ANALOGUES OF HUMAN CALCITONIN
I. INFLUENCE OF MODIFICATIONS IN AMINO ACID POSITIONS 29 AND 31 ON HYPOCALCAEMIC ACTIVITY IN THE RAT

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1. Introduction

The principal sites of calcitonin biosynthesis are the C-cells of the thyroid gland in mammals and the ultimobranchial body in birds, reptiles and fish. The amino acid sequences of the thyrogenic calcitonins from four species are known and two, the human [1] and the porcine [2] hormones, have been synthesized [3,4,5]. Attempts to elucidate the sequence of and synthesize ultimobranchial peptides, on the other hand, have up to now only succeeded in the case of salmon calcitonin [6,7], though others have been isolated in highly purified form. The calcitonins so far obtained from ultimobranchial glands exhibit greater biological activity in rat assays than those of thyroid origin [8]. This must be attributable to some particular amino acids, since the sequential differences between the thyroid hormones, which are all roughly equal in activity, are as great or even greater than the differences between them and the ultimobranchial peptides.

In contrast to that of other peptide hormones, e.g. corticotropin, parathyroid hormone or glucagon, the biological potency of the calcitonin peptides depends to a great extent on the presence of an intact C-terminus. As was shown earlier, curtailment of the porcine thyrocalcitonin chain [9] by a single amino acid, or even by the removal of the amide group at C-terminal proline, drastically reduced the hypocalcaemic activity of the peptide. Similar findings were made with HCT* (unpublished observations). One of the conspicuous differences between HCT and SCT 1 is the more hydrophilic character of the C-terminal region of the latter (formulae fig. 1). In view of the remarkably potent biological activity of SCT 1 it was therefore of interest to study the influence of particular amino acids close to the C-terminus.

* *Abbreviations:* HCT: human calcitonin = calcitonin M [1]; SCT 1: salmon calcitonin as first isolated; SCT 2: iso hormone; SCT 3: iso hormone.

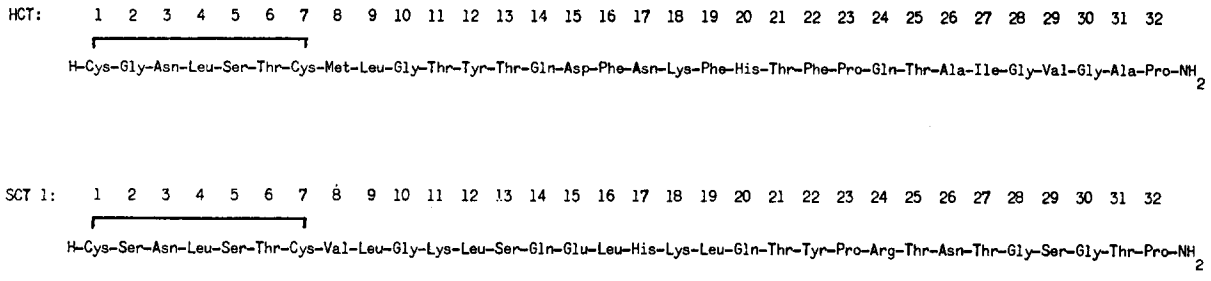

Fig. 1. Chemical structures of human calcitonin (HCT) and salmon calcitonin (SCT 1).

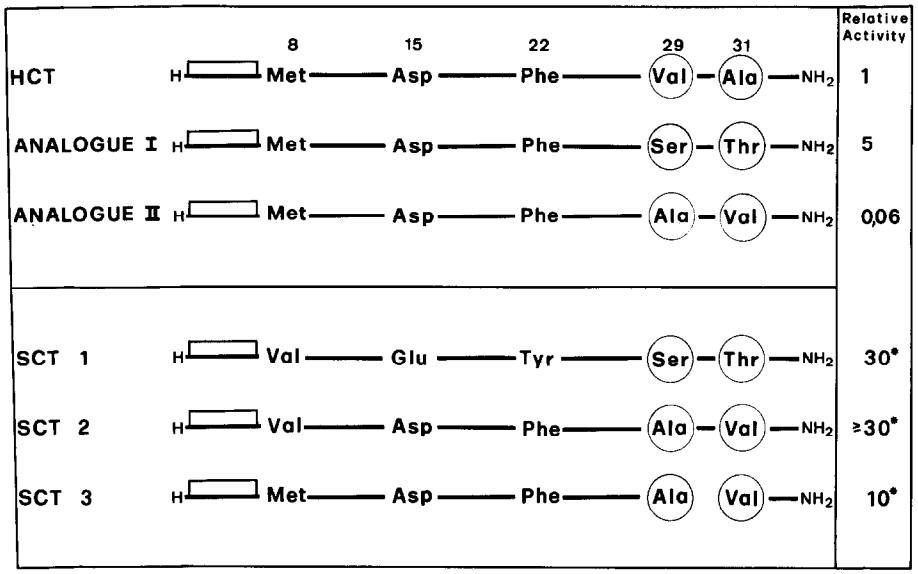

Fig. 2. Abbreviated formulae of calcitonin preparations tested (upper panel) and discussed (lower panel). HCT and its two analogues were tested in 6-point assays as shown in fig. 2. The activities of SCT 1 and the two iso hormones SCT 2 and SCT 3 were taken from ref. [9]. All activities were expressed in relation to HCT, which was arbitrarily set at 1.

In this communication, the significance of the amino acids in positions 29 and 31 of the HCT chain is examined. In this peptide these positions are respectively occupied by valine and alanine. A synthetic analogue in which these amino acids had been replaced by the corresponding amino acids of SCT 1, i.e. serine and threonine showed five times greater hypocalcaemic activity than natural HCT and a slightly longer duration of action. Reversal of their sequence to Ala^{29} and Val^{31}, as they occur in two isohormones of SCT 1 [10,11], yielded a synthetic analogue of HCT with very much weaker hypocalcaemic potency. It displayed less than 10% of the activity of HCT, and its duraction of effect was also shorter. The importance of these two positions is discussed by reference to SCT 1 and its isohormones (formulae fig. 2).

2. Materials and methods

The two analogues [Ser^{29}, Thr^{31}]HCT and [Ala^{29}, Val^{31}]HCT were synthesized by fragment condensation procedures, closely following the pathway described for the synthesis of HCT [3]. Both preparations were obtained in form of the hydrochlorides. They were injected in 0.4 ml acetate buffer solution (pH 4.6) into female rats of the Sprague-Dawley strain (Tierfarm, Sisseln) weighing 100–120 g. The animals had been fed a standard laboratory diet and kept under controlled lighting conditions. Prior to each experiment, they were starved for 16 hr, but allowed water. The hypocalcaemic potency of the substances was determined by the method of Kumar et al. [10]. Plasma calcium concentrations were measured by flame photometry. The areas covered by the concentration curves were determined by planimetry, one hour being plotted on the abscissa as 2 cm, and 0.1 mEq Ca^{++} per litre of plasma on the ordinate as 1 cm.

3. Results

3.1. *Hypocalcaemic potency (fig. 3)*

The substitution of Ser^{29} and Thr^{31} for Val^{29} and Ala^{31} in the HCT molecule produced an analogue ([Ser^{29}, Thr^{31}]HCT) with a hypocalcaemic potency

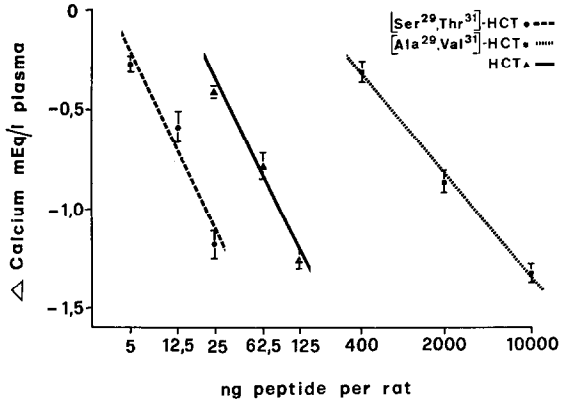

Fig. 3. Dose-response regression lines of the hypocalcaemic activity measured 50 min after an intravenous injection of HCT and the two analogues [Ser^{29}, Val^{31}]HCT. Each point represents the means for 15–20 rats ± SEM.

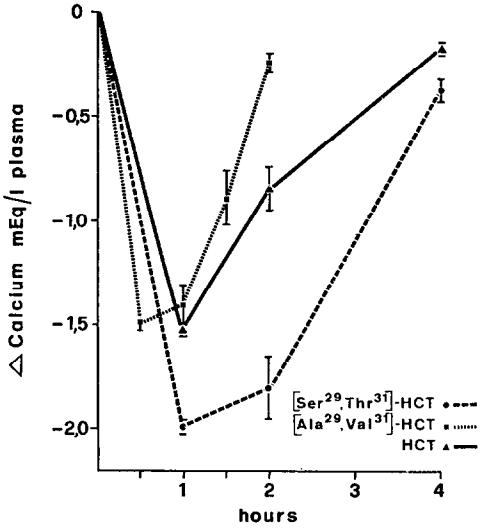

Fig. 4. Time-course of hypocalcaemia elicited by a single subcutaneous dose of 30 μg/kg HCT or the two analogues [Ser^{29}, Thr^{31}]HCT and [Ala^{29}, Val^{31}]HCT. Each point represents the mean for 20–30 rats ± SEM.

about five times greater than that of HCT. Reversal of the sequence of the amino acids to Ala^{29} and Val^{31} yielded an analogue ([Ala^{29}, Val^{31}]HCT) which only exerted a maximal hypocalcaemic effect at a very high dose. Since it showed a flatter regression line than HCT, the two peptides are not strictly comparable. It is noteworthy that at threshold doses [Ala^{29}, Val^{31}]HCT only displayed about 5% of the activity of HCT'

3.2. *Duration of hypocalcaemic (fig. 4)*

A subcutaneous dose of 30 μg/kg of HCT provoked hypocalcaemia persisting for slightly more than 2 hr. The plasma calcium concentration reached its minimum 1 hr after the administration of the peptide, and thereafter increased again rapidly. [Ser^{29}, Thr^{31}]HCT (30 μg/kg,s.c.) evoked a hypocalcaemic effect of about 2 hr duration. Minimum values were reached 1 hr after administration and persisted for another hour. Total hypocalcaemia was about 25% greater than that elicited by the same dose of HCT. [Ala^{29}, Val^{31}]HCT (30 μg/kg,s.c.) evoked a hypocalcaemic effect of about 2 hr duration. Minimum values were reached ½ hour after administration and persisted for a further ½ hr. The decrease was similar to that produced by HCT, but basal values were regained more quickly than after the injection of either HCT or [Ser^{29}, Thr^{31}]HCT. The areas covered by these time-curves were 65 cm^2 for HCT, 106 cm^2 for [Ser^{29}, Thr^{31}]HCT and 40 cm^2 for [Ala^{29}, Val^{31}]-HCT giving a ratio of about 1:1.6:0.6.

4. Discussion

Our results revealed that replacement of Val^{29} and Ala^{31} in HCT by Ser^{29} and Thr^{31}, the corresponding amino acids of the SCT 1 sequence, led to a 5-fold increase in potency. On the other hand, when Val^{29} and Ala^{31} were substituted for Ser^{29} and Thr^{31} in the SCT 1 molecule, its potency was reduced by half [11]. Thus, these two amino acid positions appear to be partly responsible for the potent hypocalcaemic activity of SCT 1; the fact that both analogues – that of HCT and that of SCT 1 – are less active than SCT 1 suggest that other regions besides the C-terminal are also involved.

Through the work of Keutmann et al. [12] the structure of two iso hormones of SCT 1 (SCT 2 and SCT 2) became known, and they were subsequently synthesized [13]. As is shown in the lower half of fig. 2, in their amino acid sequence and composition, they are somewhat closer to HCT than SCT 1 is. It is interesting to note that the two iso hormones also

differ from SCT 1 in positions 29 and 31, which are occupied respectively by alanine and valine, the same amino acids as in HCT but in reverse order. It is remarkable that this reversal leads to an HCT-analogue displaying only a fraction of the activity of the human peptide. In contrast, the calcitonins of salmon origin are much less affected if the order of these amino acids is changed. The two synthetic analogues of SCT 1 with either sequence (valine and alanine, or alanine and valine) were reported by Pless et al. [11] to exhibit equal activity, both being half as potent as SCT. Moreover, the iso hormone SCT 2, with Ala^{29}, Val^{31} sequence, has the same degree of activity as SCT 1.

These authors [11] have also shown that another analogue of SCT 1, [Asp^{15}, Phe^{22}]SCT 1 which contains the two other amino acids in which the iso hormone SCT 2 differs from SCT 1, synthesized to unmask a possible compensatory effect of the two amino acids, exhibited equal activity as SCT 1, indicating that these two amino acids per se had no influence on hypocalcaemic potency.

The iso hormone SCT 3 proved to be three times less potent than SCT 1 [11]. Its weaker activity can be attributed to the presence of methionine in position 8 instead of valine, since our own studies revealed an increased activity, when in HCT Met was replaced by Val in position 8 (unpublished).

At present, it is impossible to draw any definitive conclusions about the relevance of positions 29 and 31 as regards the hypocalcaemic potency of CT peptides. Although it is certain that the C-terminus is an important part of the molecule, it must be considered in conjunction with other essential regions.

Further work is needed to reveal other centres affecting biological activity.

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