 106 and UMR 108 cells was not significant whether or not photolysis was carried out (Table II). Polyacrylamide gel electrophoresis of extracts from UMR 106 cells after binding and photolysis was carried out in the absence and presence of 3 × 10−7 M sCT. Whereas the M, = 85,000 peak of specific binding was only seen in T47D extracts, the peak of non-specific binding corresponding to a molecular weight of approximately 70,000 was present in both extracts (Fig. 8).

DISCUSSION

In the case of several peptide hormones (22–26), labeling with an aryl azido group has allowed photoaffinity identification of a specific receptor on target cells or cell membranes. However, application for this purpose of a hormone with the azido group linked to the C-carboxamide side chains of glutamine is novel. Chemical modification of side chain functions including the ε-amino lysine (22), the carboxyl of glutamic and aspartic acids (22), the indole of tryptophan (26) have been used to link the aryl azido group to peptide hormones. Terminal amino (27) and carboxyl groups (28) have also served as linkage positions. The transglutaminase-mediated enzymatic method employed here for linkage of the aryl azido group to the C-carboxamide side chain of glutamine was assessed in an earlier publication using glucagon (1–6) and substance P as model glutamine-containing peptide hormones (15). In addition to expanding the choice of labeling procedures, the transglutaminase method has an advantage over some of the chemical methods in that modification is achieved without alteration of charge at the site of linkage. Maintenance of charge may be an important factor in preserving...
biological activity of photosensitive hormone derivatives. It remains to be determined to which of the two glutamine residues of salmon calcitonin the aryl azido group is linked. However, preferred specificity of guinea pig liver transglutaminase for glutamine 14 of salmon calcitonin has previously been observed using monodansylcadaverine as the amine substrate (29) and incubation conditions identical with those used in the present work. Only one fluorescent peptide was detected in tryptic digests of the purified fluorescent derivative of salmon calcitonin. Furthermore, only the expected number of tryptic peptides (four) were detected, indicating that no c-γ-glutamyl)-lysine cross-links were formed during incubation of salmon calcitonin with guinea pig liver transglutaminase and saturating levels of amine substrate. Consistent with the lack of cross-links and labeling solely at glutamine 14 were the facts that tyrosine and arginine were detected only in a single tryptic peptide and histidine only in the fluorescent peptide.

Clearly, the derivative of salmon calcitonin produced by this method has retained biological activity. This photoactive derivative exhibits the same biological potency as synthetic salmon calcitonin as indicated by the response of adenylate cyclase and competition for binding to T47D cells. In previous work with these (6) and with other (4, 5) calcitonin-responsive cell lines, several analogues of calcitonin have been used in experiments noting that ability to stimulate adenylate cyclase relates closely to relative binding competition, and these in turn parallel known biological activities in the peptides. Moreover, the dose dependence of binding competition and the appearance of a cross-linked component in a time-dependent fashion is consistent with the characteristics of binding of salmon calcitonin to the breast tumor cell receptor previously reported (6, 7, 19).

Identification of a specific calcitonin receptor component has not been previously reported. Previous attempts to identify calcitonin receptor, relying on the integrity of the natural receptor-ligand binding, have resulted in inconsistent recovery of a single component of similar molecular weight to that reported here. The derivative described here has allowed consistent recovery of a component possibly associated with the calcitonin receptor. The failure of biologically inactive 8-glycine human calcitonin to prevent binding of the photoactive calcitonin is consistent with specific binding of the derivative, since the single amino acid substitution in this peptide abolished its biological activity (4). Furthermore, binding preventable by active salmon, human, or eel calcitonins was abolished its biological activity, since the single amino acid substitution in this peptide was the fact that tyrosine and arginine were detected only in a single tryptic peptide and histidine only in the fluorescent peptide.

The use of calcitonin labeled with aryl azido groups at different residues may provide a means to confirm the binding specificity of the observed component and possibly to identify additional receptor subunits, should they exist. The calcitonin-responsive cancer cells are an appropriate cell to use for this purpose, since they possess abundant calcitonin receptors with properties to be expected of a physiological receptor for the hormone. The best known normal target cell for calcitonin (the osteocyte) is not available for such studies, but reagents developed out of this work could provide an approach to identification of isolated osteoclasts.

Acknowledgments—The skilled technical assistance of P. Smith and the provision of synthetic salmon calcitonin by Dr. R. Orłowski are gratefully acknowledged.

REFERENCES

Covalent cross-linking of a photoactive derivative of calcitonin to human breast cancer cell receptors.

J M Moseley, D M Findlay, T J Martin and J J Gorman


Access the most updated version of this article at http://www.jbc.org/content/257/10/5846

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/10/5846.full.html#ref-list-1