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# Distribution of calcitonin-sensitive adenylate cyclase activity along the rabbit kidney tubule

(nephron microdissection/enzyme microdetermination)

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ABSTRACT **The adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] sensitivity to salmon calcitonin in 11 different segments of the rabbit nephron was investigated using a micromethod for enzyme activity measurements in samples, each containing a single piece of tubule. The required segments were isolated by microdissection from collagenase-treated rabbit kidneys. The results were expressed as femtomoles of adenosine 3′:5′-cyclic monophosphate formed per mm of tubular length per 30 min of incubation time. In the presence of 0.1 μg/ml of synthetic salmon calcitonin, it was found that eight segments exhibited no hormonal sensitivity whereas maximal responses were induced in three segments, the "bright" portion of the distal convoluted tubule, the cortical and the medullary portions of the thick ascending limb of the loop of Henle (stimulated over control activity ratios were 32, 11, and 27). The very high sensitivity to calcitonin of the adenylate cyclase contained in these three segments (0.01 ng/ml of salmon calcitonin inducing a 2-fold stimulation; half-maximal stimulation corresponding to about 0.3 ng/ml of salmon calcitonin) suggests that the distal convoluted tubule, as well as the cortical and medullary portions of the thick ascending limb of the loop of Henle represent physiological target structures of calcitonin action within the kidney.**

It has been shown several times that calcitonin is able to stimulate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in homogenates or in membrane fractions prepared from kidney cortex (1–9) as well as from outer medulla (3–5, 7, 8). Such preparations contain a heterogeneous mixture of membranes from all cell types present in the original tissue. It is impossible, with this type of technique, to establish which cell types or structures actually contain the enzyme responsive to this hormone.

To overcome this major limitation, we recently developed a micromethod (10) in which different segments of the nephron were isolated by microdissection from collagenase treated rabbit kidneys. Their respective enzyme activity was measured separately in samples containing a single piece of tubule. This technique made it possible to investigate the location of the adenylate cyclase activity responsive to parathyroid hormone (11, 12), vasopressin (13, 14), and catecholamines (15) along the rabbit nephron.

Abbreviations: AC, adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]; SCT, synthetic salmon calcitonin; cAMP, adenosine 3′:5′-cyclic monophosphate; PCT, proximal convoluted tubule; PR, pars recta of the proximal tubule; TDL, thin descending limb of the loop of Henle; TAL, thin ascending limb; MAL, medullary portion of the thick ascending limb of the loop of Henle; CAL, cortical portion of the thick ascending limb of the loop of Henle; DCTb, bright appearing portion of distal convoluted tubule; DCTg, granular appearing portion of distal convoluted tubule; CCTg, granular portion of cortical collecting tubule; CCTl, light portion of cortical collecting tubule; MCT, medullary portion of collecting tubule; PTH, parathyroid hormone.

In this paper, we report the distribution of the salmon (SCT) calcitonin sensitive activity of adenylate cyclase.

## MATERIALS AND METHODS

**Methods.** The technique used to prepare samples containing one single piece of kidney tubule and to measure their adenylate cyclase activity has been reported in detail elsewhere (10). The main steps only will briefly be recalled here. The kidneys were removed from pentobarbital anesthetized rabbits (1–2 kg of body weight); pieces of kidney tissue were first incubated in "collagenase" solution for 35–45 min at 35°. After a short wash, these pieces were transferred into ice-cold modified Hanks' solution. Microdissection of the required tubular structures was then performed by hand under stereomicroscopic observation. Well defined segments (or portions of segments) were obtained by sectioning the microdissected tubules according to precise anatomical and morphological criteria (10, 16). The characteristics and location of the different nephron segments used are specified at the beginning of the *Results* section.

Once isolated, each selected piece of tubule was photographed in order to measure its length. An osmotic shock plus a freezing step was applied to all samples in order to ensure permeabilization of the tubular cells to nucleotides.

Adenylate cyclase activity measurement was started by adding 2 μl of "incubation solution" to each sample. After 30 min incubation at 30°, the reaction was terminated by adding 150 μl of buffer solution containing a large excess of cold ATP and adenosine 3′:5′-cyclic monophosphate (cAMP) with a tracer amount of [$^3$H]cAMP for recovery calculation. The [$^{32}$P]cAMP formed was separated from the other $^{32}$P-labeled nucleotides by passing the mixture through a Dowex column, and then through an aluminium-oxide column, as described by Salomon *et al.* (17). The results were expressed as femtomoles ($10^{-15}$ mol) of cAMP formed per 30 min incubation period per mm of tubular length. We previously checked that under the conditions used, the amount of cAMP formed increased proportionally with the incubation time (up to 60 min) and with the length of the piece of tubule contained in the samples (10).

**Materials.** The microdissection solution was a modified Hanks' solution as previously described (10). The "collagenase" solution was of the same composition as the microdissection solution except that the calcium concentration was 1 mM and collagenase and bovine serum albumin were added to a final concentration of 0.1% each. The incubation solution was similar to that used by Bockaert *et al.* (19) as regards regenerating system, ionic concentration, buffer, ATP, and cAMP concentrations; modifications concerned only $MgCl_2$ concentration which was 3.8 mM and [$^{32}$P]ATP which was used at a higher specific activity (1–2 mCi/μmol).

The salmon calcitonin used was a synthetic hormone (batch

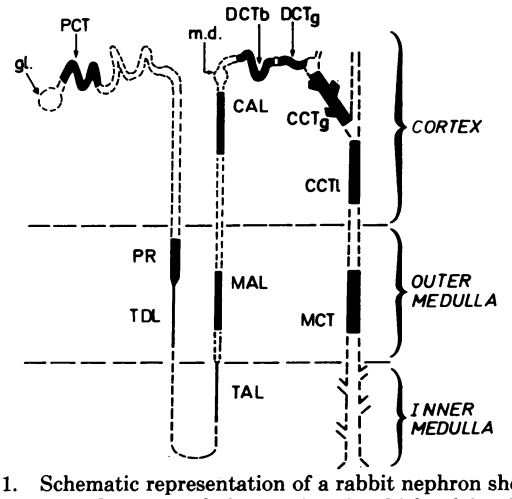


FIG. 1. Schematic representation of a rabbit nephron showing (black parts) the different tubular portions in which calcitonin-dependent adenylate cyclase activity was measured. PCT, proximal convoluted tubule; PR, pars recta; TDL, thin descending limb and TAL, thin ascending limb of the loop of Henle; MAL and CAL, medullary and cortical portions of the thick ascending limb, respectively; DCTb and DCTg, "bright" and "granular" portions of the distal convoluted tubule, respectively; CCTg and CCTl, "granular" and "light" portions of the cortical collecting tubule, respectively; MCT, medullary collecting tubule (gl. refers to the glomerulus and m.d. to the macula densa).

20-051 from Sandoz with a specific activity of 4000 units/mg). Molarities were calculated by assuming a molecular weight of 3400 for salmon calcitonin.

## RESULTS

The adenylate cyclase (AC) sensitivity to calcitonin was tested in 11 different portions of the rabbit nephron. The precise localization of these portions within the kidney is schematically depicted by Fig. 1. The samples were prepared according to the following anatomical criteria: proximal convoluted tubule (PCT), early portion, 1–1.5 mm of tissue immediately after the glomerulus; pars recta of the proximal tubule (PR), late portion, about 1 mm of tissue isolated from the outer medulla, and ending at the transition with the thin segment; thin descending limb (TDL) of the loop of Henle, about 1 mm of tissue isolated from the outer medulla (TDL samples were often paired with PR samples from the same nephrons); thin ascending limb (TAL), 0.5 to 1 mm of tissue isolated from the inner medulla together with the corresponding thick ascending limb segment (this made it possible to pair TAL samples with samples of thick ascending limb segment from the same loops); medullary portion of the thick ascending limb of the loop of Henle (MAL), about 1 mm of tissue isolated from the outer medulla at the level where transitions between PR and TDL were observed; cortical portion of the thick ascending limb of the loop of Henle (CAL), 1–1.5 mm of tissue microdissected from the outer cortex, and ending at the macula densa, which is always easy to recognize in microdissected tubules; in collagenase-treated rabbit kidneys as used in this study, the distal convoluted tubule, that nephron segment between the macula densa and the first branching with another tubule, is of a nonuniform appearance along its full length as observed under a stereomicroscope (15, 16); the first portion is of a tortuous and "bright" appearance (DCTb), whereas the later portion is of a "granular" appearance (DCTg). Differences in adenylate cyclase sensitivity to hormones were observed to correspond to these differences in morphological appearance (ref. 16) and, therefore, the DCTb and the DCTg portions were separated from each other and pairs of samples

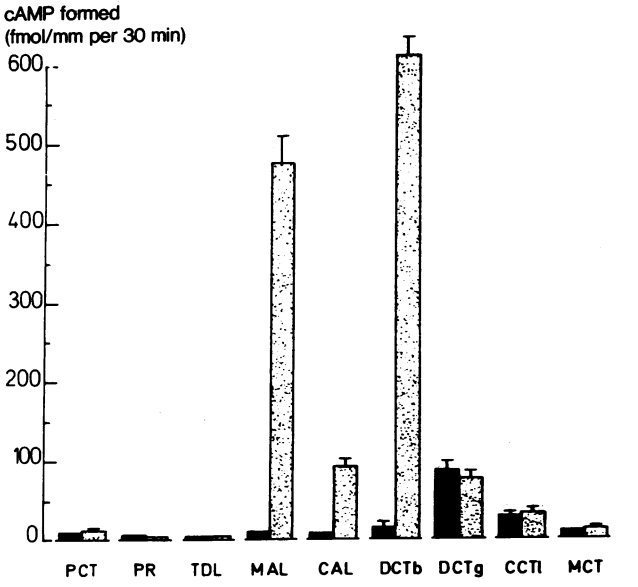


FIG. 2. Distribution of cacitonin-dependent AC activity along the rabbit kidney tubule, as measured in one single experiment. Each bar is the mean value (±SEM) of three to four samples. For explanation of the abbreviations used, refer to the legend of Fig. 1 and to the *text*. Note that the hormone stimulated AC activity in MAL, CAL, and DCTb. Incubation conditions are given in the *text;* as for the other figures and tables, the results are expressed as femtomoles ($10^{-15}$ mol) of cAMP formed per mm of tubular length per 30 min incubation time at 30°. Control, ■; SCT, 100 ng/ml, □.

(0.2–0.4 mm each of tissue) were prepared from most of the microdissected distal convoluted tubules; the cortical portion of the collecting tubule was also observed to be of heterogeneous appearance (16) and to contain a branched, "granular" portion (CCTg) resembling the DCTg, and a straight poorly branched portion of a "light" appearance (CCTl) and separate samples (about 1 mm of tissue) were prepared for each of these portions; medullary portion of the collecting tubule (MCT), 0.5–1 mm of tissue microdissected from the outer medulla at the same level as the TDL and MAL samples.

The adenylate cyclase activity measured in the absence of hormonal stimulation (controls, Table 1) is in good agreement with the pattern we previously reported (10, 16); this pattern is characterized by a lower AC activity in the pars recta (PR) than in the pars convoluta (PCT) of the proximal tubule, and by a maximal enzyme activity per mm of tubular length in the "granular" portions of both the distal tubule (DCTg) and the collecting tubule (CCTg).

The distribution along these different segments of the rabbit nephron which was observed for the calcitonin-sensitive activity of adenylate cyclase is depicted by a typical experiment on Fig. 2 and summarized by mean values on Table 1. It is clear from the data that SCT, 100 ng/ml, stimulated AC activity in three of the eleven tested segments. Very high responses (about 30-fold as compared to control values) were obtained in the medullary portion of the thick ascending limb (MAL) and in the "bright" portion of the distal convoluted tubule (DCTb). The cortical portion of the thick ascending limb (CAL) responded to a lesser extent both in absolute and relative values. As for the other segments tested, none, including the "granular" portion of the distal tubule (DCTg), i.e., the portion continuing the "bright" portion, exhibited a statistically significant response to the high SCT concentrations used.

Two or three different SCT dose-response curves were established for each responsive segment. Fig. 3 shows the curves

Table 1. Distribution of calcitonin-sensitive adenylate cyclase activity along the rabbit kidney tubule

| Segment | No. | Adenylate cyclase activity (fmol of cAMP/mm per 30 min) | | P | Stimulated / Control |
|---|---|---|---|---|---|
| | | Control | SCT-stimulated | | |
| PCT | 2 | 11.5 ± 3.3 (6) | 13.1 ± 1.5 (7) | N.S. | 1.1 |
| PR | 2 | 4.6 ± 0.8 (7) | 4.2 ± 1.6 (7) | N.S. | 0.9 |
| TDL | 2 | 10.6 ± 4.6 (5) | 6.7 ± 1.8 (7) | N.S. | 0.6 |
| TAL | 2 | 8.5 ± 0.6 (3) | 9.5 ± 5.9 (6) | N.S. | 1.1 |
| MAL | 5 | 14.4 ± 3.9 (11) | 389.9 ± 35.2 (19) | <0.001 | 27.1 |
| CAL | 3 | 8.3 ± 2.3 (10) | 92.9 ± 7.4 (12) | <0.001 | 11.2 |
| DCTb | 4 | 25.0 ± 4.8 (12) | 814.6 ± 60.3 (16) | <0.001 | 32.6 |
| DCTg | 3 | 89.7 ± 6.1 (10) | 87.1 ± 6.8 (10) | N.S. | 1.0 |
| CCTg | 3 | 67.5 ± 5.1 (9) | 67.7 ± 7.9 (12) | N.S. | 1.0 |
| CCTl | 2 | 30.9 ± 4.2 (7) | 31.3 ± 6.1 (7) | N.S. | 1.0 |
| MCT | 2 | 13.3 ± 2.6 (7) | 14.3 ± 2.5 (7) | N.S. | 1.1 |

This table gives the mean value ± SEM of the AC activity measured in 11 different segments of the rabbit nephron in the presence of 100 ng/ml of synthetic salmon calcitonin (SCT), as well as the corresponding control values measured in the same experiments in the absence of hormone. The exact definition of the abbreviations used for the different segments is given in the *text* and their location along the nephron is shown on Fig. 1. No. indicates the number of experiments in which the corresponding structure was studied. The number of samples used for calculating each mean value is indicated in parentheses. *P* indicates comparisons between SCT-stimulated and corresponding control mean values, as calculated according to the Student *t* test. N.S. indicates values are not significant.

obtained from one of these experiments for MAL and DCTb. An average curve using CAL samples from three experiments is plotted in Fig. 4. The different curves obtained were quantitatively comparable from one experiment to another and qualitatively similar for the three structures. A statistically significant stimulation, about 2-fold, was observed on each structure with the 0.01 ng/ml of SCT concentration. Half-maximal responses were found to correspond to SCT concentrations of 0.25 and 0.30 ng/ml with MAL, 0.27, 0.25 and 0.35 ng/ml with DCTb, and 0.36 and 0.35 ng/ml with CAL. In these experiments, the 1 ng/ml of SCT concentration induced nearly maximal responses which indicates that the 100 ng/ml concentration used in the experiment summarized in Table 1 would have been enough to stimulate calcitonin receptors coupled to AC activity if present in other segments.

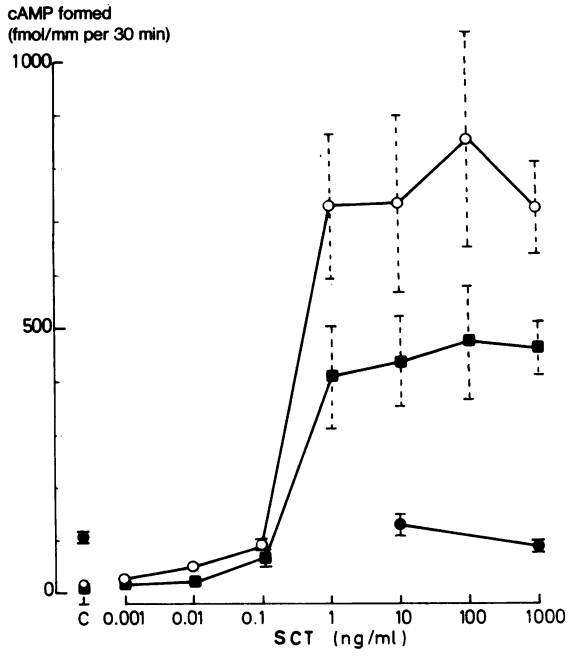


FIG. 3. Examples of calcitonin dose-response curves obtained in one experiment for two different SCT-responsive portions of the nephron. MAL, medullary portion of the thick ascending limb (■); DCTb (○) and DCTg (●), "bright" and "granular" portions of the distal convoluted tubule, respectively. C, control activity measured without hormone added. Each point is the mean value (±SEM) of three to four replicate samples. All samples were microdissected from the same rabbit kidney. DCTb and DCTg samples were paired from the same distal tubules. Note that DCTg samples had a high control AC activity and exhibited no response to SCT. Where not indicated, SEM values were smaller than the size of the corresponding points.

## DISCUSSION

The results demonstrate that, under the conditions used, synthetic salmon calcitonin stimulates AC activity in three well defined portions of the rabbit nephrons, namely the MAL, CAL, and DCTb portions. The sensitivity of these segments to the hormone was high, since half-maximal stimulations corre-

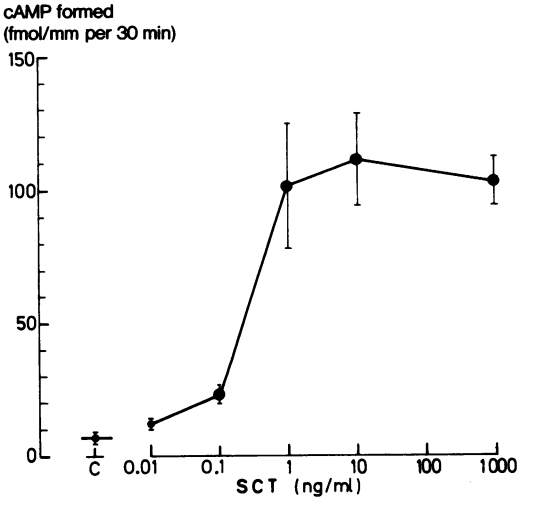


FIG. 4. Average calcitonin dose-response curve for the cortical portion of the thick ascending limb (CAL). Each point is the mean value (±SEM) of 8 to 13 samples from three different rabbit kidneys. C, controls without hormone. When compared to Fig. 3, note that CAL had a sensitivity to SCT similar to that measured for MAL and DCTb, but exhibited responses of a lower magnitude per mm of tubular length.

sponded to SCT concentrations ranging between 0.07 and 0.1 nM. A statistically significant 2-fold stimulation was elicited at 3 pM SCT. Such a sensitivity is about 100 times greater than that measured for salmon calcitonin on rat kidney homogenates by Heersche *et al.* (6) and Loreau *et al.* (7). The apparent $K_m$ for AC activation we have observed falls in the range of the values reported by Marx *et al.* (4, 8, 20) for $^{125}$I-labeled SCT binding to high affinity sites in renal plasma membranes from the rat.

Although the thick ascending limb and DCTb constitute together only a small fraction of the whole kidney tissue, the stimulation factor induced by SCT was high enough in these segments (up to 30-fold) to account for the stimulation ratios in kidney cortex homogenates reported in the literature (1–9). Therefore, it is not necessary to postulate an action of calcitonin on AC in proximal tubules, although differences may exist among animal species.

Table 1 and Fig. 2 indicate that samples from both the early part of the proximal convolution and the late part of the pars recta did not contain AC responsive to SCT. It appeared necessary to check if this also holds true for the portions of the proximal tubule included between these two end parts. This control experiment was carried out by microdissecting convoluted tubules and straight portions over their full length and by preparing series of successive samples from these complete structures. In the presence of SCT (100 ng/ml) the results [PCT, $8.6 \pm 1.4$ ($N = 15$); PR, $5.7 \pm 1.0$ ($N = 9$) fmol/mm per 30 min] were not statistically different from those measured in the corresponding control samples. This demonstrates that AC is nonresponsive to calcitonin along the full length of the proximal tubule.

Since MAL (one of the segments responsive to SCT) is located in the outer medulla, it is possible to explain the observation, reported by Marx *et al.* (4, 5), that homogenates not only from cortex but also from outer medulla contained calcitonin-sensitive AC.

In the present study, MAL and CAL samples corresponded to the respective ends of the thick ascending limb; the portion in between, i.e., the portion located in the deep cortex and at the cortico-medullary junction was not used (see Fig. 1). To test the sensitivity to SCT of this intermediate portion we performed an additional experiment in which thick ascending limbs were microdissected over their full length, i.e., from MAL up to the macula densa, and subdivided into four successive samples. The results obtained in the presence of SCT (10 ng/ml) on three such series of samples clearly established that AC contained in the thick ascending limb is SCT-responsive over its full length. However, it should be noted that the response elicited in CAL was much less than that induced in MAL, a result in agreement with those reported in Table 1.

The responses elicited in MAL and DCTb were definitely more pronounced than that induced in the segment located in between (CAL). This is clearly shown by the ratio of stimulated over control values calculated for the three segments (Table 1). This was also apparent from the results expressed as percent of the responses induced by 5 mM fluoride on the same structures, in the experiment depicted in Fig. 1 (data not shown); SCT maximal response represented 75% of the fluoride response in MAL, 75% in DCTb, and only 45% in CAL.

It should also be stressed that the transition, in respect to responsiveness, between the thin and thick portions of the ascending limb, as well as the transition between the "bright" and the "granular" portions of the distal convoluted tubule were quite sharp as evidenced by the data obtained from many paired samples of successive portions from the same nephron. The results obtained with calcitonin further substantiate the striking difference in AC sensitivity with hormones, which we observed along the successive portions of the distal convoluted tubules when using parathyroid hormone, vasopressin, and isoproterenol (16).

The DCT "bright" portion (which contains SCT-sensitive AC) exhibited no response to the other hormones we have tested so far, whereas the DCT "granular" portion (which does not contain SCT-sensitive AC) responded to parathyroid hormone (PTH) and isoproterenol (15, 16).

Furthermore, we previously reported that CAL contains PTH sensitive AC (11, 12) and MAL contains vasopressin-sensitive AC (13, 14). It thus appears that the segments mainly sensitive to calcitonin (MAL and DCTb) are insensitive to PTH, whereas those sensitive to PTH (PCT, PR, DCTg, and CCTg) (11, 12) are insensitive to SCT. CAL only responded to these two hormones and only to a limited extent. The observation that the main sites of calcitonin and PTH actions within the kidney are separated may explain why most authors (5–7, 9) but not all (2, 3, 21) found that the effects of these two hormones on cAMP generation were additive when tested together.

The overall action of calcitonin on the mammalian kidney includes natriuric, calciuric, and phosphaturic effects (22–24), the magnitude of which depends on experimental conditions, animal species and the origin of the hormonal preparation used. In this respect, the salmon hormone has repeatedly been observed to be the most potent calcitonin available, both in *in vivo* (25) and *in vitro* studies (4, 8). It is generally accepted that when administered to mammals, salmon calcitonin mimics the effects produced by the endogenous hormone. In the present study, according to the sensitivity of the response elicited by SCT, the magnitude of the maximal effect achieved, as well as to the characteristic distribution of the responsive segments along the nephron, we are allowed to conclude that MAL, CAL, and DCTb should be target structures for the physiological action of calcitonin within the rabbit kidney; such a conclusion, of course, does not exclude the possibility that the hormone may act on other segments of the nephron, insofar as mechanisms different from AC stimulation are involved.

Finally, it would be of importance to discuss the data reported here in terms of the nature of the physiological effects induced by the hormone on its responsive segments. Unfortunately, most of the data available in the literature concerning the permeability and transport properties of MAL, CAL, and DCT are related to salt and water; there is only one preliminary report (26) demonstrating the presence of active calcium reabsorption in MAL by using *in vitro* microperfused tubules. In addition, the overall effect of calcitonin on the rabbit kidney has not been fully investigated. Consequently, this part of the discussion should be deferred to the moment when the physiological function of these nephron portions are better known.

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