Effects of calcitonin on function of intercalated cells of rat cortical collecting duct

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Siga, Esteban, Beatrice Mandon, Nicole Roinel, and Christian de Rouffignac. Effects of calcitonin on function of intercalated cells of rat cortical collecting duct. Am. J. Physiol. 264 (Renal Fluid Electrolyte Physiol. 33): F221–F227, 1993.—In the rat cortical collecting duct (CCD), the presence of highly specific receptors to calcitonin (CT) coupled to a sensitive adenylate cyclase system suggests that this segment is a target site for CT. Our aim was to explore the effects of CT on the rat CCD microperfused in vitro. The hormone failed to alter the osmotic water permeability and did not affect net Na+ transport but generated a luminal-positive transepithelial potential difference (PDlum), which under control conditions was close to zero. This response was dose dependent and was still observed in the presence of luminal amiloride, despite the luminal positivity generated by the Na+ channel blocker (PDlum increased from 4.0 ± 0.8 to 9.5 ± 1.1 mV). In contrast, the nominal absence of CO2/HCO3− or the use of a low-Cl− solution totally prevented the PDlum changes caused by CT. The CT-induced luminal-positive PDlum was reduced by 2.3 ± 0.8 mV after the basolateral addition of the Cl− channel inhibitor dipherylamine-2-carboxylate. 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid and 8-bromo-cyclic AMP (cAMP), predominantly in the principal cells (13). Because the cell type determines the nature of the response, an action of CT on principal cells should therefore be similar to that of ADH on the rat CCD, i.e., an increase of water and electrogenic Na+ reabsorption and generation of a lumen-negative transepithelial potential difference (PDint) (27, 28). Thus our first goal was to test the effects of CT on water permeability, Na+ transport, and PDint. These studies showed that neither Na+ transport nor osmotic water permeability was affected but that addition of CT established a luminal-positive PDint. These results indicated that CT and ADH act on different cell types in the rat CCD. The other cell population, namely the intercalated cells (ICC), participate in the regulation of the acid-base homeostasis. We therefore decided to examine the CT-mediated PDint changes under conditions known to alter the functions of the ICC (2, 32, 35). For this purpose the effects of inhibitors, of ionic substitution, and of acid-base homeostasis alterations were tested. The data indicate that CT exerts an influence over the cells which transport protons and HCO3− in the rat CCD, i.e., the ICC.

METHODS

Experiments were performed in CCD of Sprague-Dawley rats (male and female, 70–100 g), obtained from Charles River Laboratory (Cléon, France). Control animals had free access to standard diet and tap water until the day of experimentation. Animals were killed by cervical dislocation. The left kidney was then removed and placed in ice-cold dissection solution containing (in mM) 9 NaHCO3, 14 KCl, 44 K2HPO4, 14 KH2PO4, 160 sucrose (pH 7.4), and bovine serum albumin (6% wt/vol). As recently reported (18), the use of this intracellular fluidlike solution is favorable for maintaining the transepithelial voltage and intracellular Ca2+, Mg2+, and NaCl transports in the CCD. Slices of renal tissue were transferred to a thermostatically controlled (5°C) dissection chamber and CCD were dissected from the middle part of the cortex, transferred to the perfusion chamber, mounted on concentric pipettes, and perfused in vitro as previously described (5). To keep the pH between 7.35 and 7.45, the bath solution was bubbled with 95% O2–5% CO2 and delivered to the perfusion chamber at a rate close to 10 ml/min. This high perfusion rate maintained a constant pH in the chamber, which was continuously monitored by a customer-designed pH microelectrode.

Ismotic Water Permeability

In these experiments, CO2/HCO3− Ringer solution (Table 1) was utilized on both sides but the luminal fluid was made hypsomotic (220 mosmol/kg H2O) by reduction of its NaCl concentration. After a 40-min equilibration period, control collections were made. Salmon CT (Miacalcic, Sandoz Laboratory) was then added to the bath, and after a further equilibration...
Table 1. Compositions of solutions

<table>
<thead>
<tr>
<th></th>
<th>Ringer</th>
<th>Ringer</th>
<th>Low Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂/HCO₃⁻</td>
<td>CO₂/HCO₃⁻ free</td>
<td>CO₂/HCO₃⁻ free</td>
</tr>
<tr>
<td>NaCl</td>
<td>125</td>
<td>125</td>
<td>25</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Na gluconate</td>
<td>25</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Ca gluconate</td>
<td>1.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.5</td>
<td></td>
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</tr>
</tbody>
</table>

All values are in mM. All solutions also contained 4 l-alanine, 8 glucose, 2 phosphates (except Ringer low Cl⁻), 5 Na acetate, 6 urea, and 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

period of 20 min, samples were collected. Osmotic water permeability (Pₒ) was measured as described previously (33), with [¹⁴C]limulin as volume marker (Service des Molécules Marquées, Centre d’Etude de Saclay, Commissariat à l’Energie Atomique, France).

Na⁺ Net Flux

To determine Na⁺ net flux (JNa⁺), identical CO₂/HCO₃⁻ Ringer solutions were used on the two sides. The action of CT on JNa⁺ was tested using rat CT at 10⁻⁵ M (Hissendorf, Hannover, FRG). This can be considered as a physiological concentration, since during maximal stimulations of endogenous secretion the circulating levels of CT may reach values as high as 10⁻⁵ M (6). The perfusion was initiated at the latest 45 min after the rats were killed. After mounting, the tubules were warmed to 37°C and then equilibrated for 30 min at this temperature. Three to four samples were collected during each of three subsequent 30-min collection periods. The first was a control period, the second period began 20 min after addition of rat CT to the bath, and the third period began 40 min after removal of the hormone. The concentration of Na⁺ in the bath, perfusate, and collected fluid samples were measured by electron microprobe analysis (24). The experimental procedures for collecting the tubular fluid and measuring its ionic concentration with an electron microprobe have been given in detail elsewhere (36). Net rates of ion transport were calculated as: Jx = V₁(Cx - C₀)/L, where V₁ is the collection rate (nl/min) determined by timed collections into a calibrated volumetric pipette, C is the concentration (mM) of the element x measured in the perfused (p) and collected (c) fluids, and L is the length (mm) of the tubule, measured with an eyepiece micrometer. The use of this equation is justified, since we had verified that Pₜ was very low in our experimental conditions, and consequently the net water flux through the rat CCD, in both control and CT periods, is negligible (see results). The PDₑ was simultaneously measured with calomel electrodes, via a salt bridge (3% agar in 0.16 NaCl) connected to the perfusion pipette. PDₑ was referenced to the bath and recorded continuously.

Effects of CT on PDₑ

The same CO₂/HCO₃⁻ Ringer solutions were used on both sides, unless otherwise indicated, and a high luminal perfusion rate (>10 nl/min) was used. After mounting, PDₑ was allowed to stabilize for 30 min before addition of CT to the bath. A new and very stable PDₑ was reached after ~20 min, which was compared with the value measured just before hormonal addition. Salmon CT was used initially and then rat CT when it became available. Both hormones gave similar lumen-positive PDₑ responses with identical kinetics. The PDₑ response to CT was evaluated under various conditions.

With salmon CT, dose dependence. The effects of salmon CT added to the bath at different concentrations were studied. Each tubule was exposed to only one concentration of CTʹ (n = 5 in each group).

Segment specificity. This was investigated with 10⁻¹⁰ M salmon CT in CCD and outer medullary collecting tubules (OMCD) dissected from the same kidneys and perfused in an order selected at random.

Alkali and acid loads. Rats were subjected to an in vivo alkali or acid load by the addition of 1.5% of NaHCO₃ or NH₄Cl to their drinking water 72 h before experiments.

With rat CT at 10⁻⁷ M, amiloride. The Na⁺ channel inhibitor, amiloride (10⁻⁴ M; Sigma, St. Louis, MO), was present in the luminal fluid throughout the experiments.

ionic substitution. The CCD were bathed and perfused throughout the experiments with either Ringer low-Cl⁻ or Ringer CO₂/HCO₃⁻-free solutions (Table 1).

Effects of Cl⁻ and bicarbonate transport inhibitors. To evaluate the effect of the inhibitors on PDₑ, each tubule was transiently exposed to the agent first under control conditions and then 20 min after CT addition. Each substance was used on different tubules at 10⁻⁴ M in the bath. The Cl⁻ channel blocker (8), diphenylamine-2-carboxylate (DPC), was provided by EGA Chemie (Steinheim, FRG). Acetazolamide (ACZ) and 4-acetamido-4'isothiocyanostilbene-2,2'-disulfonic acid (SITS) were from Sigma.

Statistics

Data are presented as means ± SE, and n is the number of tubules. Statistical significance was tested with the Student's paired t test when appropriate. Intergroup comparisons were made by analysis of variance (ANOVA); when variance was significant (P > 0.05), the Bonferroni t test was then applied.

RESULTS

The mean length and inner diameter of the 91 rat CCD perfused in this study were 432 ± 19 and 21 ± 0.3 mm, respectively. In the experiments where PDₑ was measured exclusively, perfusion flow rate was always >10 nl/min to avoid the participation of a dilution potential in the PDₑ (31). On the other hand, water and Na⁺ transports were studied at slow perfusion rates, as usual for flux determinations (27, 28).

Effects of CT on PDₑ

Addition to the bath of salmon CT always induced a lumen-positive PDₑ, which began to increase 2–3 min after CT addition and reached a maximal and stable value within the subsequent 15–20 min. This CT-induced lumen-positive PDₑ was dose-dependent (Fig. 1). The maximal effect was observed at 10⁻⁸ M, and half-maximal effect was around 10⁻⁹ M. The use of rat CT produced similar effects on the PDₑ, with identical kinetics, but higher concentrations were needed: 10⁻⁶ M of rat CT induced changes in PDₑ roughly similar to those of 10⁻⁻⁸ M of salmon CT, in accordance with their respective potencies (rat CT: 400 MRC U/mg, salmon CT: 4,000 MRC U/mg peptide) (15).

Segment specificity. We decided to investigate the effects of CT on CCD and OMCD because CCD is known to have an adenylate cyclase system sensitive to CT, whereas OMCD lacks this system (22). Figure 2 shows that the control PDₑ of OMCD was lumen positive (2.9 ± 0.5 mV, n = 7), whereas that of CCD was not different
from zero (−0.1 ± 0.9 mV; n = 4), OMCD vs. CCD: P < 0.05. The PD_{Te} was not altered by 10^{-10} M of salmon CT in the OMCD (Fig. 2) but was increased to 4.75 ± 1.05 mV in the CCD (P < 0.02 vs. control).

**Effects of CT on P_{f} and Na⁺ Transport**

The effect of CT on water transport was explored by addition of salmon CT to the bath. Collection rates were 3.2 ± 1.6 and 3.3 ± 1.5 nl/min in the control and CT periods, respectively. Under basal conditions, P_{f} was low (11.2 ± 2.9 μm/s) as already known (27). Neither 10^{-10} M (n = 11) nor 10^{-9} M (n = 4) CT significantly modified P_{f} (26.8 ± 6.9 and 21.7 ± 9.1 μm/s, respectively). The effects on P_{f} of 10^{-10} M arginine vasopressin (AVP) were tested on three of these tubules after the recovery period. AVP increased P_{f} from 7.1 ± 1.2 to 286 ± 22 μm/s, indicating that the principal cells were responsive to AVP.

The action of CT on J_{Na} was tested using the peptide normally present in the rat. Collection rates were 2.2 ± 1.1 and 1.9 ± 1.4 nl/min in the control and CT periods, respectively. The basal J_{Na} was not significantly different from zero (3.7 ± 3.7 pmol·min^{-1}·mm^{-1}, n = 6) and did not change significantly upon addition of 10^{-9} M rat CT to the bath (15.9 ± 9.4 pmol·min^{-1}·mm^{-1}). Time-control experiments showed that, under our conditions, the rat CCD had stable J_{Na} (data not shown) and PD_{Te} values (Table 2). The PD_{Te} was not significantly different from zero during the control period (−0.25 ± 0.7 mV), increased consistently with 10^{-9} M rat CT by ~4 mV (see Fig. 3) and became lumen positive (3.8 ± 0.8 mV, CT vs. control: P < 0.05, n = 6). This effect was reversible, although not totally. These PD_{Te} changes toward positive values, together with the absence of significant alterations of J_{Na}, strongly suggest that the PD_{Te} response to CT is independent from Na⁺ transport. To confirm this assumption, this PD_{Te} response was next investigated in the presence of 10^{-4} M amiloride in the luminal fluid. The Na⁺ channel blocker itself generated a lumen-positive PD_{Te} (Fig. 4). Addition of 10^{-8} M rat CT to the bath caused a PD_{Te} increase of 5.5 ± 0.6 mV (Table 2). There was thus an increase of PD_{Te} upon addition of rat CT in the amiloride-treated tubules as great as in tubules not treated with luminal amiloride.

**Characteristics of CT-Induced Positive PD_{Te}**

**Ionic substitution.** The observation that both salmon and rat CT induced a lumen-positive PD_{Te}, without affecting the water permeability and independently of Na⁺ transport, suggested that CT does not act on principal cells. It was therefore relevant to test the possible participation of ICC in this response. As already known (2), both H⁺ and HCO₃⁻ secretory processes are Na⁺ independent but Cl⁻ dependent. The Cl⁻ dependence of the PD_{Te} response to CT was examined on CCD perfused with Ringer low-Cl⁻ solution (Cl⁻ = 2 mM, n = 4) on both sides. We also examined this response in the nominal absence of CO₂/HCO₃⁻ on both sides (Ringer CO₂/HCO₃⁻ free, n = 6). Figure 5 depicts the results of these experiments. Although control PD_{Te} values were similar to those measured on tubules perfused with CO₂/HCO₃⁻ Ringer solution, rat CT at 10^{-8} M generated no significant lumen-positive PD_{Te} either with Ringer low-Cl⁻ solution or in the absence of CO₂/HCO₃⁻. These experiments indicate that both Cl⁻ and CO₂/HCO₃⁻ are required to observe the action of CT on PD_{Te}, suggesting the participation of a Cl⁻/HCO₃⁻ antipporter in the response of CCD to CT. Cl⁻/HCO₃⁻ exchangers are generally associated with Cl⁻ channels to eliminate from the cell the Cl⁻.

**Table 2. Effects of CT on transepithelial potential difference**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>n</th>
<th>PD_{Te}, mV</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time control</td>
<td>6</td>
<td>0.15±0.3</td>
<td>0.75±0.3</td>
</tr>
<tr>
<td>CT</td>
<td>5</td>
<td>-0.44±0.9</td>
<td>4.0±0.8</td>
</tr>
<tr>
<td>Amiloride</td>
<td>6</td>
<td>4.0±0.8</td>
<td>9.5±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of tubules. PD_{Te}, transepithelial potential difference measured before (control) and after 20 min of basolateral presence of rat calcitonin (CT) at 10^{-8} M (exp1). Time control, no CT was added during the experimental period. Amiloride, CO₂/HCO₃⁻ Ringer solution on both sides and 10^{-4} M amiloride in the luminal throughout the experiment. P, level of significance of the experimental vs. control PD_{Te} (paired t test).
introduced from the extracellular space by these exchangers. These Cl- channels are located basolaterally in the ICC (25). Addition of the Cl- channel blocker DPC (10^{-4} M) to the bath had no effect on the basal PD_{te} but decreased the PD_{te} response to 10^{-8} M rat CT by 34% (PD_{te} before DPC: 6.3 ± 0.8 vs. DPC: 4.0 ± 0.7 mV, P < 0.05, n = 7). These data suggest that the Cl- channels participate in this response.

Effects of inhibitors of H+ secretion. It has been reported (19, 34) that SITS and ACZ suppress the lumen-

positive PD_{te} in the OMCD by inhibiting proton secretion. We therefore examined the effects of these agents on the control and CT-stimulated transepithelial voltage. Under control conditions, the basolateral addition of SITS, but not ACZ (both at 10^{-4} M), induced a small change of the PD_{te}, which became slightly lumen negative (Fig. 6). By contrast, the PD_{te} response to rat CT at 10^{-8} M was greatly decreased by the presence of either SITS (116%) or ACZ (69%) in the bath. PD_{te} changed from 3.9 ± 0.9 to -0.6 ± 0.7 mV with SITS and from 7.5 ± 1.9 to 2.5 ± 1.1 mV during ACZ application (P < 0.001, n = 5 in each group). As shown in Fig. 6, effects of inhibitors were reversible.

Effects of acid-base status of rat on CCD response to CT. To test whether the acid-base status of the animals influences the response to CT, tubules from kidneys of rats subjected to an acid (n = 5) or alkali load (n = 7) were perfused. Values of pH of vesical urine collected at the moment of the death were 5.5 ± 0.05 for acid load and 7.7 ± 0.08 for alkali load (P < 0.05). Both values are significantly different from the urine pH of control rats (6.5 ± 0.03, n = 6). CCD from acid-loaded rats responded to 10^{-10} M salmon CT to the same extent as those dissected from control animals (Fig. 7), but the hormonal action was significantly attenuated when the CCD were from alkali-loaded rats (PD_{te} increases: acid load 4.0 ± 0.3 vs. alkali load 1.6 ± 0.6 mV, P < 0.01).

**DISCUSSION**

This study was designed to investigate the effects of CT on the rat CCD microperfused in vitro. CT failed to alter the CCD osmotic water permeability and did not affect...
mone. It may be therefore assumed that CT acts via the distribution of the cyclase sensitivity to this hor-
ing limb (7), CT increased PDt, also in accordance with 21) and mouse cortical (but not medullary) thick ascend-
observed in the OMCD, which lacks an adenylate cyclase but addition of CT established a sustained dose-depen-
dent lumen-positive PDt,. The response to CT was not reabsorption but acts on ICC, which transport HCO; and icipal cells responsible for the transcellular Na+ and water ulation in CCD from alkali-loaded rats. Therefore the bulk nder of evidence indicates that CT does not act on the prin-
cells are the target cells for CT, the antidiuretic ility. Because it now seems improbable that the prin-
cells are responsible for H+ and HCO; secretions, respectively. In the α-subtype, the H+-ATPase is apically located and the Cl-/HCO; exchanger is on the basolateral membrane, but the opposite distribution is observed in the β-subtype (1).

Therefore, regardless of which subtype is activated by CT, HCO; or Cl- removal from both sides or carbonic anhydrase inhibition, should block the PDt, response to CT if the CT-induced lumen-positive PDt, is the result of stimulation of ICC functions. Indeed, our experiments showed that the nominal absence of CO2/HCO; or the use of a low Cl- solution prevented the PDt, response to CT. In addition, the CT-induced lumen-positive PDt, was considerably inhibited by basolateral ACZ (a carbonic anhydrase inhibitor) and by DPC. The target cells of this Cl- channel blocker should be the ICC only, be-
cause the basolateral membrane of rat principal cells have no Cl- conductance (30). SITS is a known inhibitor of Cl-/HCO; exchangers (19). Addition of SITS to the bath inhibited the CT-mediated response of PDt, Furthermore, it is known that the acid-base status of the animal influences ICC function (32), and our results show that the CT-mediated response was significantly lower in the alkali-loaded than in the acid-loaded rats. Thus the bulk of evidence presented here indicates that the ICC are the cells responsible for the CT-induced lumen-positive PDt, recorded in rat CCD.

Lack of Effect of CT on Principal Cell Functions

CT did not change the low basal values of Na+ net transport. It could be argued however, that the scattering of the data prevents the identification of moderate responses. Actually, increases in Na+ transport were always accompan-
ied by changes in PDt, that became more lumen negative (28). Conversely, inhibition of active Na+ reab-
sorption lead to a lumen-positive PDt, (3, 20). This was the case in our experiments with luminal amiloride. In the presence of amiloride, however, CT caused an addi-
tional increase in the PDt, by 5.5 mV, as under control conditions, showing that the response to CT was in no way modified by blocking the luminal Na+ channels of the principal cells. These findings argue against the participation of the principal cells in the PDt, response to CT.

The principal cells are also the site of the AVP-medi-
ated hydromotic response of the CCD (27). Our results indicate that salmon CT failed to induce any change in water permeability, providing a further argument against the participation of principal cells in the CCD response to CT. It has been shown, however, that administration of either salmon or human CT to rats undergoing water diuresis reduces the urinary flow rate and increases the urine osmolality (0, 29). These effects were interpreted as resulting from an effect of CT on the CCD water permeability. Because it now seems improbable that the principal cells are the target cells for CT, the antidiuretic effect of this hormone observed in vivo in the rat remains to be explained.

Effects of CT on ICC Functions

In the CCD, the vectorial transport of acid or base is accomplished by the ICC. The transport components in-
clude a H+-adenosine triphosphatase (ATPase) and a Cl-/HCO; exchanger. Cl- that penetrates into the cell through the Cl-/HCO; exchanger leaves it via a Cl-
channel located in the basolateral membrane (25). There are at least two subtypes of ICC, the α- and β-cells, which are responsible for H+ and HCO; secretions, respectively. The Cl-/HCO; exchanger is on the basolateral membrane, but the opposite distribution is observed in the β-subtype (1).

Therefore, regardless of which subtype is activated by CT, HCO; or Cl- removal from both sides or carbonic anhydrase inhibition, should block the PDt, response to CT if the CT-induced lumen-positive PDt, is the result of stimulation of ICC functions. Indeed, our experiments showed that the nominal absence of CO2/HCO; or the use of a low Cl- solution prevented the PDt, response to CT. In addition, the CT-induced lumen-positive PDt, was considerably inhibited by basolateral ACZ (a carbonic anhydrase inhibitor) and by DPC. The target cells of this Cl- channel blocker should be the ICC only, be-
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Possible Physiological Consequences of Effects of CT on CCD

It is well recognized that the net K+ secretion by the CCD is increased by lumen-negative potentials and decreased by positive transepithelial voltages (14). An indirect effect of CT should therefore be a reduction of K+...
secretion in the rat CCD. Indeed, micropuncture studies revealed that CT inhibited K+ secretion in the distal tubule (10) and decreased medullary K+ recycling and K+ excretion rate (9).

In the rabbit CCD, norepinephrine and isoproterenol stimulate net HCO3− secretion/Cl− reabsorption (16). Glucagon was also shown to influence the distal HCO3− transport in the rat (26). Together with our results, these data indicate that ICC are under multiple hormonal control. Although the physiological significance of the new findings reported here awaits further studies, one can wonder whether CT participates in the fine regulation of the acid-base balance. In this regard, a recent study reported that CT shifts the acid-base status to metabolic alkalosis in osteoporotic women receiving large doses of the hormone (12).

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