Cellular localization of type 5 and type 6 ACs in collecting duct and regulation of cAMP synthesis

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Two cell types have been described in kidney collecting duct: principal cells, involved in water homeostasis (30), are the predominant cell type in the collecting duct. In rat kidney, type A intercalated cells, involved in proton secretion and bicarbonate reabsorption, are found in cortical and outer medullary collecting ducts, whereas type B intercalated cells, mostly found in cortical collecting ducts, function in the other direction (5). Type 5 and type 6 AC mRNAs are both expressed in the rat kidney outer medullary collecting duct (OMCD) (6), where principal and type A intercalated cells are the two major cell types. In microdissected OMCDs, AC activity is stimulated by arginine vasopressin (AVP) and glucagon (8, 22). Physiological and biochemical results support the conclusion that AVP stimulates AC activity in principal cells, whereas glucagon is active in intercalated cells (6, 8, 21).

An inhibitory effect of Ca\(^{2+}\) on the cAMP pathway in the rat OMCD was first suggested by the negative regulation of a Ca\(^{2+}\) ionophore on AVP-dependent cAMP accumulation (19). PGE\(_2\) and carbachol, a muscarinic agonist of the acetylcholine receptor, both induce a Ca\(^{2+}\)-dependent inhibition of hormone-stimulated intracellular cAMP accumulation (1, 6), which is probably linked to the Ca\(^{2+}\)-sensitive calcium-inhibitable adenylyl cyclase (AC) type 5 and type 6 ACs. The simultaneous superfusion of carbachol and PGE\(_2\) elicited an additive increase in the intracellular Ca\(^{2+}\) concentration, suggesting that the Ca\(^{2+}\)-dependent regulation of these agents occurs in different cell types. Glucagon-dependent cAMP synthesis was inhibited by both a pertussis toxin-sensitive PGE\(_2\) pathway (63.7 ± 4.6% inhibition, \(n = 5\)) and a Ca\(^{2+}\)-dependent carbachol pathway (48.6 ± 3.3%, \(n = 5\)). The simultaneous addition of both agents induced a cumulative inhibition of glucagon-dependent cAMP synthesis (78.2 ± 3.3%, \(n = 5\)). The results demonstrate a distinct cellular localization of type 5 and type 6 AC mRNAs in OMCD and the functional expression of these Ca\(^{2+}\)-inhibitable enzymes in intercalated cells.

In many cell types, the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) regulates cAMP levels through interactions of Ca\(^{2+}\) on cAMP synthesis and/or cAMP hydrolysis. These effects of [Ca\(^{2+}\)]\(_i\) are linked to the presence of Ca\(^{2+}\)-sensitive adenylcyclases (ACs) and/or Ca\(^{2+}\)/calmodulin-dependent phosphodiesterases (7, 13, 17). Among AC isoforms, the enzymatic activity of type 5 and type 6 AC is directly inhibited by submicromolar concentrations of Ca\(^{2+}\) (25, 31, 33). Ca\(^{2+}\)-inhibitable AC isoforms are sensitive to [Ca\(^{2+}\)]\(_i\), typically achieved in intact cells as a consequence of phospholipase C activation and/or Ca\(^{2+}\) channel activation (4).
Localization of type 5 and/or type 6 functional AC proteins in the OMCD.

The purpose of the present experiments was to study the potential role of Ca\(^{2+}\)-inhibitable AC isoforms in the regulation of cAMP synthesis in the rat OMCD cells.

The localization of type 5 and type 6 AC mRNAs at the cellular level was performed by in situ hybridization. Only type 6 mRNA was detected in rat kidney collecting duct principal cells, whereas both type 5 and type 6 Ca\(^{2+}\)-inhibitable AC isoforms were found in intercalated cells. In superfused OMCDs, the effects of PGE\(_2\) and carbachol on [Ca\(^{2+}\)]\(_i\) were additive, suggesting that the Ca\(^{2+}\)-inhibitable AC isoforms are regulated by independent Ca\(^{2+}\) and Goi-mediated processes, leading to a cumulative inhibition of AC activity (7, 14, 13, 29). This property cannot be verified accurately in principal cells because the Ca\(^{2+}\)-inhibitory effect of PGE\(_2\) was not observed (80). In our study, the simultaneous addition of carbachol and PGE\(_2\) induced a cumulative inhibition of glucagon-dependent cAMP synthesis, demonstrating that these agents inhibit the same AC catalytic units. The results therefore localize Ca\(^{2+}\)-inhibitable AC mRNAs in OMCD and demonstrate the functional expression of Ca\(^{2+}\)-inhibitable AC isoforms in intercalated cells.

METHODS

Unless otherwise specified, reagents used were purchased from Merck (Darmstadt, Germany), Sigma Chemical (St. Louis, MO), and Calbiochem (San Diego, CA). Experiments were performed in male Sprague-Dawley rats (140–180 g body wt, Ifa-Credo), maintained on a standard diet with free access to water.

Northern blots. Hybridization probes for type 5 and type 6 AC were prepared by random primed labeling of the regions described for in situ hybridization experiments using [\(\alpha\)-\(^{32}\)P]dCTP. A multiple rat tissue Northern blot (Clontech Laboratories) was hybridized, as per the manufacturer's instructions, in 5 ml of ExpressHyb solution at 68°C for 30 min. The probe was added to 5 ml of fresh ExpressHyb solution and incubated at 68°C for 1 h. Washes were performed as follows: 40 min (with 3 changes of the solution) in solution 1 (2X SSC-0.05% SDS) at room temperature, followed by an incubation of 40 min at 50°C in solution 2 (0.1X SSC-0.1% SDS). The blot was then wrapped in plastic and exposed to X-ray film with an intensifier screen at −80°C for 3 days.

In situ hybridization and immunostaining. Probes specific for type 5 and 6 ACs were designed in the most divergent regions of AC cDNA regions. A 376-bp fragment (Pou II-Sphi I, nucleotides 3965–4342) of type 6 AC cDNA, located in the 3'-untranslated region, was subcloned in pGEM3Zf (+) (Promega Biotech, Madison, WI). A 1,080-bp fragment corresponding to nucleotides 1205–2285 (EcoR I, Pou II) of type 5 AC cDNA coding region was subcloned in BSSK + (Stratagene). Sense and antisense cRNA probes were in vitro transcribed with T3, T7, or SP6 RNA polymerases (Promega Biotech) according to the manufacturer's instructions, in the presence of [\(^{35}\)S]UTP (200,000 cpm, Amersham, Les Ulis, France).

Rat kidneys were fixed in 4% paraformaldehyde in PBS, washed in PBS, and dehydrated with a graded series of ethanol and butanol. Tissues were paraffin embedded, and 4-μm sections were collected on silane-coated slides. In situ hybridization was performed as described by Sibony et al. (26). Briefly, slides were deparaffinized in toluen and rehydrated by a graded series of ethanol (100–30%). After boiling in a solution of 0.01 M citric acid in a microwave oven, slides were treated with 0.1% H\(_2\)O\(_2\) in PBS. After fixation in 50% formamide, 10% dextran sulfate, 2X SSC, 70 mM dithiothreitol (DTT) and 0.5–1.10^6 counts · min⁻¹ radiolabeled probe · tissue section⁻¹. Hybridization was performed overnight at 50°C in a humidified chamber. Slides were rinsed in 5X SSC-100 mM EDTA followed by an incubation in 50% formamide-1X SSC-12.5 mM DTT. They were further treated by RNase A (20 μg/ml). For immunostaining, the slides were then incubated for 5 min in PBS containing 1% BSA (PBS/BSA), followed by overnight incubation of a 1:200 dilution of anti-aquaporin-3 (AQP3) or anti-vacuolar H⁺-ATPase 56-kDa subunit (kindly provided by Dr. D. Brown, Massachusetts General Hospital, Charlestown, MA) antiserum in PBS/BSA. The sections were then washed 3 × 10 min in PBS, followed by a 2-h incubation in horseradish peroxidase-conjugated mouse anti-rabbit antibodies (6 μg/ml) in PBS/BSA. The sections were washed 2 × 10 min in PBS. Staining was revealed with diaminobenzidine, and slides were washed overnight in 50 mM Tris · HCl, pH 8. Finally, the slides were exposed for 3–5 wk to Kodak NTB2 liquid emulsion, counterstained with toluidine blue and examined under the microscope (Olympus Van OX).

Isolation of rat kidney OMCDs. The experimental procedure used to microdissect intact segments from collagenase-treated rat kidney has previously been detailed (8, 9). After the rats were anesthetized (pentobarbital, 6 mg/100 g body wt), the left kidney was perfused with microdissection medium containing 0.1% collagenase (Serva, Boehringer Mannheim). After hydrolysis of the kidney (20 min at 30°C in 0.12% collagenase solution), single pieces (0.3–1.5 mm length) of collecting duct were microdissected at 4°C from the outer medulla. The standard microdissection medium was composed of (in mM) 137 NaCl; 5 KCl; 0.8 MgSO\(_4\); 0.33 K\(_2\)HPO\(_4\); 0.44 KH\(_2\)PO\(_4\); 1 MgCl\(_2\); 4 NaHCO\(_3\); 10 CH\(_3\)COONa; 1.0 or 2.0 CaCl\(_2\) (see below); 5 glucose; and 20 nM HEPES, pH 7.4, and 0.1% (wt/vol) BSA (fraction V, Pentex, Miles, Kankakee, IL).

Measurement of [Ca\(^{2+}\)]\(_i\). [Ca\(^{2+}\)]\(_i\) was measured in single OMCD samples by using the calcium-sensitive fluorescence probe acetoxymethyl ester of fura 2 (fura 2-AM, Molecular Probes, Eugene, OR) as previously described (1, 10, 27). Briefly, the samples were loaded for 60 min with 10 μM fura 2-AM. Each tubule was then transferred to a superfusion chamber fixed on an inverted fluorescence microscope (Zeiss IM 35, Oberkochen, Germany). Tubules were superfused at 37°C at a rate of 10–12 ml/min, corresponding to ~10 exchanges/min. The superfusion medium (microdissection medium without serum albumin) contained either 2 mM Ca\(^{2+}\) or no Ca\(^{2+}\) (nominally Ca\(^{2+}\)-free medium without CaCl\(_2\) and containing 0.1 mM EGTA). After a 5- to 10-min equilibration period, agonists were added to the medium and superfused over tubules. Because of the dead space of the superfusion setup, the time necessary to achieve a full equilibration was
15–20 s. A circular area of 60-μM diameter was selected over the tubule (×400 magnification). The fluorescence intensity emitted from this area (during brief excitation periods at 340 and 380 nm alternatively, at a maximal rate of 30 cycles/min), was recorded every 2 s.

Tubule autofluorescence was subtracted from the fluorescence intensities measured at 340 and 380 nm. [Ca\(^{2+}\)]\(_{\text{min}}\), was calculated by using a dissociation constant of fura 2 for calcium of 224 nM as previously reported (1, 20, 27). Results obtained from different tubules (n) microdissected from several rats were expressed as means ± SE. Statistical analysis by one-way analysis of variance was followed by Fisher’s least significant difference test.

Measurement of glucagon-dependent cAMP synthesis. Hormone-dependent cAMP synthesis in an intact single segment was measured as previously reported (1, 9). Microdissection medium (1 mM Ca\(^{2+}\)) was supplemented with 5 μM indomethacin and 0.5 unit/ml adenosine deaminase (Boehringer Mannheim) to prevent the endogenous synthesis of prosta-glandins and the release of adenosine, which interfere with the regulation of cAMP levels in rat OMC (9). The incubation medium similar to the microdissection medium, included 0.1% (wt/vol) bacitracin (to inhibit peptidase activity) and 1 mM IBMX, an inhibitor of all phosphodiesterases in rat kidney (16). Microdissected tubules were transferred in 2 μl of incubation medium on glass slides (1 or 2 pieces/slide) and photographed to measure their length. Each sample was preincubated for 10 min at 30°C. After the addition of 2 μl incubation medium containing 1 μM glucagon (Neosystem Laboratoire, Strasbourg, France), with or without other agonists, samples were incubated for 4 min at 35°C. All agents were used at concentrations inducing maximal effects (1, 9, 20). The reaction was stopped by rapidly transferring the tubule together with 1 μl incubation medium into a polypropylene tube containing a 20 μl mixture of formic acid in absolute ethanol (5% vol/vol). Samples were evaporated to dryness overnight at 40°C and then kept at −20°C until cAMP assay. The amounts of cAMP were measured in acetylated samples by radioimmunoassay (Sanofi Diagnostics Pasteur, Marne-la-Coquette, France, or NEN Life Sciences Products, Le Blanc Mesnil, France). Under our conditions, the basal level of cAMP present in one single piece of tubule was similar to, or below, the sensitivity threshold of the assay (1, 9). Thus only hormone-induced cAMP synthesis could be measured. Results were expressed in femtomoles of cAMP accumulated per millimeter of segment per 4-min incubation time (fmol · mm\(^{-1}\) · 4 min\(^{-1}\)). In each experiment, all experimental conditions were tested in six to nine tubule samples from the same rat kidney. The mean cAMP value from each condition was expressed in absolute value or in a percentage of inhibition calculated from the mean value obtained with glucagon alone. Results are given as means ± SE calculated from n experiments. Statistical analysis by the one-way analysis of variance was followed by Fisher’s least significant difference test.

RESULTS

Northern blotting. The specificity of probes used for in situ hybridization was checked by Northern blotting of several rat tissues (Fig. 1). The probe for type 5 AC produced a strong hybridization signal in heart and brain (Fig. 1A), a weaker signal in kidney and lung and a very weak signal in spleen, liver, skeletal muscle, and testis, consistent with previous reports for type 5 AC (23). Similarly, type 6 AC was detected in nearly all tissues (Fig. 1B), consistent with previous localization (18, 23).

In situ hybridization. By in situ hybridization on 4-μm-thick rat kidney sections, both type 5 (Fig. 2a) and type 6 AC (Fig. 2b) mRNAs were relatively abundant in glomeruli. The labeling for type 5 AC mRNA was strongest in small arteries and blood vessels (Fig. 2a, arrows), where little labeling was observed for type 6 AC (not shown). Significant labeling for type 5 and type 6 AC was also observed in the interstitium, between kidney tubules. No significant labeling was observed in kidney with either type 5 or type 6 sense probes (Fig. 2c and d, respectively). In kidney tubules, as previously reported (14), type 6 AC mRNA was abundant in thick ascending limbs and collecting ducts (Fig. 3, c and d), but proximal tubules were weakly labeled (Fig. 3c). Consistent with previous reports by quantitative RT-PCR (6), type 5 AC was not detected in proximal tubules or thick ascending limbs (Fig. 3, a and b).

The characterization of cell types in which type 5 and type 6 AC mRNAs were expressed in collecting tubules was achieved by immunoperoxidase staining with anti-
AQP3 or anti-vacuolar H^+-ATPase 56-kDa-subunit rabbit polyclonal antibodies after the in situ hybridization. As previously reported (see Ref. 30), anti-AQP3 antibodies specifically stained basolateral plasma membranes of collecting duct principal cells (Fig. 3, a and c), whereas staining for the proton pump was localized to intercalated cells in collecting ducts (Fig. 3, b and d). In these tubules, labeling for type 6 AC was abundant in both AQP3-positive principal cells (Fig. 3c) and proton pump-positive intercalated cells (Fig. 3d). In contrast, labeling for type 5 AC was primarily observed in collecting duct intercalated cells, negative for AQP3 (Fig. 3a) but positive for the vacuolar proton ATPase (Fig. 3b). Accordingly, no labeling for type 5 AC mRNA was observed in inner medullary collecting ducts, devoid of intercalated cells (Fig. 4a), whereas these tubules were still labeled for type 6 AC (Fig. 4b). Although there was no evidence of intercalated cells negative for either type 5 or type 6 AC in the OMCD, labeling with the type 5 AC probe in cortical collecting duct intercalated cells was usually weaker than in the outer medulla, often undetectable (not shown). Altogether, these results in rat kidney OMCD therefore suggest that type 6 AC is expressed in both principal and intercalated cells, whereas type 5 AC is only found in intercalated cells. In the OMCD, most intercalated cells were labeled with both type 5 and type 6 AC, suggesting that both mRNAs could be expressed in type A intercalated cell. Type 5 AC-negative intercalated cells were only observed in cortical collecting ducts, where type B intercalated cells are more abundant than in the OMCD. This result may suggest a greater expression of type 5 AC in type A intercalated cells.

Quantification of in situ hybridization labeling. The in situ observations above were confirmed by quantification of labeling in cells unambiguously detected as either principal or intercalated cells by antibody (anti-AQP3 or anti-proton pump) staining. Results are reported in Table 1 and show no statistically significant differences of labeling for type 6 AC in OMCD principal and intercalated cells. In contrast, labeling for type 5 AC was much greater in intercalated cells and significantly different from principal cells, where labeling was not significant.

Effect of carbachol and PGE_2 on [Ca^{2+}]_i increases in rat OMCD. As underlined at the beginning of this study, [Ca^{2+}]_i increases induced by PGE_2 and carbachol in rat OMCD (1, 20) appear a prerequisite condition to observe the inhibition elicited by PGE_2 on AVP-dependent cAMP accumulation (1) and the inhibition elicited by carbachol on glucagon-dependent cAMP synthesis (6). These observations led to the hypothesis that a PGE_2-mediated [Ca^{2+}]_i increase might be mainly effective in the vasopressin-sensitive cells, whereas a carbachol-mediated [Ca^{2+}]_i increase might be located in the glucagon-sensitive cells. This hypothesis was tested by comparing [Ca^{2+}]_i variations induced by the addition of both agents to the responses obtained with each agent added alone to the superfusion medium.

Carbachol and PGE_2 were used at concentrations inducing maximal [Ca^{2+}]_i increases and did not elicit homologous desensitization (20 and data not shown). In a same tubule, the superfusion of 0.3 μM PGE_2 followed by the superfusion of 100 μM carbachol, or conversely, did not give evidence of a heterologous
desensitization in 2 mM Ca\(^{2+}\) medium (Fig. 5). Both agents induced a peak of \([\text{Ca}^{2+}]_i\) of a comparable magnitude, and carbachol elicited a pronounced plateau phase (Fig. 5).

The simultaneous superfusion of PGE\(_2\) and carbachol induced a higher increase in \([\text{Ca}^{2+}]_i\) than the response observed with the superfusion of only one agonist (Fig. 6A). The same observation was made...

Fig. 3. In situ hybridization in kidney outer medulla. In kidney tubules, type 5 AC mRNA was only detected in collecting ducts (★), where principal cells were stained by anti-aquaporin-3 (AQP3) antibodies (a) and intercalated cells were stained by anti H\(^+\)-ATPase antibodies (b). In these tubules, labeling for type 5 AC was localized in intercalated cells (arrowheads) but absent from principal cells (arrows). Type 6 AC mRNA (c, d) was abundant in both collecting duct (★) and thick ascending limbs (T). Weak labeling was also detected in proximal tubules (P). In the collecting duct, both principal cells (arrows) stained with anti-AQP3 antibodies (c) and intercalated cells (arrowheads) stained with anti-H\(^+\)-ATPase antibodies (d) were labeled by the type 6 AC mRNA probe. Both type 5 and type 6 AC mRNA were detected in the interstitium. Bars = 20 μm.

Fig. 4. In situ hybridization in kidney inner medulla. No labeling was observed in inner medullary collecting ducts (★) with the type 5 AC mRNA probe (a). In contrast, type 6 AC was still detected in inner medullary collecting duct cells (b). Sections were stained with anti-AQP3 antibodies. Bars = 20 μm.
Table 1. Quantification of in situ hybridization labeling

<table>
<thead>
<tr>
<th>Probe</th>
<th>Intercalated Cells</th>
<th>Principal Cells</th>
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<tbody>
<tr>
<td>Type 5 AC</td>
<td>15.0 ± 0.7(65)</td>
<td>2.6 ± 0.2(61)</td>
</tr>
<tr>
<td>Type 6 AC</td>
<td>9.9 ± 0.6(37)</td>
<td>9.7 ± 0.6(34)</td>
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</table>

Values are means ± SE; no. of cells are in parentheses. The average number of grains per intercalated cell and principal cell in the outer medullary collecting duct (OMCD) was quantified after in situ hybridization with probes specific for either type 5 or type 6 adenyl cyclase (AC). Cell types were identified by staining with anti-aquaporin-3 (AQP3) antibodies (principal cells) or anti-H-ATPase (intercalated cells). The difference in labeling for intercalated cells and principal cells was significant for type 5 AC (P < 10^{-5}), but not for type 6 AC (P > 50%). Labeling for type 5 AC in principal cells was comparable to background in the lumen of tubules (6 grains/100 μm²).

whatever the order of superfusion of the agonists. Intracellular Ca^{2+} concentrations were calculated from the maximal peak values that allowed accurate determinations of [Ca^{2+}]_i increases (Fig. 6A). As shown by the mean data, the [Ca^{2+}]_i increase obtained with the addition of both PGE_2 and carbachol was statistically higher than the response observed with each agonist (Table 2). In addition, the experimental value obtained with the superfusion of both PGE_2 and carbachol was not different from the theoretical value calculated by assuming a full additivity of the individual responses (Table 2).

Additional experiments were conducted in a Ca^{2+}-free medium. As usually observed on a same tubule superfused with a Ca^{2+}-free medium, the [Ca^{2+}]_i increase elicited by one given agonist was no longer observed with a second superfusion of the same agonist (data not shown). In contrast, the superfusion of PGE_2 followed by that of carbachol, or conversely, led to successive [Ca^{2+}]_i increases (Fig. 6B). The simultaneous superfusion of PGE_2 and carbachol induced a response statistically higher than the individual responses, and this response was not different from the theoretical value calculated by assuming a full additivity of the increases of [Ca^{2+}]_i elicited by PGE_2 and carbachol (Fig. 6B and Table 2). Altogether, these data establish that PGE_2 and carbachol release Ca^{2+} from different Ca^{2+} pools located in either the same cell or different cells of rat OMCD.

**Multiple combined inhibition of glucagon-dependent AC activity.** Glucagon-dependent cAMP synthesis is inhibited by both carbachol through a Ca^{2+}-dependent process (6) and PGE_2 through a Ca^{2+}-independent, Gα_i-mediated process (3). If present in a same cell, these regulations suggest that different mechanisms may inhibit the same AC enzymatic activity. This hypothesis was tested in multiple, combined inhibition experiments by using criteria previously defined (1). PGE_2 or carbachol inhibited to a comparable extent, close to 50–60%, the response to glucagon (Table 3). The simultaneous addition of both agents led to a residual cAMP value lower than that obtained with each agent alone, but the response to glucagon was not fully abolished (Table 3). This result establishes that PGE_2 and carbachol were active in the same glucagon-sensitive cells. The results were further analyzed by comparing the values measured to those that could be expected if a different mechanism of inhibition on AC activity accounted for carbachol- and PGE_2-mediated regulation, i.e., if these two agents elicited a cumulative inhibition of cAMP synthesis. The measured value (8.7 ± 0.9 fmol · mm^{-1} · 4 min^{-1}) (Table 3) was not different from the theoretical value calculated assuming an hypothesis of cumulative inhibition (7.5 ± 0.6 fmol · mm^{-1} · 4 min^{-1}). These results therefore demonstrate that PGE_2 and carbachol inhibit the same pool of glucagon-sensitive AC catalytic units in rat OMCD by different and independent mechanisms.

**DISCUSSION**

In this study, Ca^{2+}-inhibitable AC isoforms were localized by in situ hybridization at the cellular level in the rat OMCD to further define the regulation of cAMP content in this segment. Hormone-dependent cAMP accumulation is inhibited by Ca^{2+} in both cell types of the OMCD, but the agonist involved and the mechanism of Ca^{2+} inhibition are cell specific. Indeed, the muscarinic agonist carbachol induces a Ca^{2+}-dependent inhibition of glucagon-mediated cAMP synthesis but has no effect on AVP-stimulated cAMP synthesis (6) or cAMP accumulation (2). On the other hand, PGE_2 inhibits AVP-dependent cAMP accumulation, mainly by an increase in cAMP hydrolysis, through a
**Table 2. Additive effect of PGE₂ and carbachol on \([Ca^{2+}]_i\) increases**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>([Ca^{2+}]_i) Increases, nM</th>
<th>(2) mM Ca²⁺ medium</th>
<th>(2) mM Ca²⁺-free medium</th>
</tr>
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<tbody>
<tr>
<td>PGE₂</td>
<td>87 ± 10⁺</td>
<td>95 ± 8⁺</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>106 ± 13⁺</td>
<td>85 ± 12⁺</td>
<td></td>
</tr>
<tr>
<td>Exp (PGE₂+carbachol)</td>
<td>202 ± 16†</td>
<td>165 ± 12†</td>
<td></td>
</tr>
<tr>
<td>The (PGE₂+carbachol)</td>
<td>193 ± 16</td>
<td>180 ± 14</td>
<td></td>
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</table>

Values are means ± SE obtained from different recordings (n), examples of which are given in Fig. 6. The intracellular Ca²⁺ concentration (\([Ca^{2+}]_i\)) variations elicited at the peak in either 2 mM Ca²⁺ or Ca²⁺-free medium (0 Ca²⁺ + 0.1 mM EGTA) are expressed as maximal increases over basal value. The mean basal value of \([Ca^{2+}]_i\), was of 60 ± 4 nM (n = 14 tubules microdissected from 4 kidneys) and 104 ± 3 (n = 10 tubules microdissected from 4 kidneys) without and with Ca²⁺ in the superfusion medium, respectively. PGE₂ (0.3 μM in 2 mM Ca²⁺ and 3 μM in Ca²⁺-free medium) and carbachol (100 μM) were added alone or in combination to the superfusion medium. The order of superfusion of the agonists was modified from one tubule to another. The mean value obtained with each agonist was compared with the experimental value obtained with the simultaneous addition of both agonists (Exp) and to the theoretical value (Th) calculated by assuming a full additivity of \([Ca^{2+}]_i\), increases. *P < 0.01 compared with the experimental or theoretical value of additivity. †Not significant compared with the theoretical value of additivity.

**Fig. 6. Additive effect of PGE₂ and carbachol on \([Ca^{2+}]_i\) increases in rat outer medullary collecting duct (OMCD). A: representative traces of \([Ca^{2+}]_i\) variations elicited by different agonists added in 2 mM Ca²⁺ medium. The tubule was superfused with 100 μM carbachol, then the combination of both agonists, and finally 0.3 μM PGE₂. Horizontal bars, superfusion period of each solution. The magnitude of the responses was independent from the order of the superfusion (see Table 2). B: representative traces of \([Ca^{2+}]_i\) variations elicited by different agonists added in Ca²⁺-free medium. The tubule was superfused with 2 mM Ca²⁺ and then with Ca²⁺-free medium. Rapid superfusion of PGE₂ (3 μM) and then carbachol (100 μM) elicited successive \([Ca^{2+}]_i\) increases (left). The magnitude of carbachol- or PGE₂-mediated \([Ca^{2+}]_i\) increase was independent from the order of superfusion [carbachol = 48 ± 8 nM (n = 8) and 38 ± 5 (n = 5) with the 1st and 2nd superfusion, respectively; PGE₂ = 121 ± 15 nM (n = 5) and 111 ± 11 (n = 8) with the 1st and 2nd superfusion, respectively]. Note that, in contrast to successive superfusions of different agonists, a second superfusion with the same agonist did not elicit a second response (data not shown). The combination of both agonists added in Ca²⁺-free medium induced a response higher than that elicited by either carbachol or PGE₂ (right). Note that, after the treatment with PGE₂ followed by the superfusion of carbachol, it was necessary to superfuse the tubule with 2 mM Ca²⁺ medium to refill the intracellular Ca²⁺ stores. The magnitude of the different responses was independent from the order of superfusion (see Table 2).

Ca²⁺-mediated process that is insensitive to pertussis toxin (1, 3). In addition, PGE₂ inhibits glucagon-dependent cAMP synthesis through a Ca²⁺-independent, Gα₁₃-mediated process (3). These mechanisms are summarized in Fig. 7.

In the OMCD, carbachol and PGE₂ increase \([Ca^{2+}]_i\), through an interaction with the m1 subtype of the

**Table 3. Cumulative inhibition of PGE₂ and carbachol on glucagon-dependent cAMP synthesis**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>cAMP Synthesis, fmol · mm⁻¹ · 4 min⁻¹</th>
<th>%Inhibition of Response to Glucagon</th>
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<tr>
<td>Glucagon</td>
<td>43.7 ± 8.1</td>
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<tr>
<td>Glucagon + PGE₂</td>
<td>14.9 ± 2.0⁺</td>
<td>63.7 ± 4.6</td>
</tr>
<tr>
<td>Glucagon + carbachol</td>
<td>21.9 ± 3.3⁺</td>
<td>48.6 ± 3.3</td>
</tr>
<tr>
<td>Glucagon + PGE₂ + carbachol</td>
<td>8.7 ± 0.9⁺†</td>
<td>78.2 ± 3.33</td>
</tr>
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</table>

Values are means ± SE calculated from 5 experiments. In each experiment, 0.3 μM PGE₂ and 100 μM carbachol were used alone and in combination in the presence of 1 μM glucagon and 1 mM IBMX; the percentage of inhibition was calculated from the corresponding response obtained with glucagon. *P < 0.05 compared with the mean value obtained with glucagon. †Not significant compared with the response obtained with either PGE₂ or carbachol added alone. ‡Not significant compared with the experimental value calculated by assuming a cumulative inhibition hypothesis (7.5 ± 0.6 fmol · mm⁻¹ · 4 min⁻¹ and 81.5 ± 2.4% inhibition, respectively).
muscarinic receptor (20) and, very likely, with the EP1 subtype of the PGE2 receptor (3), respectively. These receptor subtypes are usually coupled to the phospholipase C pathway (12, 15). In our experiments, simultaneous superfusion of PGE2 and carbachol, in either 2 mM Ca\(^{2+}\) or Ca\(^{2+}\)-free medium, produced [Ca\(^{2+}\)]\(_i\) peaks corresponding to a full additivity of the effects of both agonists. This result and the observation of a Ca\(^{2+}\)-dependent inhibition of cAMP content elicited by either PGE2 in vasopressin-sensitive cells (1) or carbachol in glucagon-sensitive cells (6) strongly suggest a cell-specific [Ca\(^{2+}\)]\(_i\) increase induced by PGE2 and carbachol in principal and intercalated cells, respectively (Fig. 7).

Type 5 and type 6 AC mRNAs have been detected by quantitative RT-PCR in the OMCD, and functional data have suggested that this localization corresponds to the expression of functional proteins (6). Consistently with previous RT-PCR (6), type 6 AC mRNA was highly expressed in thick ascending limbs and collecting ducts. Colocalization of type 6 AC mRNA by in situ hybridization and cells positive for AQP3 as well as cells positive for vacuolar H\(^{+}\)-ATPase by immunocytochemistry demonstrated that type 6 AC mRNA is present in both collecting duct principal and intercalated cells, respectively (Fig. 7).

Extracellular Ca\(^{2+}\), or carbachol-mediated [Ca\(^{2+}\)]\(_i\) increases, inhibit glucagon-dependent AC activity by ~50% (6). By comparison, in OMCD principal cells or in the cortical thick ascending limb (cTAL), where the Ca\(^{2+}\)-inhibitable type 6 AC is also expressed, PGE2 or angiotensin II induces a Ca\(^{2+}\)-mediated inhibition of cAMP synthesis of only 10–20% (7, 9, 14). Ca\(^{2+}\)-inhibitable AC can be inhibited by either [Ca\(^{2+}\)]\(_i\) peaks or Ca\(^{2+}\) entry (11, 14). The following two major hypotheses can be discussed to explain the high sensitivity of OMCD glucagon-sensitive cells AC activity to Ca\(^{2+}\).

Role of Ca\(^{2+}\) in intercalated cell AC activity. The activation of the Ca\(^{2+}\)-sensing receptor RaKCaR can inhibit cAMP synthesis by up to 90% in the cTAL, in contrast to the small inhibitory effect of angiotensin II in this segment. This high inhibition involves both a
[Ca^{2+}]_i peak and a capacitive Ca^{2+} entry (14). Although there is no evidence for the expression of RaK-CaR in OMCD basolateral plasma membranes (10, 24, 32), the presence of a yet unknown Ca^{2+}-sensing receptor in intercalated cells could account for the great sensitivity of intercalated cell AC to extracellular Ca^{2+}. The inhibition of glucagon-dependent, but not AVP-dependent, AC activity (6) by extracellular Ca^{2+} supports also the hypothesis that Ca^{2+} channels are specifically expressed in intercalated cells. Accordingly, the presence of non-voltage-gated Ca^{2+} channels has been demonstrated in rat OMCD (10). In addition, the [Ca^{2+}]_i increase elicited by carbachol in glucagon-sensitive cells is characterized by a plateau phase of markedly larger amplitude than that observed with PGE_2 in AVP-sensitive cells (Refs. 1 and 20 and this study) or with angiotensin II in cTAL (14). This plateau reflects Ca^{2+} entry triggered by a [Ca^{2+}]_i release (1, 20) and could also result from the activation of Ca^{2+} channels. It can be noted that in some cell types, carbachol was described to induce a direct activation of Ca^{2+} channels (15, 28). A carbachol-induced Ca^{2+} entry could therefore account for the high inhibition of glucagon-dependent AC activity by Ca^{2+}.

Role of AC isoforms in intercalated cell sensitivity to Ca^{2+}. Type 5 AC is expressed only in intercalated cells. The rabbit type 5 AC isoform was previously reported to be more sensitive to Ca^{2+} than type 6 AC (31). However, recent results with the canine type 5 AC isoform did not confirm this property (25). Additional experiments are therefore necessary to demonstrate a different sensitivity to Ca^{2+} of type 5 and type 6 AC that might account for the high Ca^{2+}-inhibitable AC activity observed in intercalated cells.

In conclusion, our results in rat kidney demonstrate the localization of type 6 AC mRNA in both OMCD principal and intercalated cells. In contrast, type 5 AC was only detected in intercalated cells, where both AC mRNA isoforms are therefore expressed. Functional data establish the expression of Ca^{2+}-inhibitable AC proteins, which allow the cumulative inhibition of glucagon-dependent AC synthesis by both PGE_2, through a G_0,-mediated process (3), and carbachol, through an increase of Ca^{2+} (6, 20). The simultaneous action of these two inhibitory pathways therefore can deeply decrease the physiological functions achieved by glucagon in intercalated cells of the rat collecting duct, i.e., proton secretion and/or bicarbonate reabsorption (21).

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