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

CÉCILE HÉLIÈS-TOUSSAINT,1 LOTFI AARAB,1 JEAN-MARIE GASCE2
JEAN-MARC VERBAVATZ,1 AND DANIELLE CHABARDÉS1
1Service de Biologie Cellulaire, Commissariat à l’Énergie Atomique/Saclay, 91191 Gif-sur-Yvette,
and 2Laboratoire de Médecine Expérimentale, Collège de France, 75005 Paris, France

Received 20 September 1999; accepted in final form 29 February 2000

IN MANY CELL TYPES, the intracellular Ca2+ concentration ([Ca2+]i) regulates cAMP levels through interactions of Ca2+ on cAMP synthesis and/or cAMP hydrolysis. These effects of [Ca2+]i are linked to the presence of Ca2+-sensitive adenyl cyclases (ACs) and/or Ca2+-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 Ca2+ (25, 31, 33). Ca2+-inhibitable AC isoforms are also sensitive to [Ca2+]i, typically achieved in intact cells as a consequence of phospholipase C activation and/or Ca2+ channel activation (4).

Two cell types have been described in the kidney collecting duct: principal cells, involved in water homeostasis (30), and 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 Ca2+ on the cAMP pathway in the rat OMCD was first suggested by the negative regulation of a Ca2+ ionophore on AVP-dependent cAMP accumulation (19). PGE2 and carbachol, a muscarinic agonist of the acetylcholine receptor, both induce a Ca2+-dependent inhibition of hormone-stimulated intracellular cAMP accumulation (1, 6), which is probably linked to the [Ca2+]i increases elicited by PGE2 and carbachol in rat OMCD (1, 20). However, the effect of these agents on cAMP accumulation is cell type and agonist dependent. Indeed, PGE2 induces only a small inhibition of AVP-stimulated cAMP synthesis, and its Ca2+-dependent inhibitory effect on AVP-dependent cAMP accumulation is due mainly to an increase in cAMP hydrolysis (1, 9). In contrast, carbachol, which has no effect on the response to AVP (2, 6), induces a marked Ca2+-dependent inhibition of glucagon-stimulated AC activity (6). In addition, glucagon-stimulated cAMP synthesis is inhibited by extracellular Ca2+, whereas AVP-dependent cAMP synthesis is not (6). These observations therefore show that the inhibitory effect of either extracellular Ca2+ or agonist-mediated [Ca2+]i changes on intracellular cAMP are cell type dependent. These differences could be accounted for, at least partly, by the cellular local-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajprenal.org 0363-6127/00 $5.00 Copyright © 2000 the American Physiological Society F185
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 superused OMCDs, the effects of PGE\(_2\) and carbachol on [Ca\(^{2+}\)]\(_i\) were additive, suggesting that the Ca\(^{2+}\) increase elicited by these agents occurs in different cell types. One fundamental property of Ca\(^{2+}\)-inhibitable AC isoforms is to be regulated by independent Ca\(^{2+}\) and G\(_\alpha\)-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\(^{2+}\)-dependent inhibition of PGE\(_2\) on AVP-stimulated cAMP synthesis is of very low magnitude (1, 7, 9). In contrast, such a dual regulation can be studied in OMCD intercalated cells, where about one-half of glucagon-dependent AC activity is inhibited by either a Ca\(^{2+}\)-dependent carbachol pathway (6) or a G\(_\alpha\)-sensitive PGE\(_2\) pathway (3). 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 (Damstadt, 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 [\(\alpha\)-\(\text{32P}\)]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 (2× SSC-0.05% SDS) at room temperature, followed by an incubation of 40 min at 50°C in solution 2 (0.1× 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-Sph 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 \([\text{32P}]\text{UTP}, \text{S} (>1,000 \text{Ci/mmole, 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 4% paraformaldehyde-PBS, and proteinase K treatment, slides were covered with hybridization buffer [final concentrations: 50% formamide, 10% dextran sulfate, 1 mg/ml SS-2DNA, 2× 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 5× SSC-0.1% SDS followed by an 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 antibody (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 AX).

Isolation of rat kidney OMCDs. The experimental procedure used for 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 0.16% 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 Na\(_2\)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 HEPES, pH 7.4, and 0.1% (wt/vol) BSA (fraction V, Pentex, Miles, Kankakee, IL).

Measurement of \([\text{Ca}^{2+}]_i\). \([\text{Ca}^{2+}]_i\) 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 55, 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. [Ca²⁺], was calculated by using a dissociation constant of fura 2 for calcium of 224 nM as previously reported (10, 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 Ca²⁺) was supplemented with 5 μM indo- methacin and 0.5 unit/ml adenosine deaminase (Boehringer Mannheim) to prevent the endogenous synthesis of prostaglandins 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 Laboratories, 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. 2, c 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\textsuperscript{+}-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\textsubscript{2} on \([\text{Ca}^{2+}]_{i}\) increases in rat OMCD. As underlined at the beginning of this study, \([\text{Ca}^{2+}]_{i}\) increases induced by PGE\textsubscript{2} and carbachol in rat OMCD (1, 20) appear a prerequisite condition to observe the inhibition elicited by PGE\textsubscript{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\textsubscript{2}-mediated \([\text{Ca}^{2+}]_{i}\) increase might be mainly effective in the vasopressin-sensitive cells, whereas a carbachol-mediated \([\text{Ca}^{2+}]_{i}\) increase might be located in the glucagon-sensitive cells. This hypothesis was tested by comparing \([\text{Ca}^{2+}]_{i}\) variations induced by the addition of both agents to the responses obtained with each agent added alone to the superfusion medium.

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

The simultaneous superfusion of PGE\textsubscript{2} and carbachol induced a higher increase in [Ca\textsuperscript{2+}]\textsubscript{i} than the response observed with the superfusion of only one agonist (Fig. 6A). The same observation was made...
Localization of Ca\(^{2+}\)-inhibitable AC in Kidney

### Table 1. Quantification of in situ hybridization labeling

<table>
<thead>
<tr>
<th>Probe</th>
<th>Intercalated Cells</th>
<th>Principal Cells</th>
</tr>
</thead>
<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>
</tr>
</tbody>
</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 \(\mu\)m\(^2\)).

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 \([\text{Ca}^{2+}]_i\) increases

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>([\text{Ca}^{2+}]_i) Increases, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 mM Ca²⁺ medium (n = 10)</td>
</tr>
<tr>
<td>PGE₂</td>
<td>87 ± 10*</td>
</tr>
<tr>
<td>Carbachol</td>
<td>106 ± 13*</td>
</tr>
<tr>
<td>Exp (PGE₂+carbachol)</td>
<td>202 ± 16†</td>
</tr>
<tr>
<td>The (PGE₂+carbachol)</td>
<td>193 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± SE obtained from different recordings (n), examples of which are given in Fig. 6. The intracellular Ca²⁺ concentration ([Ca²⁺]) variations ([Ca²⁺]) 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 [Ca²⁺], 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 (The) calculated by assuming a full additivity of [Ca²⁺], increases. *P < 0.01 compared with the experimental or theoretical value of additivity. †Not significant compared with the theoretical value of additivity.

Ca²⁺-mediated process that is insensitive to pertussis toxin (1, 3). In addition, PGE₂ inhibits glucagon-dependent cAMP synthesis through a Ca²⁺-independent, Ga₁₉-mediated process (3). These mechanisms are summarized in Fig. 7.

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>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td>43.7 ± 8.1</td>
<td></td>
</tr>
<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 ± 3.3‡</td>
</tr>
</tbody>
</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. †P < 0.05 compared with the response obtained with either PGE₂ or carbachol added alone. ‡Not significant compared with the theoretical values 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).

Fig. 7. Schematic summary of intracellular cAMP regulations in rat kidney OMCD. PLC, phospholipase C; PDE, phosphodiesterase; ag, agonist; R, receptor.

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$$^{2+}$$-dependent inhibition of 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 Go$_q$-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/bicarbonate reabsorption (21).

This work was supported by the Commissariat à l’Énergie Atomique and the Centre National de la Recherche Scientifique (URA 1859). C. Helies-Toussaint was supported by a postdoctoral fellowship from the Commissariat à l’Énergie Atomique, and L. Aarab was supported in part by a grant from the Commissariat à l’Énergie Atomique.

**REFERENCES**

1. Aarab L, Montegut M, Siaume-Perez S, Imbert-Teboul M, and Chabardès D. PGE$_2$-induced inhibition of AVP-dependent cAMP accumulation in the OMCD of the rat kidney is cumulative with respect to the effects of $\alpha_2$-adrenergic and $\beta_1$-adrenoceptor agonists, insensitive to pertussis toxin and dependent on extracellular calcium. *Pflügers Arch.* 423: 397–405, 1993.


