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Research Article

Proinsulin C-peptide and its analogues induce intracellular Ca^{2+} increases in human renal tubular cells

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Abstract. Based on the findings that proinsulin C-peptide binds specifically to cell membranes, we investigated the effects of C-peptide and related molecules on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human renal tubular cells using the indicator fura-2/AM. The results show that human C-peptide and its C-terminal pentapeptide (positions 27–31, EGSLQ), but not the des (27–31) C-peptide or randomly scrambled C-peptide, elicit a transient increase in $[Ca^{2+}]_i$. Rat C-peptide and rat C-terminal pentapeptide also induce a $[Ca^{2+}]_i$ response in human tubular cells, while a human pentapeptide analogue with Ala at position 1 gives no $[Ca^{2+}]_i$ response, and those with Ala at positions 2–5 induce responses with different amplitudes. These results define a species cross-reactivity for C-peptide and demonstrate the importance of Glu at position 1 of the pentapeptide. Preincubation of cells with pertussis toxin abolishes the effect on $[Ca^{2+}]_i$ by both C-peptide and the pentapeptide. These results are compatible with previous data on C-peptide binding to cells and activation of Na^+,K^+ATPase. Combined, all data show that C-peptide is a bioactive peptide and suggest that it elicits changes in $[Ca^{2+}]_i$ via G-protein-coupled pathways, giving downstream enzyme effects.

Key words. Proinsulin C-peptide; Intracellular Ca^{2+}; Hormonal response; Peptide analogues; Fura-2/AM; Pertussis toxin; Species cross-reacitivity.

The proinsulin C-peptide has a structural role in the folding of the insulin precursor and is secreted together with insulin in equimolar amounts into the blood stream [1], where it has previously been considered to have no further effects. A view has prevailed for decades that C-peptide is biologically inactive by itself, but data now show that it is a physiologically active peptide [2, 3]. It binds specifically to cell membranes [4], elicits a number of enzyme effects, including activation of Na^+,K^+ATPase [5], MAP kinase [6, 7], and nitric oxide synthase [8]. Several of the effects are abolished after pretreatment with pertussis toxin [4–6, 8]. In addition, C-peptide is now known to exert a number of in vivo effects in animal models of type I diabetes, and in patients, for example, it increases skeletal and skin blood flow, diminishes glomerular hyperfiltration, reduces microalbuminuria, and improves nerve conduction velocity [9–12].

The terms of C-peptide binding to cell membranes, fluorescence correlation spectroscopy has indicated a binding constant in the low nanomolar range [4, 13], which is also the physiological concentration of C-peptide. Hence,

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binding sites may normally be sufficiently saturated to explain the lack of response to exogeneous C-peptide administration in healthy subjects. In both studies of binding and ATPase activation, the C-terminal pentapeptide segment of C-peptide can exert the full-length C-peptide effect [4, 14]. Similarly, a Glu residue in the C-terminal part has been ascribed important functions in binding, indicated by lack of displacement of bound C-peptide with a pentapeptide in which that Glu has been exchanged to Ala [15], and by structural conservation of the Glu in species variants [9]. N-terminal Glu residues also appear to be functionally important [9]. Several of the C-peptide effects appear to be mediated through Ca^{2+}-dependent signalling pathways, and studies have indicated a C-peptide effect on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [5, 16]. In the present work, we therefore studied the effects of C-peptide, the terminal pentapeptide, pentapeptide analogues, species variants, and scrambled C-peptide on $[Ca^{2+}]_i$ in human renal tubular cells, using the fluorescent indicator fura-2/AM. The results provide new data on C-peptide action and specificity.

Materials and methods

Peptides

The following peptides were used, all prepared by solid-phase peptide synthesis and purchased from Sigma Genosys (Cambridge, UK): human C-peptide, scrambled C-peptide (with the same residues, but randomly ordered), des(27−31)C-peptide, C-terminal pentapeptide (EGSLQ), and pentapeptide analogues with step-wise exchanges to Ala (AGSLQ, EASLQ, EGALQ, EGSAQ, EGSLA). In addition, rat C-peptide II and rat C-terminal pentapeptide (EVARQ) were used.

Cell culture

Human renal tubular cells were obtained from the outer cortex of renal tissues obtained from non-diabetic patients undergoing elective nephrectomy for renal cell carcinomas. The collection of the tissue samples during surgery was approved by the Ethics Committee of the Karolinska Hospital. The cells were cultured in RPMI 1640 (Life Technologies, Grand Island, N. Y.) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, benzylpenicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were grown to confluency on cover slips in a six-well plate and starved in serum-free medium for 4 h before experiments.

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured on cells attached to cover slips. The cells were loaded with 2 µM of the fluorescent Ca^{2+} indicator fura-2/AM at 37 °C for 30 min in a buffer containing 115 mM NaCl, 24 mM $NaHCO_3$, 4.7 mM KCl, 1.26 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_2$, 11.1 mM glucose, 20 mM HEPES, and 5 mg/ml bovine serum albumin, pH 7.4. Cover slips were mounted as the bottom of an open chamber placed on a thermostatically controlled stage of an inverted epifluorescence microscope (Zeiss, Axiovert 135). The microscope was connected to a SPEX fluorolog-2 system for dual-wavelength excitation fluorimetry. The emissions of the two excitation wavelengths of 340 and 380 nm were used to calculate the fluorescence ratio (F340/F380) reflecting changes in $[Ca^{2+}]_i$ [17]. Treatments during recordings were made using a perifusion system attached to the chamber. Changes in $[Ca^{2+}]_i$ were measured using an attached CCD camera that allowed recording of individual cells in the cell clusters. The influence of pertussis toxin was studied by preincubation of the cells with the toxin at 1 µg/ml for 4 h at 37 °C.

Results

Human renal tubular cells loaded with the indicator fura-2/AM were exposed in different series of experiments to the peptides studied. Changes in $[Ca^{2+}]_i$ were recorded. The effect of 5 nM human C-peptide is shown in figure 1, both as the F340/F380 ratio trace and as images of the cells taken at different time points. In the images, the $[Ca^{2+}]_i$ is expressed in a pseudocolour scale. The most reproducible responses were obtained at 5 and 10 nM. Hence, comparative measurements were performed at these peptide concentrations. Results similar to those with human C-peptide were obtained with the C-terminal pentapeptide of the human C-peptide, while scrambled C-peptide and des(27−31)C-peptide, lacking the C-terminal pentapeptide part of C-peptide, gave no effect (end of fig. 2 A). Successive exposures of the same cells to 5 nM of human C-peptide, a scrambled C-peptide, and the C-terminal pentapeptide, EGSLQ, establish the specificity of the C-peptide effect (fig. 2 A). Rat C-peptide II and the rat pentapeptide C-terminal fragment, EVARQ, also increased $[Ca^{2+}]_i$ in these human renal tubular cells (fig. 3).

Preincubation of the cells with pertussis toxin at 1 µg/ml eliminated the responses with both C-peptide and the C-terminal pentapeptide. This confirms that the Ca^{2+} effect is dependent on G-protein-coupled signal transduction pathways. When cells were perifused with a buffer without Ca^{2+}, the effects of the peptides were abolished, indicating that extracellular Ca^{2+} is required for the response. To evaluate the importance of the different pentapeptide residues for the responses, we tested all five C-terminal pentapeptide analogues in which one residue each had been exchanged to Ala. This is shown in figure 2 by the additions of 5 nM of four different Ala-substituted pentapeptide analogues at the first four arrows in figure 2 A

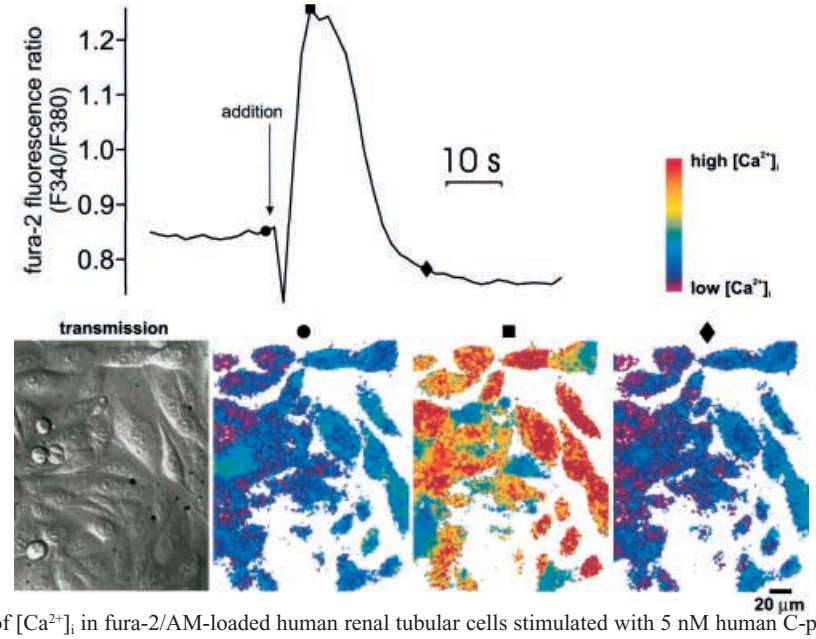

Figure 1. Monitoring of $[Ca^{2+}]_i$ in fura-2/AM-loaded human renal tubular cells stimulated with 5 nM human C-peptide. Top, the trace of the 340/380 fluorescence ratio. Bottom, images of the cells in transmission light (first panel) and in a colour code (three next panels) representing $[Ca^{2+}]_i$ at the time points shown by spot indications in the trace above.

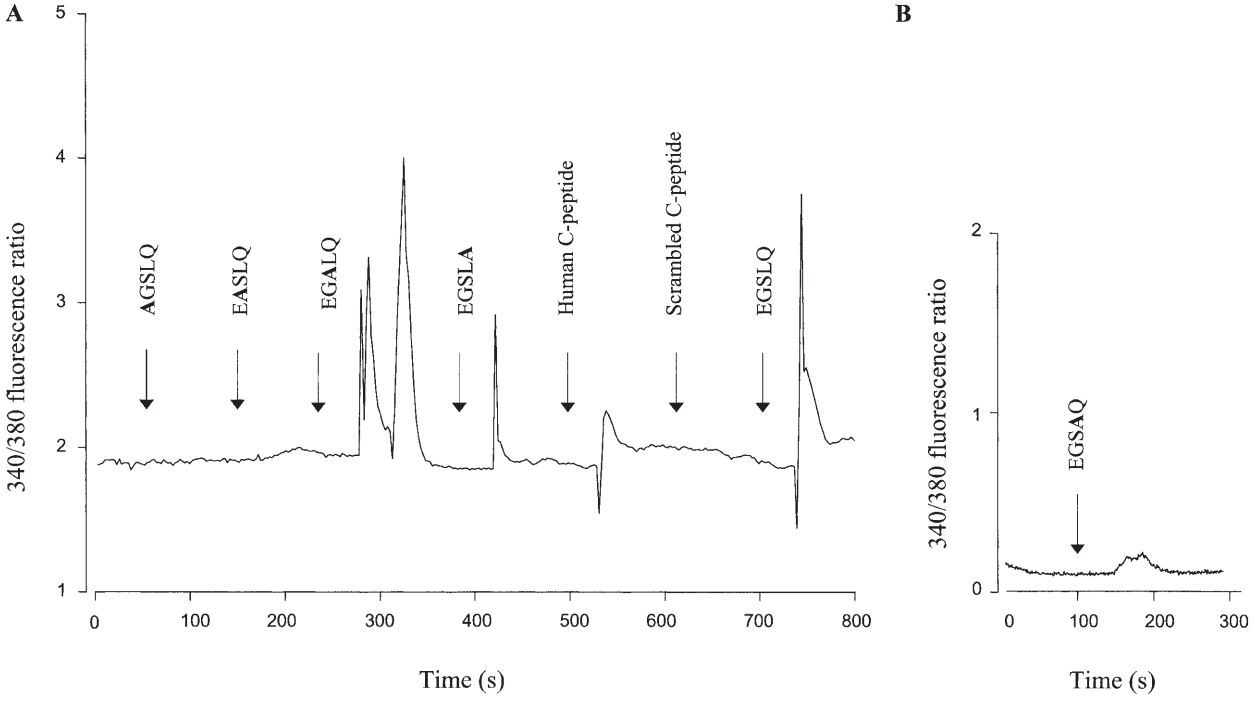

Figure 2. Changes in $[Ca^{2+}]_i$ as measured by fluorescence from the indicator fura-2/AM in human renal tubular cells stimulated with five different Ala-substituted analogues of human C-peptide C-terminal pentapeptide (**A**GSLQ, E**A**SLQ, EG**A**LQ, EGSL**A** (*A*) and EGS**A**Q (*B)* and of human C-peptide, scrambled human C-peptide, and the human C-peptide C-terminal pentapeptide (*A*) at concentrations of 5 nM. In *A*, the same set of cells was used for successive additions. The response of the second analogue, E**A**SLQ, is weak, but was also present to some extent also in other experiments. Consequently, it appears to affect $[Ca^{2+}]_i$, in contrast to **A**GSLQ, but less than wild-type EGSLQ.

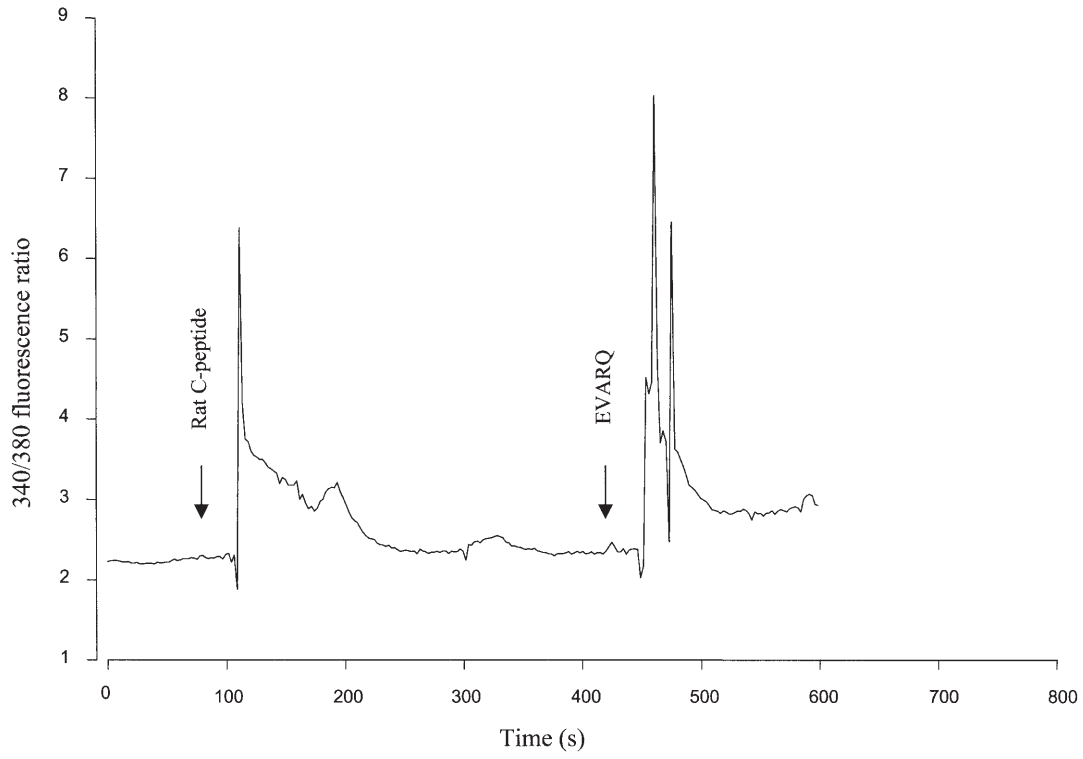

Figure 3. Effects on $[Ca^{2+}]_i$ in human tubular cells stimulated with rat C-peptide II (left) and its C-terminal pentapeptide, EVARQ (right), at concentrations of 10 nM.

and of the fifth analogue in figure 2B. The Glu1Ala analogue, AGSLQ (first arrow, fig 2A) did not affect $[Ca^{2+}]_i$, the second analogue, EASLQ (second arrow, fig. 2A), did so weakly, while the other three alternatives (the third and fourth arrows in fig. 2A, and the arrow in fig. 2B) induced changes in $[Ca^{2+}]_i$.

Discussion

The present results establish that human C-peptide elicits changes in $[Ca^{2+}]_i$ in human renal tubular cells, suggesting that C-peptide can affect the bioactivity of these cells. The effects can be seen at the physiological C-peptide concentration, which is about 1 nM, but are more stably measured at a somewhat higher concentration of 5 nM. Lack of effect with the scrambled C-peptide establishes the specificity of the response, while the inhibition with pertussis toxin suggests that the $[Ca^{2+}]_i$ response is G-protein dependent. The lack of effect in the absence of extracellular Ca^{2+} suggests that the response is caused by Ca^{2+} influx into the cells, and not primarily by emptying of intracellular stores. Much higher C-peptide concentrations, above 100 nM, also gave non-optimal reproducibility, presumably because of false-positive results from unspecific effects. All these facts, combined with a previous demonstration of saturable C-peptide binding to cells at physiological concentrations [4, 14], suggest that C-peptide has hormonal effects by attaching to cellular receptors, eliciting a G-protein-coupled signal and a calcium influx.

The results further show that the C-terminal pentapeptide appears sufficient for the $[Ca^{2+}]_i$ response, in agreement with the results from binding displacements of this peptide studied with fluorescence correlation spectroscopy [4], and from stimulation of Na^+,K^+ATPase activity [14]. Consequently, different molecular assays for monitoring C-peptide effects in biological systems have now been defined, and the C-terminal pentapeptide apparently exhibits full bioactivity in three different assays.

The species cross-reactivity now observed with the rat C-peptide and pentapeptide in the human system is noteworthy, since binding experiments with C-peptide and its C-terminal pentapeptide using fluorescence correlation spectroscopy do not show this species cross-reactivity [15]. However, C-peptide functional interactions with cells may depend on precisely spaced Glu residues, compatible with the species cross-reactivity, since human and rat C-peptides and their fragments have identically positioned Glu residues.

Effects on binding to cells studied with fluorescence correlation spectroscopy and on changes in $[Ca^{2+}]_i$ can also be directly compared with the set of Ala-substituted pentapeptides. This demonstrates the absolute requirement

for Glu1 in the pentapeptide, corresponding to Glu27 in the intact C-peptide, in both systems. However, the relative effects of the other Ala-substituted analogues, which all show some activity (fig. 2), are similar but not fully identical when measured as binding displacement and as effects on $[Ca^{2+}]_i$. This is probably explained by differences in the experimental set-ups.

Finally, the pertussis toxin effect, inhibiting the C-peptide response, is observed in all three assay systems under discussion. Thus, the present inhibition of the increase in the $[Ca^{2+}]_i$ response by the toxin is paralleled by its effect on the binding measured spectroscopically and on the stimulation of the Na^+,K^+ATPase activity. These findings provide strong indications of a specific bioactivity of C-peptide, working in a hormonal manner on cells.

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