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C-peptide stimulates rat renal tubular Na^+, K^+-ATPase activity in synergism with neuropeptide Y

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Summary This study was performed in order to test the hypothesis that the connecting peptide of proinsulin, C-peptide, might in itself possess biological activity. Renal tubular Na^+, K^+-ATPase, which is a well-established target for many peptide hormones, was chosen as a model. Rat C-peptide (I) was found to stimulate Na^+, K^+-ATPase activity in single, proximal convoluted tubules dissected from rat kidneys. C-peptide increased the Na^+ affinity of the enzyme and all subsequent studies were performed at non- saturating Na^+ concentrations. C-peptide stimulation of Na^+, K^+-ATPase activity occurred in a concentration-dependent manner in the dose range 10^{-8}–10^{-6} mol/l. The presence of neuropeptide Y, 5×10^{-9} mol/l, enhanced this effect and stimulation of Na^+, K^+-ATPase activity then occurred in the C-peptide dose range 10^{-11}–10^{-8} mol/l. C-peptide stimulation of Na^+, K^+-ATPase activity was abolished in tubules pretreated with pertussis toxin. It was also abolished in the presence of FK 506, a specific inhibitor of the Ca^{2+}-calmodulin-dependent protein phosphatase 2B. These results indicate that C-peptide stimulates Na^+, K^+-ATPase activity, probably by activating a receptor coupled to a pertussis toxin-sensitive G-protein with subsequent activation of Ca^{2+}-dependent intracellular signalling pathways. [Diabetologia (1996) 39: 199–205]

Biosynthesis of insulin proceeds via the formation of proinsulin in the endoplasmic reticulum of pancreatic beta cells and its subsequent cleavage into insulin and C-peptide [1, 2]. Insulin and C-peptide are both retained in secretory granules within the beta cell and eventually released in equimolar amounts into the blood circulation [3]. C-peptide fulfills an important function in the assembly of the two-chain insulin structure [4], but has otherwise been considered to be biologically inactive. However, several studies have recently indicated that in patients with insulin-dependent diabetes who lack endogenous C-peptide, its administration improves renal function, stimulates muscle circulation and glucose utilization, and improves blood-retinal barrier function [5–7].

The mechanism behind the observed effects of C-peptide is not apparent, but several of the processes stimulated by C-peptide relate to membrane permeability and transport, events which are in part dependent on Na^+, K^+-ATPase activity. Na^+, K^+-ATPase is expressed in almost all eukaryotic cells. It couples the hydrolysis of ATP to the vectorial transport of Na^+ and K^+ across the cell membrane. The cation gradients by Na^+, K^+-ATPase are of fundamental importance for such vital functions as control of cell volume, nutrient uptake, membrane excitability and transepithelial transport of sodium. Experimental diabetes is known to be accompanied by altered Na^+, K^+-ATPase activity in skeletal muscle, myocar-

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Abbreviations: PTX, Pertussis toxin; NPY, neuropeptide Y; PCT, proximal convoluted tubule; BSA, bovine serum albumin; dB cAMP, dibutyryl cyclic adenosine monophosphate; PP2B, Ca^{2+}/calmodulin-dependent protein phosphatase 2B; PKC, protein kinase C; [Ca^{2+}]; intracellular calcium concentration

dium, nerve tissue and renal tubular segments [8–10]. Na^+, K^+-ATPase is particularly abundant in the renal tubules, where it has been shown to be the target for many hormones. Insulin [11] and neuropeptide Y (NPY) [12] belong to the group of hormones that increase the activity of renal Na^+, K^+-ATPase.

The above considerations prompted us to investigate the possible influence of C-peptide on Na^+, K^+-ATPase in proximal tubular segments from the rat nephron, a well-defined model [13, 14]. Having then found an effect of C-peptide, we subsequently studied the subcellular mechanisms for its action as well as its possible interaction with other hormones known to stimulate Na^+, K^+-ATPase activity.

Materials and methods

Animals. Male Sprague-Dawley rats (ALAB, Sollentuna, Sweden) aged 40–45 days and weighing between 150 and 200 g were used. They were fed ordinary rat chow (Ewos, Södertälje, Sweden) and tap water ad libitum.

Preparation of proximal convoluted tubule (PCT) segments. Tubular segments were obtained from collagenase-perfused rat kidneys. The procedure generally followed the method described by Doucet et al. [14]. The rats were anaesthetized with Mebumal vet (Nord Vacc, Stockholm, Sweden; 5–6 mg per 100 g body weight i.p.). After a midline incision, the left kidney was exposed and perfused with a modified Hanks' solution of the following composition (in mmol/l): 137 NaCl, 5 KCl, 0.8 $MgSO_4$ 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Tris-HCl, to which 0.05 % collagenase (Sigma Chemical Co., St. Louis, Mo., USA) and 0.1 % bovine serum albumin (BSA) (Behringwerke, Marburg, Germany) were added. The kidney was removed and cut along its corticopapillary axis into small pyramids that were incubated for 20 min at 35 °C in the perfusion solution containing 10^{-3} mol/l butyrate to optimize mitochondrial respiration [15] and bubbled with 95 % O_2 and 5 % CO_2. After incubation, the tissue was rinsed three times with fresh perfusate without collagenase and BSA (microdissection solution), which has the same composition as a modified Hanks' solution but with 0.25 mmol/l $CaCl_2$.

Microdissection was performed on ice under a stereomicroscope. The single PCT segments were manually dissected (tubular segment length 0.5–1.0 mm) from the outer cortex, and individually transferred to the concavity of a bacteriological slide in a drop of the microdissection solution and photographed for determination of length in an inverted microscope at 100 × magnification. Tubules were stored on ice until dissection was completed after a maximum of 30 min.

Incubation of tubules with peptides and/or drugs. The tubule segments were incubated for 30 min at room temperature either in 1 µl of microdissection solution alone (control tubules) or in 1 µl of microdissection solution containing one or more of the drugs mentioned below (experimental tubules). The ratio of cell volume to buffer volume was approximately 1:1000. In most protocols the Na^+ concentration of the solution was 20 mmol/l, but in some protocols it was varied from 1 to 70 mmol/l. To keep the osmolality of the solution constant at 290 mOsm/kg of water, choline chloride was added in equivalent millimolar amount to the amount of sodium chloride removed.

In one protocol, tubules were pretreated with or without 1 µg/ml pertussis toxin (PTX) at 37 °C for 4 h under 95 % O_2 and 5 % CO_2 with a moist atmosphere [16] before incubation with C-peptide. In the experiment with calphostin C, tubules were preincubated under ultraviolet-light activation [17].

Determination of Na^+, K^+-ATPase activity. The preincubation period was stopped by reducing the temperature of the tubular segments to 4 °C. The segments were then made permeable by hypotonic shock, freezing and thawing in order to make it possible for labelled ATP to enter the cells and to change the sodium concentration of the cells. They were then incubated at 37 °C for 15 min in a medium containing (in mmol/l): 20 NaCl (unless otherwise stated), 5 KCl, 10 $MgCl_2$, 1 EGTA, 100 Tris-HCl, 10 Tris-ATP, and [γ-^{32}P] ATP (NEN, Boston, Mass., USA); 2–5 Ci · $mmol^{-1}$ in tracer amounts (5 nCi · μl^{-1}) in the absence or presence of 2 mmol/l ouabain (USB, Cleveland, Ohio, USA). For determination of ouabain-insensitive (Mg-dependent) ATPase activity NaCl and KCl were omitted and Tris-HCl was 150 mmol/l. The ^{32}P-phosphate liberated by hydrolysis of ATP was separated by filtration through a millipore filter after absorption of the unhydrolysed nucleotide on activated charcoal and radioactivity was determined in a liquid scintillation spectrometer.

In each study, total ATPase activity and ouabain-insensitive ATPase activity were measured on each of six to ten different tubule segments. This procedure was repeated on tubules from at least three animals. Na^+, K^+-ATPase activity (pmol of $^{32}P_i$ hydrolysed · mm of $tubule^{-1}$ · h^{-1}) was then calculated as the difference between the mean value for total ATPase and ouabain-insensitive ATPase activity, and expressed either as absolute values or as per cent above the corresponding control measurement. Kinetic analyses of $K_{0.5}(Na^+)$ were carried out as described by Shyjan et al. [18].

Measurement of intracellular calcium in cultured rat PCT cells

Preparation of cells. Primary cultures of PCT cells were prepared using a modification of the method described by Larsson et al. [19]. Male Sprague-Dawley rats 40 days old were anaesthetized with Mebumal vet. 5–6 mg · 100 g^{-1} body weight i.p. (Nord Vacc) and the kidneys were perfused with 0.05 % collagenase (Sigma type 1) in Hanks' medium. The outer 150 µm of the renal cortex was removed in a microtome and incubated in the collagenase solution at 37 °C for 15 min. A tissue suspension was centrifuged and rinsed twice with 0.01 % soybean trypsin inhibitor-solution (Gibco Laboratories, Grand Island, N.Y., USA) and a concentrate of PCT fragments and PCT cells were plated onto glass cover slips. The cells were cultured in Dulbecco's Modified Eagle's Medium [DMEM, 20 mmol/l 4-(2-hydroxyethyl)-1-piperazineethane sulphoric acid (Hepes), 24 mmol/l $NaHCO_3$, 50,000 IU/l penicillin and 50 mg/l streptomycin, pH 7.4] with 10 % fetal bovine serum (Gibco) in an incubator at 37 °C with 95 % O_2 and 5 % CO_2. After 28 h in culture the medium was changed to DMEM with 1 % fetal bovine serum. The cells were examined approximately 18 h later.

Quantitative fluorescence microscopy. Single-cell intracellular calcium $[Ca^{2+}]_i$ was quantitated by using Fura-2 (Fura-2/AM, Molecular Probes, Eugene, Ore., USA). After loading the cells with this dye, the petri dish was placed on a 37 °C stage of a Zeiss IM 405 inverted microscope (Oberkochen, Germany) and a single cell was focused. A computerized spectrophotometer system (DM3000 cm; SPEX Inc., Edison, N.J., USA)

was used and emitted light was measured by photon counting (Hamamatsu R 928, Hamamatsu, Japan). All experimental solutions were kept at 37 °C and equilibrated with 5 % CO_2 and 95 % O_2. The $[Ca^{2+}]_i$ was measured using a balanced salt solution (concentration in mmol/l: 109 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 1 Na_2HPO_4, 25 $NaHCO_3$, 20 Hepes, 5 glucose and 1 Na-butyrate). The cells were exposed to C-peptide by completely removing the 2.5-ml solution in the petri dish (exept for a thin layer covering the cells) and adding solution with C-peptide.

The cells were excited at 345 and 380 nm and emitted light was measured at 510 nm. In order to calculate intracellular Ca^{2+}-concentrations from experimental (345/380)-ratios, the following equation was used [20]:

$$[Ca^{2+}]; = (R-R_{min})/(R_{max}-R) \cdot K_d \cdot F_0/F_1$$

R is the ratio from the cell measurements; R_{max} and R_{min} represent the ratios for Fura-2 in the presence of saturating and zero calcium, respectively; K_d is the dissociation constant for the Fura-2-Ca complex, assumed to be 224 nmol/l at 37 °C; F_0 and F_1 are the fluorescence intensities at 380 nm for free and Ca-saturated Fura-2, respectively. R_{min} and R_{max} were measured using Fura-2 potassium salt dissolved in media with 1 mmol/l Ca (R_{max}) or in Ca-free media with 1 mmol/l EGTA (R_{min}). The background was obtained by recording the signal without any cell in the field of measurement. In pilot experiments the background values obtained in this way deviated less than 5 % from the values obtained with a cell in the field and the Fura-2 signal quenched with $MnCl_2$.

Drugs and peptides. Synthetic rat C-peptide (I) and randomly scrambled C-peptide were obtained from Ferring AB (Malmö, Sweden). The scrambled C-peptide included the same amino-acid residues as the native peptide but placed in a random sequence. The purity of the peptides established by HPLC was greater than 99 %. Porcine NPY, angiotensin II, bovine insulin, dibutyryl cyclic adenosine monophosphate (dB cAMP) and PTX were from Sigma Chemical Co. FK 506 was from Fujisawa Pharmaceutical Co. (Osaka, Japan); calphostin C was from Kamiya Biomedical Co. (Thousand Oaks, Calif., USA).

Statistical analysis

Values are given as mean ± SEM. Statistical analysis was performed with Student's *t*-test and analysis of variance. A value of p less than 0.05 was considered significant.

Results

It was found in pilot studies that C-peptide did not have any effect on tubular Na^+, K^+-ATPase activity when the ATPase assays were carried out under V_{max} conditions for all substrates. However, C-peptide was found to stimulate Na^+, K^+-ATPase activity at non-saturating Na^+ concentrations. The dose-response effects of C-peptide were therefore evaluated at a Na^+ concentration of 20 mmol/l, which does not saturate proximal tubular Na^+, K^+-ATPase. In this and all subsequent experiments each data point represents the average of 18–30 measurements of both total and ouabain-insensitive Na^+, K^+-ATPase activity; the tubule segments were obtained from at least three different rats. Under these conditions C-peptide could be shown to elicit a concentration-dependent stimulation of Na^+, K^+-ATPase activity in the PCT segments (Fig. 1). Apparent half-maximal activation occurred at 5×10^{-7} mol/l. At 10^{-6} mol/l, C-peptide stimulated Na^+, K^+-ATPase by 85 ± 2 % above control values. Higher concentrations of C-peptide resulted in only a small further increase in Na^+, K^+-ATPase activity. C-peptide had no measurable effect on ouabain-insensitive ATPase. Randomly scrambled C-peptide (10^{-6} mol/l) failed to stimulate the Na^+, K^+-ATPase activity of the PCT segments (5.0 ± 4.1 % above control).

The Na^+ dependence of Na^+, K^+-ATPase activity was determined in the presence or absence of C-peptide (5×10^{-7} mol/l). All assays were carried out in the presence of 5 mmol/l K^+. In the absence of C-peptide a half-maximal increase of enzyme activity occurred

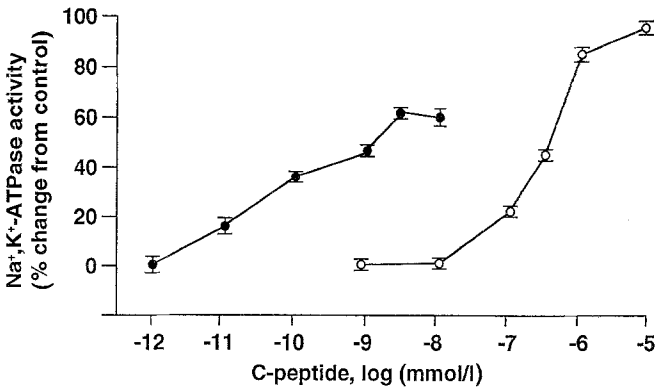

Fig. 1. Effects of C-peptide and NPY on Na^+, K^+-ATPase activity of PCT segments. Tubules were incubated for 30 min with varying concentrations of C-peptide in the presence (●) or absence (○) of NPY at its subthreshold concentration (5×10^{-9} mol/l) for stimulation of the enzyme activity. The Na^+ concentration was 20 mmol/l. Values are mean ± SEM, n = 18–30 in each experiment

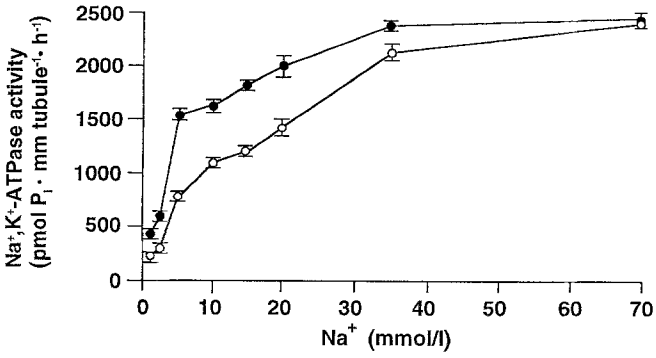

Fig. 2. Effect of C-peptide on Na^+, K^+-ATPase activity of PCT segments, as a function of Na^+ concentration in the presence of 5 mmol/l K^+. Segments were incubated for 30 min in the absence (○) or presence (●) of C-peptide (5×10^{-7} mol/l). Values are mean ± SEM, n = 18–30 in each experiment. Control values for Na^+, K^+-ATPase activity were 1350 ± 55 pmol $P_i \cdot$ mm tubule$^{-1} \cdot h^{-1}$

at a Na^+ concentration of 15 mmol/l (Fig. 2). In the presence of C-peptide, only 4 mmol/l of Na^+ was required for half-maximal increase of activity. C-peptide had no effect on Na^+, K^+-ATPase under V_{max} conditions. All subsequent experiments were performed at a Na^+ concentration of 20 mmol/l.

To study whether C-peptide interacts with the receptor coupled to PTX-sensitive G-proteins, we pretreated tubules with PTX before incubating with C-peptide. In tubules pretreated with PTX, the stimulatory effect of C-peptide (5×10^{-7} mol/l) on PCT Na^+, K^+-ATPase activity was completely blocked (Fig. 3). PTX alone had no effect on the enzyme activity indicating that PTX treatment did not in itself adversely affect cell viability.

Since hormonal stimulation of proximal tubular Na^+, K^+-ATPase activity is mediated by activity of the Ca^{2+}/calmodulin-dependent protein phosphatase 2 B (PP2B) [21], FK 506, an inhibitor of PP2B, was used to further explore the signalling pathways of C-peptide. FK 506 at 10^{-8} mol/l blocked C-peptide stimulation of Na^+, K^+-ATPase activity (Fig. 4). FK 506 alone had no effect on Na^+, K^+-ATPase activity under the present experimental conditions, i.e. non-saturating Na^+ concentration. We also tested the effects of calphostin C, a potent and specific inhibitor of protein kinase C (PKC) [22] since the PKC pathways may also be Ca^{2+}-dependent. Calphostin C did not interfere with the stimulatory effect of C-peptide. Calphostin C alone caused a slight stimulation of Na^+, K^+-ATPase activity.

The effect of cAMP on C-peptide stimulation was investigated using the cAMP analogue dB cAMP. The effect of 5×10^{-7} mol/l C-peptide on enzyme activity was almost completely abolished in the presence of dB cAMP 10^{-3} mol/l (1640 ± 125 pmol $P_i \cdot$ mm tubule$^{-1} \cdot h^{-1}$, compared with 1621 ± 167 from control segments). dB cAMP itself had no effect (1569 ± 209 pmol $P_i \cdot$ mm tubule$^{-1} \cdot h^{-1}$, compared with 1561 ± 177 from control segments) of its own.

It was considered of interest to examine whether C-peptide might act synergistically with another hormone. For this purpose we chose insulin and NPY. Insulin is of obvious importance in this context. NPY was selected, because destruction of the proinsulin-producing pancreatic beta cells may result in upregulation of NPY in many organs [23, 24]. Both NPY and insulin by themselves stimulate renal tubular Na^+, K^+-ATPase activity depending on the concentrations [11, 12]. For NPY the subthreshold effect occurs at the dose of 5×10^{-9} mol/l and the maximal effect at 10^{-5} mol/l [12]. In the present study, the activity of Na^+, K^+-ATPase was not significantly different from the control either in tubules incubated with NPY 5×10^{-9} mol/l or in tubules incubated with C-peptide 10^{-8} mol/l (Fig. 1). In contrast, tubules incubated with both NPY 5×10^{-9} mol/l and C-peptide 10^{-8} mol/l showed a Na^+, K^+-ATPase activity

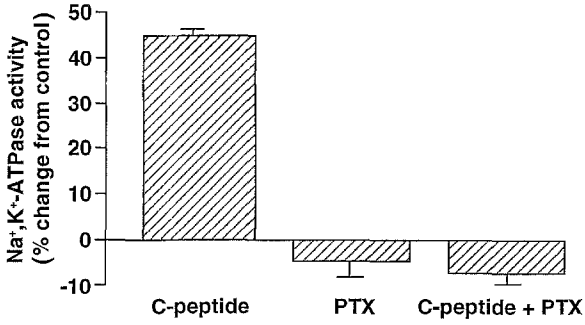

Fig. 3. Effects of PTX treatment on the stimulation of Na^+, K^+-ATPase activity by C-peptide. After PCT segments were incubated with or without PTX (1 μg/ml) at 37 °C for 4 h, they were exposed either to C-peptide (5×10^{-7} mol/l) or vehicle for 30 min at room temperature. The Na^+ concentration was 20 mmol/l. Results are expressed as% change above the control value obtained from tubules incubated with microdissection solution only without pretreatment. Data are mean ± SEM, $n = 18$–30 in each experiment

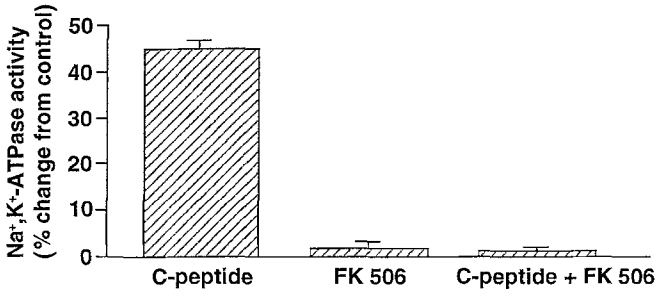

Fig. 4. Effects of FK 506 (10^{-8} mol/l), an inhibitor of protein phosphatase 2B on the stimulation of Na^+, K^+-ATPase activity by C-peptide (5×10^{-7} mol/l). The Na^+ concentration was 20 mmol/l. Data are mean ± SEM, $n = 18$–30 in each experiment

which was significantly increased above control (60.0 ± 3.0 %, $p < 0.01$). The dose-response curves for activation of Na^+, K^+-ATPase activity by C-peptide both in the presence and absence of NPY 5×10^{-9} mol/l are shown in Figure 1. In the presence of NPY, C-peptide at concentrations within or below the physiological range (10^{-10}–10^{-9} mol/l) [25] caused significant stimulation of Na^+, K^+-ATPase activity. In accordance with a recent report by Féraille et al. [11] we found in this study that insulin maximally stimulated Na^+, K^+-ATPase activity at 10^{-7} mol/l (110 % above control values) and that the subthreshold dose was 5×10^{-11} mol/l (data not shown). When tubules were incubated with the combination of insulin and C-peptide, both at subthreshold concentrations, no synergistic stimulation was observed (1678 ± 142 pmol $P_i \cdot$ mm tubule$^{-1} \cdot h^{-1}$, compared with 1673 ± 141 for control tubules).

The effect of C-peptide on the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was examined using rat PCT cells in primary culture. Exposure of the cells to C-peptide was accompanied by a marked increase in

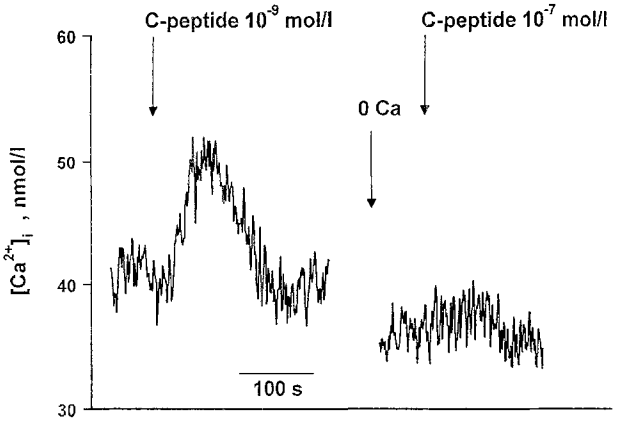

Fig. 5. The intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in single PCT-cells in primary culture. $[Ca^{2+}]_i$ was determined using Fura. The cell to the left was exposed to C-peptide 10^{-9} mol/l. Removal of Ca^{2+} from the medium by addition of 1 mmol/l EGTA abolished the C-peptide effect on $[Ca^{2+}]$ (right)

$[Ca^{2+}]_i$, (Fig. 5). $[Ca^{2+}]_i$ usually returned to its basal level within 2 min but in some cells oscillations of $[Ca^{2+}]_i$ occurred. Approximately 90 % of the cells responded to C-peptide with a rise in $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ could be elicited with C-peptide in concentrations ranging from 10^{-6} to 10^{-13} mol/l. When PCT cells were maintained in a calcium-free medium (1 mmol/l EGTA), exposure to C-peptide failed to increase $[Ca^{2+}]_i$ levels (Fig. 5).

Discussion

Recent studies in insulin-dependent diabetic patients have suggested that, contrary to the general view, C-peptide possesses biological activity [5–7]. So far there has been little evidence for an effect of C-peptide at the cellular level. Flatt et al. [26] have found evidence for a specific binding of C-peptide in cultured beta cells indicating the existence of C-peptide receptors. We have evaluated whether C-peptide might exert hormonal effects by using Na^+, K^+-ATPase as a target protein, since its activity is known to be modulated by many hormones including insulin [11, 27–32]. The effects of C-peptide on Na^+, K^+-ATPase were studied in a preparation of fresh tissue with a homogenous cell population, where many of the intracellular signalling pathways that regulate Na^+, K^+-ATPase activity have already been identified [22]. The results from this study provide strong evidence for a hormonal effect of C-peptide since it elicited a concentration-dependent stimulation of proximal tubular Na^+, K^+-ATPase activity. The C-peptide effect was abolished by PTX pretreatment, indicating that C-peptide probably activates a receptor coupled to a G_i or G_o protein [33]. Randomly scrambled C-peptide was found to have no effect on Na^+, K^+-ATPase activity, indicating that non-specific effects may be excluded.

The effects of C-peptide on Na^+, K^+-ATPase activity resemble in many aspects those of norepinephrine (acting on α-adrenergic receptors), angiotensin II, and NPY [12, 13]. Like C-peptide, all of these first messengers increase the sodium affinity of PCT Na^+, K^+-ATPase. The effect is attenuated by dB cAMP, a cAMP derivative and it was abolished in the presence of FK 506. The fact that hormones stimulating the activity of Na^+, K^+-ATPase generally act by increasing the Na^+ affinity of the enzyme, is of great physiological importance, since all mammalian cells appear to have a Na^+ concentration that is so low that it will not saturate the enzyme. In the presence of C-peptide, the $K_{0.5}$ (Na^+) for Na^+, K^+-ATPase was reduced from 15 to 5 mmol/l. By lowering the $K_{0.5}$ (Na^+), C-peptide should increase the gradient for Na^+ influx into the cell via channels and pores. Whether this only results in an increased transcellular Na^+ transport or whether there is also a resetting of intracellular Na^+ concentration, has not yet been settled.

Natriuretic hormones, such as dopamine and atrial natriuretic peptide and their second messengers, cAMP and cGMP, have been shown to oppose the stimulating effect of several antinatriuretic hormones, such as norepinephrine, angiotensin II and NPY on the Na^+ affinity of Na^+, K^+-ATPase. This results in a bidirectional regulation of tubular Na^+, K^+-ATPase activity which allows Na^+ reabsorption to be regulated with great precision [13, 34]. The finding that cAMP abolished the stimulatory effects of C-peptide may imply that C-peptide participates in this bidirectional regulation of proximal tubular Na^+ reabsorption by antinatriuretic and natriuretic hormones.

The results further indicate that C-peptide activates Ca^{2+}-dependent signalling pathways. In studies performed on cultured rat PCT cells, we observed that exposure to C-peptide rapidly and consistently increases intracellular calcium (Fig. 5). Moreover, FK 506 – a specific inhibitor of PP2B [21] – completely blocked the stimulatory effect of C-peptide. The PP2B is of central importance for the regulation of renal Na^+, K^+-ATPase activity [13, 34]. Stimulation of Na^+, K^+-ATPase activity by either α-adrenergic agonists, angiotensin II and/or NPY is completely blocked in the presence of FK 506. FK 506, which has no effects by itself at non-saturating Na concentrations, will at saturating Na concentrations cause a dose-dependent inhibition of proximal tubular Na^+, K^+-ATPase activity [34].

When C-peptide was used alone, concentrations that were higher than the circulating levels of C-peptide in the rat (10^{-10}–10^{-9} mol/l) [25] were required to stimulate tubular Na^+, K^+-ATPase activity. There are several possible explanations for this seemingly controversial finding. During preparation of the tubular segments, a procedure which involves mild col-

lagenase digestion, freezing and thawing, a fraction of putative C-peptide receptors may have been damaged. Alternatively C-peptide may have to act in synergism with other hormones in order to elicit its biological effects. The first alternative has not been evaluated, but the second possibility proved to be valid.

In the presence of a subthreshold dose of NPY, physiological concentrations of C-peptide (10^{-9} mol/l) significantly increased Na^+, K^+-ATPase activity. NPY is released following activation of sympathetic nerves and has been reported to act synergistically with several hormones, such as norepinephrine, angiotensin II and histamine [35–38]. Tissue levels of NPY are up-regulated in streptozotocin-treated and C-peptide-deficient rats [23, 24]. The present results suggest that the effects of C-peptide may be dependent on the activity of the sympathetic nervous system. Conversely, the effects of NPY may also be dependent on circulating levels of C-peptide. It is noteworthy that no synergistic interaction could be observed between C-peptide and insulin with regard to stimulation of Na^+, K^+-ATPase activity, indicating an independent action of these two hormones.

PCT cells offer a good model for studies of short-term hormonal regulation of Na^+, K^+-ATPase activity and the subcellular signalling pathways involved. However, in the context of insulin-dependent diabetes these cells may not be the most relevant physiological target for C-peptide action, since renal proximal tubular Na^+, K^+-ATPase is up-regulated in diabetes [39–42] due to high activity of the Na^+/glucose co-transporter [43]. In contrast, Na^+, K^+-ATPase activity is decreased in nerve tissue and glomeruli in diabetes [44–46] and there is evidence suggesting that decreased neuronal Na^+, K^+-ATPase is causally related to diabetic neuropathy [46]. Preliminary evidence suggests that C-peptide administration to patients with diabetic autonomic neuropathy is accompanied by significantly improved autonomic nerve function [47]. Further studies will be required to examine the role of C-peptide deficiency in the regulation of Na^+, K^+-ATPase of nerve tissue and glomeruli in the diabetic state.

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