Processing of calcitonin and epidermal growth factor after binding to receptors in human breast cancer cells (T 47D)

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¹²⁵I-labelled calcitonin and ¹²⁵I-labelled epidermal growth factor (EGF) bound to T 47D breast cancer cells at 37°C in a manner that became increasingly resistant to removal by acid pH. Bound ¹²⁵I-labelled EGF became resistant to acid removal more rapidly than did bound ¹²⁵I-labelled calcitonin. The shift from acid accessibility to acid inaccessibility was energy-dependent since it was not seen at 4°C and was inhibited in the presence of cell metabolic inhibitors. Radioactivity removed by acid represented intact hormone as assessed by trichloroacetic acid precipitation, whereas radioactivity released spontaneously by the cells was trichloroacetic acid-soluble. Inclusion of 10 mM-NH₄Cl in the incubation medium resulted in an accumulation of cell-associated radioactivity without affecting the shift to acid inaccessibility. The accumulated radioactivity was relatively more trichloroacetic acid-precipitable in comparison with that associated with control cells. These data are consistent with internalization of receptor-bound EGF and a similar though slower mechanism of processing for receptor-bound calcitonin. The predominant route of hormone release from cells seems to occur via intracellular degradation rather than dissociation from cell-surface receptors.

Binding of EGF to fibroblasts is characterized by rapid removal of the hormone from the cell surface to intracellular vesicles. These vesicles eventually fuse with lysosomes and degraded hormone products are finally released from the cells. This sequence of events has been demonstrated by exploiting a variety of fluorescent (Haigler *et al.*, 1978), electron-dense (McKanna *et al.*, 1979) or radioactive (Carpentier *et al.*, 1981) hormone-labelling techniques, the data from which correlate well with those obtained with biochemical methods discriminating between cell-surface-bound and internalized hormone (Carpenter & Cohen, 1976; Haigler *et al.*, 1980). Lysosomotropic agents have been shown to cause intracellular accumulation of EGF by both methods without affecting internalization of hormone (McKanna *et al.*, 1979; King *et al.*, 1980).

EGF binds to the human breast cancer cells (T 47D) and stimulates mitogenesis (K. W. Ng, S. A. Livesey, R. G. Larkins & T. J. Martin, unpublished work), as it does in MCF 7 cells, another human breast cancer cell line (Osborne *et al.*, 1980). CT

also binds specifically to T 47D cells (Findlay *et al.*, 1980), and stimulates adenylate cyclase activity in these cells. The CT receptor and response possess the characteristics to be expected of a 'physiological' CT receptor. Although these two peptide hormones differ in their initial actions on the cells, studies described in the present paper indicate that there are certain similarities in the ways in which receptor-bound EGF and CT are processed.

Materials and methods

Hormones and chemicals

Synthetic salmon CT (lot no. K937-151-1) was a gift from the Armour Pharmaceutical Co., Kankakee, IL, U.S.A. EGF was prepared from mouse submaxillary glands by the method of Savage & Cohen (1972) and was pure by both amino acid analysis and bioassay (Ng *et al.*, 1982). Bacitracin (65 units/mg) was obtained from Apothekerns Laboratories, Oslo, Norway. Methylamine and dinitrophenol were from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from standard suppliers. Na¹²⁵I was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Abbreviations used: EGF, epidermal growth factor; CT, calcitonin; TCA, trichloroacetic acid.

Peptide iodination

Synthetic salmon CT was labelled with ^{125}I , purified and stored as previously described (Hunt *et al.*, 1977). Using $2\mu\text{g}$ of chloramine-T for 10 s, specific radioactivities of 160–200 Ci/g were achieved. EGF was labelled with ^{125}I by the method of Greenwood *et al.* (1963), with $25\mu\text{g}$ of chloramine-T for 1 min. ^{125}I -labelled EGF was purified from the reaction mixture by gel filtration as previously described (Ng *et al.*, 1982). Specific radioactivities of 100–200 Ci/g were obtained.

Cell culture

T 47D cells were originally grown from a pleural effusion from an infiltrating ductal breast cancer (Horwitz *et al.*, 1978). Culture maintenance was in Roswell Park Memorial Institute 1640 medium containing 10% foetal-calf serum and added insulin ($0.1\mu\text{M}$; Novo Crystalline) and cortisol ($0.1\mu\text{M}$) in plastic tissue-culture flasks and maintained at 37°C in an atmosphere of CO_2/air (1:19). Antibiotics were minocyclin (1 mg/litre) and gentamycin (80 mg/litre). Subcultures were carried out by washing in 0.02% (w/v) EDTA, followed by treatment with 0.0125% (w/v) trypsin in 0.02% EDTA for 2 min at 37°C , addition of complete medium before centrifugation at 200g and suspension in complete medium. Bovine foetal-calf serum was obtained from Gibco Biocult and all other culture media from the Commonwealth Serum Laboratories, Parkville, Victoria, Australia.

Cells were subcultured 2–4 days before experiments into 12-place multiwell plates in medium containing no added insulin or cortisol. Experiments were carried out at confluence.

Preparation of cells for binding of ^{125}I -labelled CT or EGF

Medium was removed from wells and cells washed with 2 vol. of approx. 4 ml of phosphate-buffered saline at ambient temperature. Warm RPMI 1640 medium (0.5 ml) containing 0.1% (w/v) bovine serum albumin was pre-incubated with the cells for at least 15 min before any additions or manipulation. ^{125}I -labelled CT or EGF and unlabelled hormone for assessment of non-specific binding were added in $25\mu\text{l}$ of incubation medium. To terminate binding, medium was removed and cells were again washed with 2 vol. of approx. 4 ml of phosphate-buffered saline at ambient temperature. Cells were removed from wells using $2 \times 0.5\text{ ml}$ of 0.5 M -NaOH and bound radioactivity was determined by using a Packard automatic scintillation well counter with an efficiency of 70% for ^{125}I . Details of specific experiments are included in the Figure legends.

Results

EGF binding to T 47D cells

Fig. 1 shows binding of ^{125}I -labelled EGF to T 47D cells and competition for binding by increasing concentrations of unlabelled EGF. EGF was shown to bind specifically in that a range of unrelated peptides at high concentrations did not compete significantly for binding. The inset shows a Scatchard transformation of the binding data. Parameters of $K_d = 0.27 \pm 0.01\text{ nM}$ and receptor number per cell of $25\,100 \pm 2300$ were calculated from three such experiments (mean $\pm$ S.E.M.). In view of the complex interactions of EGF with T 47D cells, as described below, rather than a true attainment of equilibrium required for Scatchard analysis, calculations for these parameters represent approximations at best.

Removal of cell-bound EGF and salmon CT by acid pH

Confluent monolayers of T 47D cells in multiwell dishes were incubated with ^{125}I -labelled EGF or ^{125}I -labelled salmon CT at 4°C or 37°C . After thorough washing to remove unbound hormone, cells were treated for 1 min with ice-cold 0.15 M -NaCl/ 0.05 M -glycine, pH 2.5. Previous studies have shown that for EGF (Haigler *et al.*, 1980) and prolactin (Costlow & Hampl, 1981) a pH of 2.5 removes at least 90% of radioactively labelled hormone bound to the surface of cells. Figs. 2 and 3 show that the percentage of bound radioactivity released by acid pH after binding of EGF or salmon CT decreased as a function of time of incubation at

Fig. 1. Binding of ^{125}I -labelled EGF to T 47D cells. ^{125}I -labelled EGF (50000 c.p.m.; 50 fmol/ml) was incubated as described in the Materials and methods section with T47D cells at 37°C for 2 h. Binding was measured in the presence of increasing concentrations of unlabelled EGF. Results are expressed as means $\pm$ S.E.M. The inset shows a Scatchard transformation of the binding data.

Fig. 2. Removal of bound ¹²⁵I-labelled EGF from T 47D cells by pH 2.5

¹²⁵I-labelled EGF (130 000 c.p.m.; 130 fmol/ml) was incubated as described in the Materials and methods section with T 47D cells at 4°C (○) or 37°C (●). At the times indicated cells were washed and 1 ml of ice-cold 0.15 M-NaCl/0.05 M-glycine, pH 2.5, added for 1 min. Radioactivity removed by the pH 2.5 buffer was transferred to 1 ml of ice-cold 10% (w/v) TCA containing 1% (v/v) serum as co-precipitate. (c) shows the total mass of ¹²⁵I-labelled EGF bound at either temperature and the mass bound non-specifically i.e. in the presence of 42 pmol/ml unlabelled EGF. (b) shows the percentage of bound radioactivity removed at either temperature. (a) shows the TCA precipitability of the eluted radioactivity expressed as a percentage of that of ¹²⁵I-labelled EGF incubated in medium alone. Results are means of triplicate determinations and standard errors of the means, omitted for clarity, were in all cases less than 10% of the mean values plotted.

Fig. 3. Removal of bound ¹²⁵I-labelled salmon CT from T 47D cells at pH 2.5

¹²⁵I-labelled salmon CT (140 000 c.p.m.; 170 fmol/ml) was incubated as described in the Materials and methods section with T 47D cells at 4°C (b and d) or 37°C (a and c). At the times indicated cells were washed and 1 ml of ice-cold 0.15 M-NaCl/0.05 M-glycine (pH 2.5) was added for 1 min. (c) and (d) show the total mass of ¹²⁵I-labelled salmon CT bound at either temperature (○) and the mass bound non-specifically, i.e. in the presence of 0.6 nmol of unlabelled salmon CT/ml (●). (a) and (b) show the percentage of bound radioactivity removed at pH 2.5 at either temperature. Results represent the means $\pm$ S.E.M. of triplicate determinations.

37°C, whereas at 4°C there was no change in the percentage of acid-removable bound radioactivity for either hormone. The radioactivity released by acid pH after binding of EGF represented intact molecule as assessed by TCA precipitability (Fig. 2) and this was also true for CT as reported previously (Lamp *et al.*, 1981). Exposure of cells to pH 2.5 for 1 min did not interfere with cell membrane integrity in terms of eosin dye exclusion.

¹²⁵I-labelled salmon CT bound to membranes from disrupted T 47D cells remained equally acid-elutable over a 4 h period (results not shown).

Effects of metabolic inhibitors on acid elution of EGF and salmon CT

Since the acid-elutability of these hormones from T 47D cells did not change at 4°C but did at 37°C, several metabolic inhibitors were co-incubated with cells to investigate further the energy dependence of this phenomenon. Table 1 shows the results of co-incubation of NaN₃, dinitrophenol and potassium cyanide in buffer containing no added glucose or amino acids. With EGF all inhibitors were effective at the concentrations used in inhibiting the

Table 1. ¹²⁵I-labelled EGF or ¹²⁵I-labelled salmon CT binding to T 47D cells: effect of metabolic inhibitors on acid elution of bound hormones

T 47D cells in 12-place multiwells were pre-incubated at 37°C for 30 min with or without the listed agents in 0.5 ml of Dulbecco's phosphate-buffered saline (pH 7.4), containing 0.1% (w/v) bovine serum albumin. ¹²⁵I-labelled EGF (480 000 c.p.m.; 500 fmol/ml) or ¹²⁵I-labelled salmon CT (280 000 c.p.m.; 340 fmol/ml) were then added and incubation was continued for the times indicated. Cells were washed and 1 ml of ice-cold 0.15 M-NaCl/0.05 M-glycine (pH 2.5) was added for 1 min. The remaining cell-associated radioactivity was measured as described in the Materials and methods section. Results are expressed as percentages of bound radioactivity removed by acid and are means $\pm$ S.E.M. of triplicate determinations.

Agent	Incubation time (min)	EGF (% acid-elutability)		
		10	20	60
Control		29.8 $\pm$ 0.52	11.6 $\pm$ 0.10	9.1 $\pm$ 0.17
Bacitracin (1 mg/ml)		32.8 $\pm$ 0.49	11.0 $\pm$ 0.14	7.9 $\pm$ 0.10
Methylamine (1 mg/ml)		29.9 $\pm$ 1.13	9.5 $\pm$ 0.42	6.4 $\pm$ 0.19
NaN ₃ (10 mM)		81.5 $\pm$ 0.81	58.2 $\pm$ 1.05	46.2 $\pm$ 0.84
NaN ₃ (5 mM)		72.4 $\pm$ 1.47	70.0 $\pm$ 0.54	46.9 $\pm$ 0.75
Dinitrophenol (1 mM)		78.7 $\pm$ 0.42	77.4 $\pm$ 0.55	42.9 $\pm$ 1.30
Dinitrophenol (0.5 mM)		78.8 $\pm$ 0.87	78.1 $\pm$ 0.73	49.0 $\pm$ 1.46
Potassium cyanide (10 mM)		80.2 $\pm$ 0.58	73.1 $\pm$ 0.40	51.8 $\pm$ 1.51
Potassium cyanide (5 mM)		74.8 $\pm$ 0.94	73.1 $\pm$ 0.72	12.7 $\pm$ 0.55

Agent	Incubation time	CT (% acid-elutability)		
		15 min	1 h	4 h
Control		61.9 $\pm$ 1.08	38.7 $\pm$ 3.15	20.0 $\pm$ 0.59
Bacitracin (1 mg/ml)		69.2 $\pm$ 0.64	43.2 $\pm$ 2.19	28.1 $\pm$ 0.97
Methylamine (1 mg/ml)		58.0 $\pm$ 0.46	34.5 $\pm$ 3.82	20.2 $\pm$ 0.41
NaN ₃ (10 mM)		69.6 $\pm$ 0.99	59.8 $\pm$ 1.16	55.3 $\pm$ 0.10
NaN ₃ (5 mM)		67.2 $\pm$ 1.00	64.8 $\pm$ 1.00	51.3 $\pm$ 3.32
Dinitrophenol (1 mM)		65.1 $\pm$ 0.69	65.0 $\pm$ 1.47	56.6 $\pm$ 0.89
Dinitrophenol (0.5 mM)		61.8 $\pm$ 2.52	63.2 $\pm$ 1.41	52.8 $\pm$ 1.66
Potassium cyanide (10 mM)		66.8 $\pm$ 1.24	64.5 $\pm$ 1.37	26.8 $\pm$ 0.64
Potassium cyanide (5 mM)		72.6 $\pm$ 4.13	68.8 $\pm$ 1.42	21.9 $\pm$ 0.57

shift from acid-elutability to acid-inaccessibility of bound radioactivity over a 1 h period. For CT a significantly greater amount of cell-bound radioactivity remained acid-removable at 1 h with each inhibitor compared with control and at 4 h NaN₃ and dinitrophenol continued to inhibit the shift to acid inaccessibility. These metabolic inhibitors were not effective if RPMI 1640 medium containing 10 mM-glucose and added amino acids was used.

Table 1 also shows that neither bacitracin nor methylamine had any effect on acid-elutability of bound EGF, although bacitracin caused a small inhibition compared with control of the shift of acid accessibility of bound ¹²⁵I-labelled salmon CT, which reached significance at 15 min and 4 h ($P < 0.005$).

Effect of NH₄Cl on acid elution and cell binding of EGF and CT

Incubation of ¹²⁵I-labelled EGF or CT with T 47D cells in the presence of 10 mM-NH₄Cl at 37°C caused a significant increase in the amount of radioactivity specifically associated with the cells, which persisted for at least 8 h when label was

continuously present in the incubation (Figs. 4 and 5). NH₄Cl at 10 mM did not inhibit the decrease in acid-elutability seen for either hormone (Table 2). When control and NH₄Cl-incubated cells were dissolved in 2% (w/v) sodium dodecyl sulphate and the TCA-precipitability of cell-associated radioactivity was measured in each case, the radioactivity of NH₄Cl-treated cells was at each time relatively more precipitable by TCA than that of control cells (Figs. 4 and 5).

Dissociation of EGF and CT bound to T 47D cells

¹²⁵I-labelled EGF or ¹²⁵I-labelled salmon CT were incubated with T 47D cells for 1 h at 37°C. After thorough washing, fresh medium was added to the cells, removed at hourly intervals and replaced with fresh medium. The pattern of dissociation of radioactivity in each case is shown in Table 3. Also shown is the TCA-precipitability of the radioactivity after each hourly change, and TCA-precipitability of ¹²⁵I-labelled EGF or ¹²⁵I-labelled salmon CT incubated in medium alone for 1 h at 37°C.

Fig. 4. Effect of NH_4Cl on binding and degradation of ^{125}I -labelled EGF in T 47D cells

^{125}I -labelled EGF (130000 c.p.m.; 130 fmol/ml) was incubated as described in the Materials and methods section with T 47D cells at 37°C in the presence (●) or absence (○) of 10 mM NH_4Cl . At the times indicated cells were washed, dissolved in $200\text{ }\mu\text{l}$ of 2% (w/v) sodium dodecyl sulphate and added to 1 ml of ice-cold 10% (w/v) TCA containing 1% (v/v) serum as co-precipitate. (b) shows the total mass of ^{125}I -labelled EGF bound and the mass bound non-specifically, i.e. in the presence of 42 pmol of unlabelled EGF/ml. (a) shows the TCA precipitability of the radioactivity associated with NH_4Cl -treated cells expressed as a ratio of that associated with control cells. Results are means of triplicate determinations and standard errors of the mean, omitted for clarity, were in all cases less than 10% of the mean values plotted.

Discussion

There are now many data available exploiting both morphological and biochemical techniques, describing the subsequent events after peptide hormones bind to receptors in intact cells (for review, see Gorden *et al.*, 1980). For example, EGF-receptor interaction and post-receptor events have been described biochemically (Carpenter & Cohen, 1976; Das & Fox, 1978; Haigler *et al.*, 1980) and morphologically (Gorden *et al.*, 1978; Haigler *et al.*, 1978; Schlessinger *et al.*, 1978; McKanna *et al.*, 1979; Carpentier *et al.*, 1981). The EGF studies show that EGF binds initially to

Fig. 5. Effect of NH_4Cl on binding and degradation of ^{125}I -labelled salmon CT in T 47D cells

^{125}I -labelled salmon CT (180000 c.p.m.; 220 fmol/ml) was incubated as described in the Materials and methods section with T 47D cells at 37°C in the presence (●) or absence (○) of 10 mM NH_4Cl . At the times indicated the cells were treated as described in the legend to Fig. 4. (b) shows the total mass of ^{125}I -labelled salmon CT bound and the mass bound non-specifically, i.e. in the presence of 0.6 nmol of unlabelled salmon CT/ml. (a) shows the TCA precipitability of the radioactivity associated with NH_4Cl -treated cells as a ratio of that associated with control cells. Results are means of triplicate determinations and standard errors of the mean, omitted for clarity, were in all cases less than 10% of the mean value plotted.

cell-surface receptors, which then move in the plane of the membrane to cluster in depressed, protein-coated membrane regions called coated pits. These coated pits pinch off and form intracellular vesicles termed 'receptosomes' that accumulate in the region of the Golgi, close to the nucleus. It seems that after fusion of the receptosomes with the Golgi, lysosomes are formed; lysosomal enzymes degrade the endocytosed ligand and it is extruded from the cell as degraded fragments (Adamson & Rees, 1981).

Specific high-affinity binding of EGF to T 47D breast cancer cells is demonstrated in the present paper; CT binding and adenylate cyclase and protein kinase activation in the same cell line have been previously reported (Findlay *et al.*, 1980; Findlay *et al.*, 1981c). In addition the two hormones have been respectively shown to enhance and inhibit growth of T 47D cells in culture (Ng *et al.*, 1981).

Table 2. *Effect of NH₄Cl on the binding and acid-elutability of bound ¹²⁵I-labelled EGF or ¹²⁵I-labelled salmon CT in T 47D cells*

Cells were incubated at 37°C as described in the Materials and methods section with ¹²⁵I-labelled EGF (400 000 c.p.m.; 400 fmol/ml) or ¹²⁵I-labelled salmon CT (250 000 c.p.m.; 300 fmol/ml) in the presence or absence of 10 mM-NH₄Cl. At the times indicated, cells were washed and 1 ml of ice-cold 0.15 M-NaCl/0.05 M-glycine (pH 2.5) was added. The remaining cell-associated radioactivity was measured as described in the Materials and methods section. Results are expressed both as the total mass of hormone bound at each time and as the percentage of bound radioactivity removed by acid. Means $\pm$ S.E.M. of triplicate determinations are shown.

Time (min)	Salmon CT			
	(fmol specifically bound/10 ⁶ cells)		Proportion eluted with acid (%)	
	Control	10 mM-NH ₄ Cl	Control	10 mM-NH ₄ Cl
15	2.1 $\pm$ 0.1	1.4 $\pm$ 0.3	47.4 $\pm$ 4.0	52.1 $\pm$ 0.3
30	3.8 $\pm$ 0.4	2.2 $\pm$ 0.4	36.8 $\pm$ 0.8	37.2 $\pm$ 1.2
45	4.2 $\pm$ 0.5	3.3 $\pm$ 0.3	36.5 $\pm$ 0.7	37.1 $\pm$ 1.2
60	4.1 $\pm$ 0.4	5.6 $\pm$ 0.7	38.5 $\pm$ 1.3	36.7 $\pm$ 0.7
90	5.2 $\pm$ 0.2	5.7 $\pm$ 0.6	33.9 $\pm$ 1.2	37.5 $\pm$ 2.8
120	4.6 $\pm$ 0.5	5.2 $\pm$ 0.1	28.6 $\pm$ 0.9	25.1 $\pm$ 0.4
180	4.7 $\pm$ 0.7	5.3 $\pm$ 0.6	27.7 $\pm$ 0.9	18.3 $\pm$ 0.5
240	3.0 $\pm$ 0.5	6.4 $\pm$ 0.1	27.4 $\pm$ 0.3	16.1 $\pm$ 0.4

Time (min)	EGF			
	(fmol specifically bound/10 ⁶ cells)		Proportion eluted with acid (%)	
	Control	10 mM-NH ₄ Cl	Control	10 mM-NH ₄ Cl
5	0.47 $\pm$ 0.03	0.54 $\pm$ 0.02	70.9 $\pm$ 1.2	69.5 $\pm$ 0.8
10	0.55 $\pm$ 0.04	0.83 $\pm$ 0.05	60.6 $\pm$ 0.6	55.7 $\pm$ 1.8
20	0.81 $\pm$ 0.02	1.02 $\pm$ 0.02	18.0 $\pm$ 0.9	15.6 $\pm$ 1.2
40	1.23 $\pm$ 0.04	1.35 $\pm$ 0.06	10.7 $\pm$ 0.4	8.5 $\pm$ 0.5
60	1.96 $\pm$ 0.15	1.65 $\pm$ 0.07	7.9 $\pm$ 0.7	8.7 $\pm$ 0.5
120	3.00 $\pm$ 0.21	2.08 $\pm$ 0.37	6.4 $\pm$ 0.2	4.4 $\pm$ 0.3
180	2.62 $\pm$ 0.17	3.31 $\pm$ 0.19	6.2 $\pm$ 0.5	3.7 $\pm$ 0.2
240	3.18 $\pm$ 0.13	4.33 $\pm$ 0.06	5.2 $\pm$ 0.1	2.8 $\pm$ 0.6

The T 47D cell line therefore is a suitable model system in which to examine the events occurring after binding of CT in comparison with those determined biochemically with EGF.

¹²⁵I-labelled EGF bound to T 47D cells could initially be largely removed from the cells by low pH, but rapidly became inaccessible to acid removal. This is consistent with the observations in Balb 3T3 cells, where such a phenomenon has been clearly demonstrated as due to a 'shift' of cell-surface bound hormone to internalization into the cell (Haigler *et al.*, 1980). A shift from an acid-accessible compartment to acid-inaccessibility for CT bound to T 47D cells has been previously reported from this laboratory (Lamp *et al.*, 1981). By analogy with the EGF results this shift may be interpreted to represent internalization of the bound ¹²⁵I-labelled salmon CT. This interpretation is strengthened by the requirement of energy for this process, since (a) both EGF and CT remained elutable when binding was carried out at 4°C and (b) inhibitors of electron transport (in the absence of glucose and free amino acids in the medium) inhibited the shift from

acid-elutability to non-elutability. In the presence of these metabolic inhibitors prolactin remains on the cell surface of rat mammary tumour cells (Costlow & Hamble, 1981). Additionally, these agents were previously shown to inhibit degradation of cell-bound ¹²⁵I-labelled EGF (Carpenter & Cohen, 1976), a process that almost certainly depends on internalization of the bound hormone. Finally, there was no change in the acid elutability of ¹²⁵I-labelled salmon CT bound to membranes from disrupted T 47D cells over a 4 h period. Therefore the change in elutability in whole cells is probably not due either to the ligand becoming inaccessible in the membrane or to the nature of the surface binding changing to a predominantly higher affinity and/or covalent form.

Methylamine and bacitracin are both amines that effectively inhibit internalization of α_2 -macroglobulin (Davies *et al.*, 1980) and tri-iodothyronine (Cheng *et al.*, 1980) in cultured fibroblasts, presumably by inhibiting the protein cross-linking enzyme, transglutaminase (Davies *et al.*, 1980). However, these compounds are ineffective in inhibiting internalization of EGF into fibroblasts (Pastan

Table 3. *Dissociation of radioactivity from T 47D cells after binding of ^{125}I -labelled EGF or ^{125}I -labelled salmon CT* ^{125}I -labelled EGF (700 000 c.p.m.; 350 fmol/ml) or ^{125}I -labelled salmon CT (430 000 c.p.m.; 260 fmol/ml) were incubated in 1 ml of medium as described in the Materials and methods with T 47D cells at 37°C for 1 h in 6-place multiwells. Cells were then washed and fresh medium was replaced. At hourly intervals the medium was removed into 10% (w/v) ice-cold TCA and fresh medium was restored to the cells. Results are from triplicate determinations $\pm$ S.E.M. and are expressed as c.p.m. released from the cells in each hour interval and the TCA-precipitability of the released radioactivity. Total radioactivity bound was, for ^{125}I -labelled EGF, 55 793 $\pm$ 759 c.p.m., for ^{125}I -labelled salmon CT, 40 083 $\pm$ 914 c.p.m. Percentage precipitability in TCA of ^{125}I -labelled hormone incubated for 1 h at 37°C in medium alone was, for ^{125}I -labelled EGF, 79% and for ^{125}I -labelled salmon CT, 86%.

Dissociation interval (h)	EGF	
	(c.p.m. dissociated)	Proportion of radioactivity that was TCA-precipitable (%)
0-1	17 757 $\pm$ 703	18.4 $\pm$ 0.1
1-2	14 728 $\pm$ 1316	11.6 $\pm$ 0.1
2-3	8 269 $\pm$ 218	10.7 $\pm$ 0.2
3-4	5 291 $\pm$ 98	10.0 $\pm$ 0.2
4-5	2 774 $\pm$ 45	10.0 $\pm$ 0.7
5-6	2 489 $\pm$ 43	9.7 $\pm$ 0.5
6-7	2 161 $\pm$ 30	9.0 $\pm$ 1.0

Dissociation interval (h)	Salmon CT	
	(c.p.m. dissociated)	Proportion of radioactivity that was TCA-precipitable (%)
0-1	11 108 $\pm$ 655	33.3 $\pm$ 2.8
1-2	5 883 $\pm$ 111	20.1 $\pm$ 1.4
2-3	6 183 $\pm$ 550	21.7 $\pm$ 5.3
3-4	3 458 $\pm$ 277	27.3 $\pm$ 2.3
4-5	2 073 $\pm$ 90	25.3 $\pm$ 0.4
5-6	2 043 $\pm$ 86	39.0 $\pm$ 4.7

et al., 1981) and had no effect on EGF internalization into T 47D cells, although CT internalization was slowed slightly in the presence of bacitracin.

When ^{125}I -labelled salmon CT or ^{125}I -labelled EGF were incubated with T 47D cells and dissociation of radioactivity from the cells was followed, the dissociated radioactivity was predominantly degraded hormone as assessed by TCA-precipitation. This contrasts with the radioactivity eluted from the cell surface by acid pH, which was always intact by the same criterion. This may indicate that most of the bound hormone is spontaneously released from the cells only after internalization and intracellular degradation and not by release from cell-surface receptors. We have also shown that ^{125}I -labelled CT binds tightly to its receptor in a manner that is not competed for by subsequently added unlabelled calcitonin (Lamp *et al.*, 1981).

Lysotrophic agents inhibit degradation of cell-bound ^{125}I -labelled EGF (Carpenter & Cohen, 1976; Carpentier *et al.*, 1981) presumably by inactivating lysosomal enzymes by increasing intralysosomal pH (Ohkuma & Poole, 1978). In the present study NH_4Cl caused an accumulation of both cell-bound ^{125}I -labelled EGF and ^{125}I -labelled salmon CT. The radioactivity contained in NH_4Cl -treated cells was in

each case more TCA-precipitable than that in control cells. This result is consistent with a lysosomal degradative pathway for internalized EGF and CT in T 47D cells.

Adamson & Rees (1981) have proposed a model for EGF action on target cells with which the present work would be consistent. According to this model, EGF binds to cell-surface receptors that are present in the membrane in large excess over an effector or transducer molecule. EGF binds with high affinity to receptors linked to the transducer and leads to an activating sequence. Binding to other (spare) receptors leads to rapid internalization of the ligand-receptor complex and subsequent degradation of the ligand [although some EGF may survive sufficiently intact to interact with the nucleus (Johnson *et al.*, 1980)]. The fate of internalized receptor is not clear; the fate of the EGF-receptor-transducer complex is also a matter for speculation, although it has been found that persistent cell-surface binding of EGF is required for mitogenesis (Schechter *et al.*, 1978). Fig. 2 indicates that the majority of ^{125}I -labelled EGF bound to T 47D cells is rapidly internalized, although a small percentage remained elutable by acid.

The mechanism of processing of CT as deduced from the present study therefore resembles that

described in the EGF model. However, comparison of Figs. 2 and 3 shows that the rate of disappearance of CT from the cell surface is much lower than for EGF. Since coated-vesicle endocytosis is likely to provide the major system for the cellular uptake of most macromolecules and peptide hormones that bind to cells (Pearse & Bretscher, 1981), the difference between the two hormones in the rate of removal from the cell surface may relate to a difference in the rate or ability of diffusion of receptors through the membranes. Carpentier *et al.* (1981) have calculated that in isolated hepatocytes both the rate and extent of internalization of EGF are much greater than for insulin or glucagon, suggesting that the ligand or its specific receptor is the major determinant of the rate of internalization. In contrast with EGF, at least 20% of the ^{125}I -labelled salmon CT remains surface-bound after 4 h of incubation. The transducer molecule for CT action is the adenylate cyclase complex. We have previously shown that after a short exposure of T 47D cells to calcitonin both whole cells and disrupted cell membranes exhibit persistent activation of adenylate cyclase (Lamp *et al.*, 1981). This persistent activation was interpreted as being due to either continued occupation of a critical number of receptors or to an irreversible, cholera toxin-like action by calcitonin on the N-regulatory subunit of the adenylate cyclase complex, or a combination of these two mechanisms. The same studies also indicate that the ratio of receptor to transducer (adenylate cyclase) is much greater than, for example, for EGF, implying that in the short term more receptors would remain activated and on the cell surface. The means of termination of the calcitonin signal is not clear. However, work with T 47D cells and with a bronchogenic carcinoma cell line (BEN cells) (Ellison *et al.*, 1975) demonstrated that after exposure of cells to CT, subsequent binding of ^{125}I -labelled salmon CT to the same cells is blocked, and return from this 'down-regulated' state is dependent on protein synthesis (Findlay *et al.*, 1981a,b). It follows that after binding of CT to receptors, binding capacity only reappears after synthesis of new receptors or alternatively receptors may be re-inserted in the membrane after synthesis of carrier or of some other facilitative protein(s).

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