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RENAL UPTAKE AND DEGRADATION OF TRAPPED-LABEL CALCITONIN

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Abstract—In order to quantitate the role of the kidneys in the clearance and degradation of calcitonin, a trapped-label procedure was used to label human calcitonin. In contrast to conventional [^{125}I]calcitonin, the trapped-label preparation allows quantitative measurements of the extent of uptake as well as of degradation *in vivo* because the final degradation products do not leave the cells. Trapped-label calcitonin activated adenylate cyclase of bone cells and kidney, as did the native hormone. Ten minutes after intravenous injection into rats, 16% of a trace dose was found in the kidneys. Renal recovery increased to 20% after one hour; in addition, 14% of the injected dose was found in the urine. Eighty per cent of the radioactivity in the urine was in high-molecular weight material. After 90 min, the sum of the accumulated radioactivities in the kidneys and the urine reached 40% of the dose. More than 80% of the radioactivity was sedimentable by centrifuging in a density gradient, indicating that intact calcitonin, as well as the degradation products in the cells, were enclosed within membrane-bound vesicles. Two minutes after injection of trapped-label calcitonin, the peak of radioactivity was found in light gradient fractions associated with cell membrane marker enzymes. Between 5 and 15 min, the peak migrated from light fractions to heavy fractions containing lysosomal marker enzymes. After just 2.5 min, 61% of the renal radioactivity was in low-molecular weight degradation products, as determined by gel filtration. The kinetics of renal degradation of calcitonin indicate that substantial amounts of endocytosed calcitonin is degraded before the hormone reaches the lysosomes.

The kidneys play a major role in the elimination of small proteins and peptides. Many important hormones are peptides; for instance, calcitonin (human calcitonin has a molecular weight of 3425). Peptides with molecular weight less than 5000 pass the glomerular membrane along with water in the same concentration as in plasma. Many of these peptides are recovered only in trace amounts in the urine; the major part is reabsorbed in the tubular system.

Determination of arteriovenous differences indicate that the kidneys remove a major portion of injected calcitonin [1, 2]. However, when iodine-labelled calcitonin is injected *in vivo*, only a small fraction of the dose can be recovered in the kidney or the liver after a few minutes. It seems reasonable that the radioactive degradation products from [^{125}I]calcitonin‖ (iodide ions in the liver and iodotyrosine in the kidneys) leave the degradation sites to mix with degradation products from other organs in the general circulation. In an early study, 14.5% of a trace dose of [^{125}I]human calcitonin was recovered in the kidneys after 10 min decreasing to 4.5% after 30 min [3]. Salmon calcitonin, which has a higher affinity for calcitonin receptors, was found to an extent of 18.9% in the kidney after 10 min, decreasing to 10.1% after 60 min [4].

New information regarding total uptake in various organs can be obtained only if the degradation products are retained in the cells in which the degradation takes place. This may be achieved by radio-labelling using a trapped-label method: the radioactive atom is first introduced into a non-degradable compound which cannot cross lipid membranes and is subsequently attached to the protein. We used a moiety synthesized from cellobiose and tyramine (TC) [5]. A radioactive iodine atom may be introduced into TC by any of the methods commonly used to label proteins, and the [^{125}I]TC-moiety is covalently attached to calcitonin by means of a bifunctional coupling reagent, trichlorotriazine. The final degradation product from the TC-protein-conjugate is probably a complex molecule derived from tyramine-cellobiose and trichlorotriazine, attached to either a lysine or the amino-terminal amino acid in calcitonin. It has been shown that the [^{125}I]TC-moiety is suitable for labelling plasma proteins in turnover studies since only a minute fraction of the labelled material is recovered as fragments in urine or blood [5, 6]. In order to evaluate the binding activity of [^{125}I]TC-calcitonin to the calcitonin receptor, the calcitonin stimulated adenylate cyclase activity was determined for native calcitonin as well as for [^{125}I]TC-calcitonin.

We wanted to investigate the role of the kidneys in degrading this peptide hormone. One question is

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‖ Abbreviations: TC, tyramine-cellobiose or *N*-(1-deoxycellobiotitol-1-yl)-4-hydroxyphenyl-methylamine; ALPH, alkaline phosphatase; ACPH, acid phosphatase; AAP, alanine aminopeptidase; NAG, N-acetyl-β-glucosaminidase; AC, adenylate cyclase; [^{125}I]TC-CT, [^{125}I]TC-calcitonin (human); sCT, salmon calcitonin; PTH, parathyroid hormone.

whether the amount which is absorbed by the kidneys is commensurate with the almost complete glomerular filtration of this molecule [1]. It might be completely degraded in the kidneys, or part of the endocytosed material might be recirculated intact back to the blood after tubular reabsorption. The renal uptake of calcitonin occurs directly from the bloodstream, as well as through glomerular filtration. In the case of lysozyme (another small, basic protein) tentative evidence for recirculation has been presented [6].

MATERIALS AND METHODS

Radio-labelled proteins. TC was synthesized by the reductive amination of cellobiose with tyramine [6, 7]. TC was labelled with radioactive iodine and attached to calcitonin using *s*-trichlorotriazine as the linking agent [5, 6]: 3 μL of 10 mM TC in water, 5 μL of 0.5 M sodium phosphate buffer, pH 7.5, and 0.5 mCi of $Na^{125}I$ were mixed in a conical test-tube. Three portions of 5 μL of 1 mM sodium oxychloride in 0.05 M sodium phosphate buffer (pH 7.5) were addded at 30 sec intervals. The oxidation reaction was terminated by the addition of 10 μL of 0.05 M Na_2SO_3/0.05 M KI. Ten microliters of 3 mM *s*-trichlorotriazine in acetonitrile was added, followed by 5 μL of 0.01 M NaOH. This solution was left for 30 sec.

The final attachment step was performed as follows: 7 μL of a solution of human calcitonin Sigma Chemical Co., St Louis, MO, U.S.A.), 50 mg/mL in 10 mM EDTA-20 mM Na-phosphate, pH 7.4, and 50 μL of a 0.2 M sodium carbonate buffer, pH 9.0, were added to the activated [^{125}I]TC. After 10 min at room temperature, the reaction mixture was fractionated by passage through a Sephadex G-25 Fine column which was eluted with 0.1 M ammonium acetate and 2% bovine serum albumin at pH 5.0. [^{125}I]TC-calcitonin was stored at −20° in the same buffer. The specific activity of the product was 3×10^5 cpm/μg. The relatively low specific activity was a result of adhering to the labelling procedure, as described in previous publications [5, 6], and avoiding the formation of calcitonin molecules substituted with more than one TC-moiety.

Cells and tissue preparations. UMR 106 osteosarcoma cells were maintained in monolayer culture in Ham's F10 medium (plus penicillin, streptomycin and tylocin) with 10% fetal calf serum [8]. The cells were plated at 10^4 cells/cm^2 and grown for 8 days prior to manipulation.

For preparation of crude membrane fractions, washed cells were homogenized in a Waring blender in 10 mM Tris–HCl buffer, pH 7.5 with 1 mM EDTA (TE-buffer) as described previously [9]. The homogenate was centrifuged at 27,000 *g* for 30 min at 4° and the pellet was resuspended in TE-buffer containing 1% BSA. Tissues (bone, kidney or liver) were minced and washed repeatedly with 0.9% NaCl. These preparations were treated in the same manner as the cells, except that residual connective tissue and bone were removed by filtration through glass wool prior to the centrifugation step.

Adenylate cyclase assays. The assay was carried out in a final volume of 50 μL with 1 mM ATP (including 1.8×10^6 cpm/tube of α-[^{32}P]ATP), 40 μM GTP, 1 mM cAMP with 7200 cpm/tube of [^{3}H]cAMP, 1.4 mM EDTA, 0.1 mM EGTA (ethyleneglycolbis (aminoethylether)tetra-acetate), 3.5 mM Mg^{2+} (1.1 mM free), 0.15 mM Ca^{2+} and 25 mM Tris–HCl (pH 7.5) together with a regenerating system for ATP (20 mM creatine phosphate, 0.2 mg/mL creatine kinase and 0.02 mg/mL of myokinase). CT (1 μM) and the positive control substances PTH (1 μL), glucagon (1 μM), 5′guanylyl imidodiphosphate (Gpp[NH]*p*) (40 μM), PGE_2 (10 μg/mL) or forskolin (400 μM) were added just prior to the final incubation, which was carried out at 35° for 20 min. The reactions were stopped with 0.1 mL of a solution containing 10 mM cAMP, 40 mM ATP and 1% dodecyl sulphate, followed by mixing and immediate cooling to 0°. The [^{32}P]cAMP formed and the [^{3}H]cAMP added to monitor recovery were isolated using Dowex and alumina oxide chromatography [10, 11].

Injection of labelled calcitonin. Male Wistar rats weighing between 300–400 g were anesthetized with Inactin (Byk-Gulden, Konstanz, F.R.G.), 150 mg/kg body weight. Body temperature of the rats was maintained with a heating pad. A trace amount of labelled calcitonin (10 μg/kg of body weight) was diluted to a final volume of 200 μL with 0.9% NaCl before injection into the jugular vein. After intervals ranging from 2.5 min to 60 min, blood samples were taken by cardiac puncture and the animal was killed with an intracardiac injection of 2 mL of 10% Nembutal solution in 50% aqueous ethanol. The kidneys and liver were removed and placed in liquid nitrogen, and urine was collected from the bladder.

Gel permeation chromatography of urine and kidney homogenates. The entire procedure was carried out at 0–2°. Deep-frozen kidneys were allowed to thaw at the working temperature. Tissue samples were homogenized in a Potter-Elvehjem homogenizer in 0.25 M sucrose containing 2 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.2 and 1 mM EDTA . Triton X-100 was added to 0.1% concentration. The homogenate was centrifuged at 10,000 rpm for 30 min in a Sorvall SS-34 rotor and passed through a 0.42 μm Millipore filter. Intact and degraded calcitonin in supernatants or urine samples were separated by gel filtration on Biogel P4 columns eluted with 0.1 M ammonium acetate/2% BSA at pH 5.0. A trace of cyanocobalamin (M_r 1357) was added to serve as a molecular weight marker; the first appearance of this compound in the eluate was taken as the dividing line between high- and low-molecular weight material. Radioactivities in each fraction were determined in a Kontron gamma counter, and added up to give the "area under the curve".

Subcellular fractionation in density gradients. The rats were injected and killed as described above. The kidneys were immediately placed in ice-cold 0.25 M sucrose solution containing 2 mM HEPES and 1 mM EDTA , pH 7.2 ("buffered sucrose"). After removal of most of the connective tissue of the papilla, the kidney was homogenized in 4 mL of the same isotonic buffer in a Dounce homogenizer cooled in ice water (five strokes with the loose-fitting, followed by 10 strokes with the tight-fitting pestle).

Table 1. The effect of [^{125}I]TC-calcitonin on cell and tissue membranes known to possess receptors for calcitonin

Cells or tissues	Adenylate cyclase activities relative to basal		
	[^{125}I]TC-CT	sCT	b1-34PTH
Rat osteosarcoma (UMR 106) cells	3.0 ± 0.1*	3.9 ± 0.3*	14.8 ± 1.1*
Rat calvaria	1.4 ± 0.1*	1.4 ± 0.1*	1.9 ± 0.2*
Rat kidney cortex	1.6 ± 0.2*	1.3 ± 0.1*†	8.8 ± 0.6*

Crude plasma membranes were prepared essentially as descriped in Materials and Methods. Twenty microlitre aliquots (25–40 μg protein) were incubated for 20 min at 35° in the presence of 1.1 mM free Mg^2, 1 mM ATP and 40 μM GTP without or with the addition of 1×10^{-6}M [^{125}I]TC-CT, unlabelled sCT, or b1-34PTH (8^{nleu}, 8^{nleu}, 34^{tyr}). Results are computed as pmoles cAMP/mg protein/min and presented as stimulated AC activities relative to (= divided by) basal levels. Figures represent means ±SD of triplicate determinations.

* Designates $\alpha = P < 0.05$, Wilcoxon rank test; hormone-stimulated vs basal.

† Designates $\alpha = P < 0.05$, Wilcoxon rank test; [^{125}I]TC-CT vs sCT.

The homogenate of kidney tissue was centrifuged at 300 *g* for 5 min in order to remove cell nuclei and unbroken cells. Close to 50% of the radioactivity was recovered in the supernatant. A sample containing 320 μL of the supernatant mixed with 180 μL of 2.0 M sucrose was layered onto an 11 mL linear gradient of 0.8 M to 2.0 M sucrose containing 5 mM HEPES, 0.1 mM EDTA, pH 7.3. The gradients were centrifuged at 33,000 rpm for 14 hr in a Beckman SW 41 Ti rotor using an L-5-50 centrifuge [12]. After centrifugation, the gradients were fractionated. Refractive indices, ^{125}I activity, acid phosphatase activity, *N*-acetyl-β-glucosaminidase activity, alanine aminopeptidase activity, and alkaline phosphatase activity were measured in separate aliquots of the fractions. Eighty per cent of the radioactivity and 93–97% of the marker enzyme activities (63% of acid phosphatase) migrated into the gradient.

Acid phosphatase (EC 3.1.3.2) and *N*-acetyl-glucosaminidase (EC 3.2.1.30) were assayed in 0.1 M sodium citrate buffer, pH 4.3, containing 0.15 mM NaCl. Substrates were phenolphthalein monophosphate (1 mM) (the Sigma Chemical Co., St Louis, MO, U.S.A.) and 4-nitrophenyl-β-D-*N*-acetylglucosaminide (10 mM) (Sigma), respectively. After 30 min, the reactions were stopped by the addition of an equal volume of 0.5 M sodium carbonate. The concentratin of unesterified phenolphthalein was measured at 552 nm, and liberated 4-nitrophenol was determined from the absorbancy at 422 nm, after correction for the contribution from phenolphthalein at this wavelength. As a substrate for acid phosphatase, phenolphthalein monophosphate gives approximately 10% higher activity under these conditions than the standard procedure [13] using glycero-32-phosphate as a substrate.

Alanine aminopeptidase (EC 3.4.11.2) was assayed using 1 mM L-alanine 2-naphthylamide (Sigma) in a 0.1 M sodium phosphate buffer, pH 7.0 [14]. The reaction was stopped by the addition of 1.5 vol. of cold 0.2 M citric acid containing 2% (w/v) Brij 35 and 1 mg/mL diazotized 2-methoxy-4-nitroaniline (Fast Red B Salt, Sigma). The colour was read at 540 nm.

RESULTS

Biological activity of [^{125}I]*TC calcitonin*

In order to check that the biological activity of the trapped-label hormone was intact, its effect on adenylate cyclase activity was compared to that of native calcitonin in rat osteosarcoma (UMR 106) cells, in rat calvaria and in other tissues. Native or [^{125}I]TC-labeled calcitonin were added to the assay mixture at a concentration of 10^{-6} M (alternatively, positive control substances were added, see Materials and Methods).

In the osteosarcoma cells, [^{125}I]TC-calcitonin increased the adenylate cyclase levels to 3 times basal (3.9 times with unlabelled salmon calcitonin) (Table 1). The two preparations increased levels about equally (1.4 times) in rat calvaria. These observations show that unlabelled and [^{125}I]TC-calcitonin possess biological activity at closely similar levels.

In rat kidney, 1.3 times increase in adenylate cyclase activity of the cortex, and none in the medulla, was observed on addition of unlabelled calcitonin, in agreement with a previous study [15]. With labelled hormone, the increase was 1.6 times (Table 1). In rat liver there was no effect of calcitonin, no receptors being present.

Clearance of [^{125}I]*TC-calcitonin from the blood*

Trace doses of [^{125}I]TC-calcitonin were injected into rats and blood samples were taken at intervals up to 60 min. The initial rate of clearance was very fast, less than 5% of the dose remaining in the blood after 2 min (Fig. 1). The fast initial phase of the clearance curve was over after about 15 min. The subsequent slow decline indicated that the period of equilibrium with extravascular fluid was over.

Part of the radioactivity in the blood was bound to degradation products, as determined by gel filtration (see below). After just 2.5 min, 22% was in low-molecular weight material, increasing to 33% after 10 min, but subsequently, the amount in this fraction decreased (14% after 20 min).

Renal uptake of [^{125}I]*TC-calcitonin*

The curve showing uptake of [^{125}I]TC-calcitonin

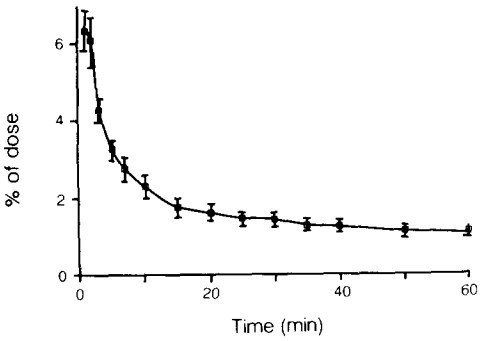

Fig. 1. Clearance of trace doses of trapped-label calcitonin in the rat. A trace dose of [^{125}I]TC-calcitonin was injected into the jugular vein of an anesthesized animal. Blood samples of 50 μL were taken from the femoral vein. Total radioactivity in the blood is shown as percentage of the initial dose. Vertical bars indicate standard errors of the mean of determinations in seven animals. The blood volume was taken as 5.0% of the body weight. [36].

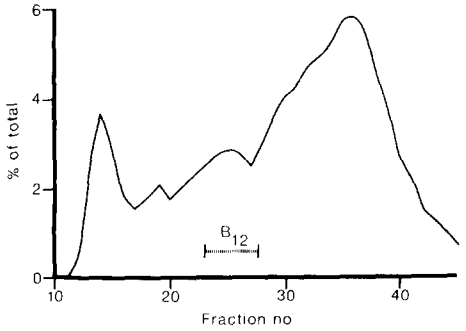

Fig. 3. Gel filtration profile of radioactivity in a kidney homogenate. A kidney was removed 10 min after the injection of [^{25}I]TC-calcitonin, chilled and homogenized. The homogenate was centrifuged and fractionated on a Biogel P4 column. The first appearance of the molecular weight marker cyanocobalamin (M_r 1357) was taken as the dividing line between high- and low-molecular weight material (see Materials and Methods).

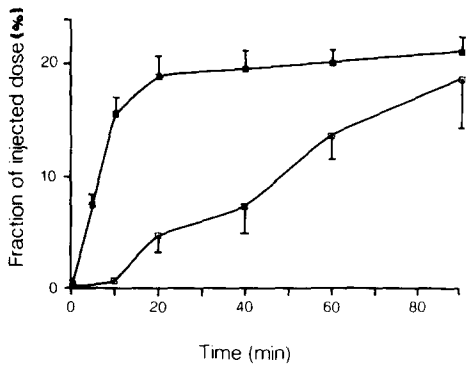

Fig. 2. Recoveries of radioactivity from [^{125}I]TC-calcitonin in kidneys (solid squares) and urine (open squares) as per cent of injected dose. The points represent the means of measurements on 5 to 11 rats. Standards errors of the mean are indicated by bars.

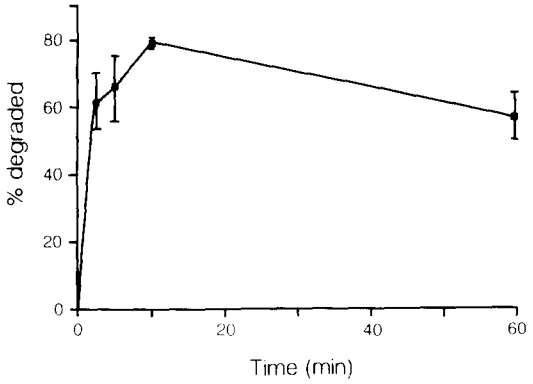

Fig. 4. Renal degradation of calcitonin. Kidneys were removed at various times after the injection of [^{125}I]TC-calcitonin and homogenized in chilled, buffered sucrose. Intact and degraded calcitonin were separated by gel filtration on Biogel P4 (see Fig. 3). Data are the means of determinations in three rats per time point. Bars indicate standard errors of the mean.

into the kidneys also showed two phases: a period of rapid increase up to about 20 min after the injection (19.0 ± 5.8% at this point), then a phase of very slow increase in radioactivity (Fig. 2). After 90 min, 21.4 ± 2.3% was recovered in the kidneys. There was a lag period of 10 min before appreciable radioactivity appeared in the urine (Fig. 2); subsequently, the amount increased steadily during the whole of the measurement period of 90 min, reaching 18.7 ± 9.6%.

Between 5 and 60 min, recovery of [^{125}I]TC-calcitonin in the liver ranged between 15.1 ± 1.1 (SD) and 16.9 ± 8.2% (not shown).

Renal degradation of [^{125}I]TC-calcitonin

Kidneys were removed at various times after the injection, homogenized, and the homogenate was fractionated by gel filtration in order to separate intact calcitonin from degradation products. The distribution profile from a typical run (10 min after the injection) is shown in Fig. 3. There was a distinct peak of high-molecular weight material, smaller quantities of degradation products of intermediate molecular weight, and a large peak of low-molecular weight material. The results of several gel filtration experiments are summarized in Fig. 4. After just 2.5 min, over 60% of the endocytosed calcitonin in the kidneys is degraded; at 10 min, close to 80% was degraded.

The radioactive material in the urine was fractionated by gel filtration. Samples were collected after 20 or 60 min (Fig. 5); at both time points, between 70 and 80% of the radioactivity was found in high-molecular weight fractions.

Intracellular distribution of [^{125}I]TC-calcitonin and its degradation products

At intervals ranging from 2.5 to 15 min after

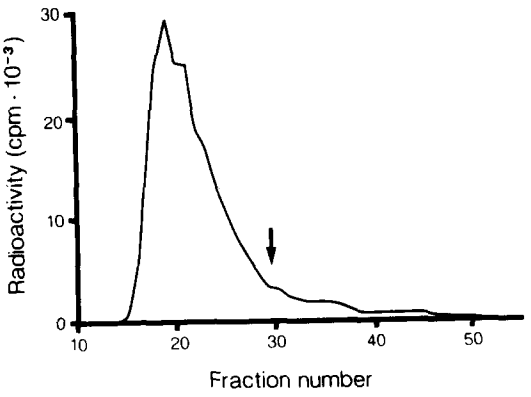

Fig. 5. Fractionation of radioactive material in the urine by gel filtration. A urine sample was taken 60 min after the injection of [^{125}I]TC-calcitonin and fractionated on a Biogel P4 column (see Fig. 3). The arrow shows the position of the peak of cyanocobalamin (M_r 1357). The appearance of this marker was taken as the dividing line between high- and low-molecular weight material.

injecting [^{125}I]TC-calcitonin into a rat, the kidneys were removed and the postnuclear particles from the cortex were fractionated by isopycnic centrifuging in an isotonic density gradient (Fig. 6). The activities of selected marker enzymes for the brush border and the lysosomes were determined (Fig. 7) in each fraction as well as in the original homogenate.

Close to 80% of the radioactivity entered the gradient, showing that at least this percentage was enclosed in subcellular vesicles. As the major portion of the renal radioactivity was acid-soluble even after 2.5 min after the injection, it may be assumed that the gradient distribution profiles mainly show degradation products of various sizes.

After 2.5 min, the radioactivity in the gradient showed a single, asymmetrical peak (Fig. 6A). The shoulder at 1.18 g/mL coincided very closely with the peaks of the brush border marker enzymes (Fig. 7A and C); the main part of the peak was at slightly lower density. Five minutes after the injection, there was a double peak of radioactivity (Fig. 6B); one peak coincided with the peak of the 2.5 min profile, and the other, at 1.19 g/mL, was found in the same general region as the lysosomes, as indicated by one of the lysosomal marker enzymes (*N*-acetyl-glucosaminidase, Fig. 7D); however, the peak did not coincide exactly with either of the lysosomal marker enzymes. Continuing transfer of material into this peak was evident in the 7.5 min distribution profile (Fig. 6C).

After 15 min, nearly all of the radioactivity was found in a single peak (Fig. 6D) which coincided very closely with the peak of the lysosomal marker

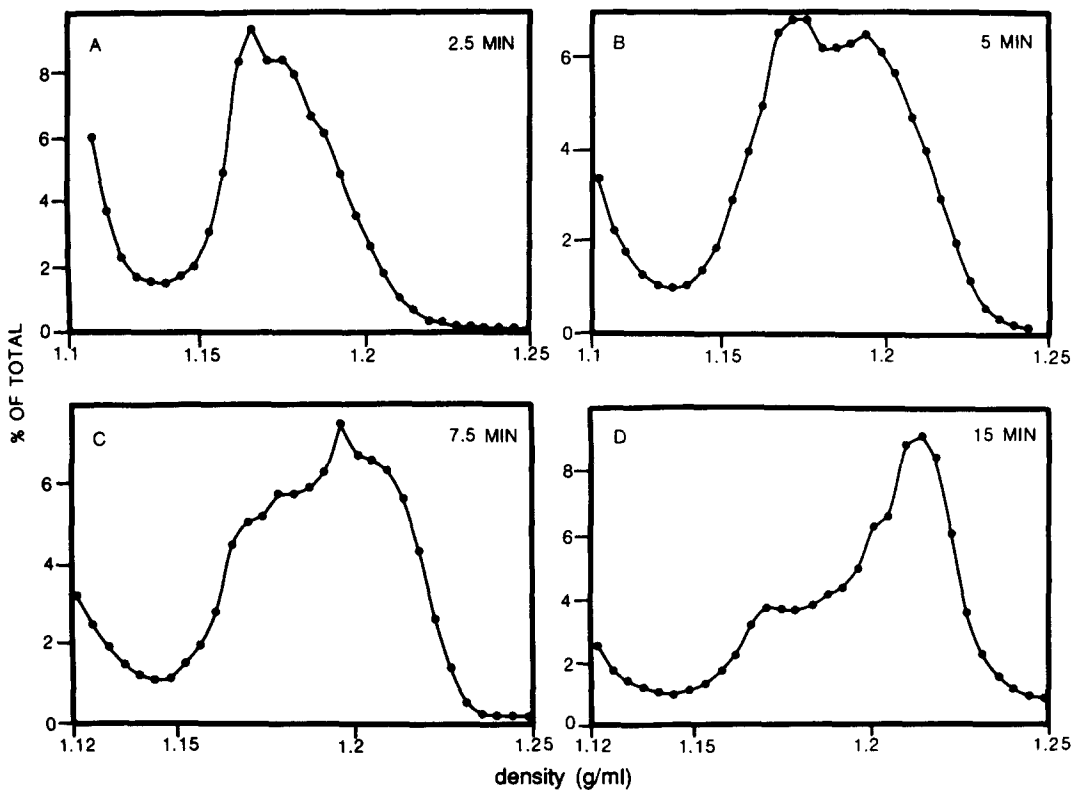

Fig. 6. Subcellular distribution of radioactivity from [^{125}I]TC-calcitonin in the kidney. Following intravenous injection of trace doses of [^{125}I]TC-calcitonin, the rats were killed at the times indicated. The kidneys were removed, chilled on ice, homogenized, and the homogenate was fractionated in sucrose gradients as described in Methods.

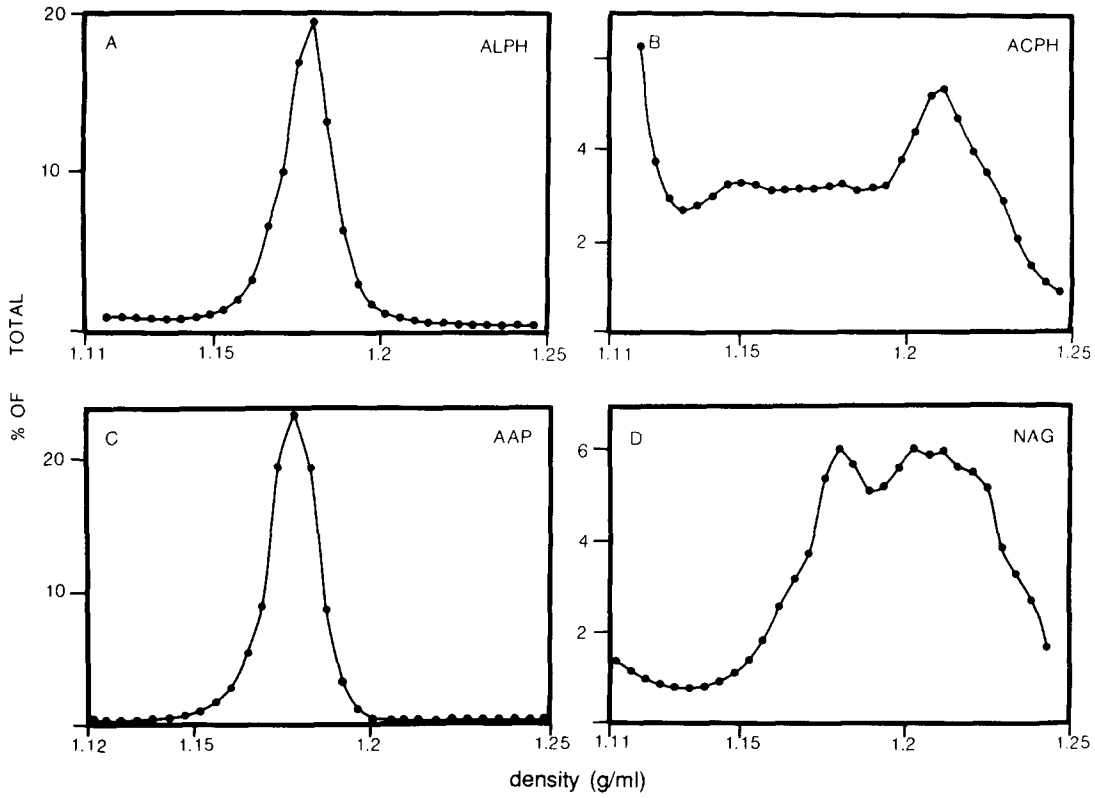

Fig. 7. Subcellular distributions of four marker enzymes in a kidney homogenate. Kidneys were removed, homogenized, the homogenate was fractionated in sucrose gradients, and activities of selected marker enzymes were determined as described in Methods. ALPH, alkaline phosphatase; ACPH, acid phosphatase; AAP, alanine aminopeptidase; NAG, *N*-acetyl-β-glucosaminidase. The gradients are the same as in Fig. 6.

enzyme acid phosphatase (Fig. 7B). At this point, nearly all of the radioactivity represented low-molecular weight degradation products (Fig. 4), which, as previously mentioned, did not leave the lysosomes because the original peptide was labelled by a trapped-label procedure. The large peak at 1.22 g/mL in Figs 6D and 7B presumably contain lysosomes which are responsible for the final degradation of labelled calcitonin.

DISCUSSION

These experiments indicate that the kidneys play an important part in the catabolism of calcitonin. This is to be expected, because low molecular weight proteins can pass the glomerular membrane in a concentration close to the concentration found in plasma. However, no previous report has described the true time course of renal uptake of calcitonin. Using conventionally iodinated hormone, kidney calcitonin recovery apparently peaked 10 min after the injection [4]. In our study, the time course of recovery of calcitonin in kidneys was shown to be biphasic. During the first 20 min after injection, the amount of calcitonin increased very rapidly. Subsequently, the kinetics of uptake changed to a slow, linear increase.

The time course of renal accumulation of radioactivity reflected the activity levels in the blood, which initially decreased rapidly, then remained at a very low level. The clearance was fast and complete: approximately 3% of the dose remained in the blood after 5 min. The low level may represent an equilibrium between elimination and re-entry into the blood stream from the extravascular space.

Activation of adenyl cyclase by calcitonin was not materially altered by coupling of the peptide with [^{125}I]TC, indicating that the labeled hormone bound to specific receptors. The TC-triazine moiety binds only to amino groups. There are two on human calcitonin: one on Lys-18, which is apparently not involved in binding to the receptor; the other one is the amino terminal. Acylation of this amino group may cause a detectable increase in affinity for the receptor [16]. Thus, retention of specific binding after conjugation with TC agrees with present knowledge of the chemistry of calcitonin.

Marx *et al.* [17] and Warshawsky *et al.* [18] demonstrated that calcitonin is bound to calcitonin receptors at the basolateral side of distal tubular cells as well as to receptors on the brush border. The high renal recovery of calcitonin may also in part reflect calcitonin which is tightly bound to calcitonin receptors at the basolateral side of distal tubular cells.

Dissociation of calcitonin from its receptors is very slow [19]. Evidence from electron microscopic studies indicates that luminal uptake predominates [18]. In luminal endocytosis, scavenger receptors [20, 21] may be involved.

Several potential sites of renal calcitonin degradation have been identified by incubation of cortical fractions with labelled calcitonin *in vitro* [22] but some of these sites may play only minor roles *in vivo*. It is not known whether calcitonin taken up from the two different poles of the proximal tubular cells eventually enters the same lysosomes. In the cell fractionation experiments, a single narrow peak of ^{125}I activity, corresponding to the position of acid phosphatase in the gradient, was observed 15 min after injection. (The shoulder at 1.15 g/mL corresponds to the endosomal peak observed at earlier time points.) The narrow peak may contain calcitonin degradation products from both poles of the cell, consistent with the idea that only a single class of lysosomes is involved [23]. No calcitonin degrading activity is associated with the basolateral membrane [22, 24].

In our study, a substantial percentage of calcitonin (approximately 60%) is found degraded in kidney homogenate 2.5 min after injection. The intracellular location of this very rapid degradation was elucidated by fractionating kidney homogenates in sucrose gradients at various time points ranging from 2.5 to 20 min. At 2.5 and 5 min after injection, the peak of radioactivity from [^{125}I]TC-calcitonin was located in fractions with density 1.17 g/mL. Peak activities of the lysosomal enzymes acid phosphatase and *N*-acetylglucosaminidase were found at density 1.20–1.21 g/mL, where peak calcitonin activity was recovered later (15 min after injection). These findings suggest that the substantial degradation of calcitonin found at 2.5–5 min after injection takes place before the intracellular transport of calcitonin has reached lysosomal compartments. Substantial calcitonin-degrading activity is associated with the brush border [24]. Significant degradation in endosomal compartments has been reported in hepatocytes [25–27] and macrophages [28, 29], but endosomal degradation is by no means universal [30, 31].

Part of the material which was filtered by the glomeruli may have been intermediate-sized peptides which were further degraded by enzymes in the brush border; however, most of the labelled material taken up by the tubular cells was probably intact calcitonin. The endocytosed calcitonin was subjected to rapid proteolysis; there was no discernible "lag period" between endocytosis of the ligand and the appearance of degradation products. In the renal degradation of lysozyme and cytochrome *c*, a lag period was observed [6, 32]. This indicates that calcitonin is more susceptible to degradation and/or transported faster than lysozyme or cytochrome *c*.

The high renal recovery of degradation products from [^{125}I]TC-calcitonin confirms the hypothesis that the degradation products of TC-labelled peptides will not leave the epithelial cells where they are reabsorbed and degraded, as only a minor amount of degradation products from [^{125}I]TC-calcitonin was found in the urine.

However, recovery of intact hormone in the urine increased linearly during the entire experimental period, starting at zero-level. The high recovery (15% of injected dose) of intact [^{125}I]TC-calcitonin in urine 60 min after injection contrasts with our previous observations with TC-labelled preparations of the cationic proteins lysozyme or cytochrome c: Less than 2% of the radioactivity from these peptides was recovered in the urine [32]. From TC-labeled insulin (an anionic peptide hormone), 5% of the dose was found as fragments in the urine after 90 min [33]. (Actually, the sum of the recoveries in the kidneys and in the urine were rather similar for [^{125}I]TC-calcitonin and [^{125}I]insulin after 60–90 min, implicating renal filtration as a major determinant of elimination of both peptides.)

An explanation of the high recovery of intact calcitonin in the urine must take into account the time course of excretion: very little intact calcitonin enters the urine while the concentration in the blood—and the filtered amount—is high. This observation excludes the hypothesis that a fraction of the filtered calcitonin remains in the urine simply because of a low affinity for the scavenger receptors. Furthermore, 80% of the activity found in urine was intact [^{125}I]TC-calcitonin and only 20% degradation products, so tubular cleavage of calcitonin can only be a minor cause of the low receptor affinity. One possibility remains: loss of intact molecules by diacytosis (retro-endocytosis), i.e. fusion of endocytic vesicles with the plasma membrane of the brush border [34, 35]. These vesicles may originate in the basolateral membrane, which has no calcitonin degrading activity [22, 24]. In this case, the hormone would be transferred to the urine by transcytosis.

These studies show that the kidneys play an even more important role in the elimination of calcitonin than previously assumed; the sum of the recoveries in the kidneys and the urine reaches 40% of the dose after 90 min, and this is close to three times higher than the highest renal recovery which was reported for this hormone previously. The degradation of the peptide is rapid and starts immediately after endocytosis, most likely in endocytic vesicles. The degradation products are transported to lysosomes of a single type, regardless of whether endocytosis occurred from the apical or basolateral side of the cell.

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