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

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Cellular localization of type 5 and type 6 ACs in collecting duct and regulation of cAMP synthesis. Am J Physiol Renal Physiol 279: F185–F194, 2000.—The cellular distribution of Ca2+-inhibitable adenylyl cyclase (AC) type 5 and type 6 mRNAs in rat outer medullary collecting duct (OMCD) was performed by in situ hybridization. Kidney sections were also stained with specific antibodies against either collecting duct intercalated cells or principal cells. The localization of type 5 AC in H+ -ATPase-, but not aquaporin-3-, positive cells demonstrated that type 5 AC mRNA is expressed only in intercalated cells. In contrast, type 6 AC mRNA was observed in both intercalated and principal cells. In microdissected OMCDs, the simultaneous superfusion of carbachol and PGE2 elicited an additive increase in the intracellular Ca2+ concentration, suggesting that the Ca2+-dependent regulation of these agents occurs in different cell types. Glucagon-dependent cAMP synthesis was inhibited by both a pertussis toxin-sensitive PGE2 pathway (63.7 ± 4.6% inhibition, n = 5) and a Ca2+-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 Ca2+-inhibitable enzymes in intercalated cells.

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 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-
ization 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 Ca2+-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 hybridiza-
tion. Only type 6 mRNA was detected in rat kidney
collecting duct principal cells, whereas both type 5 and
type 6 Ca2+-inhibitable AC isoforms were found in
intercalated cells. In superfused OMCDs, the effects of
PGE2 and carbachol on [Ca2+]i were additive, suggest-
ning that the Ca2+ increase elicited by these agents
occurs in different cell types. One fundamental prop-
erty of Ca2+-inhibitable AC isoforms is to be regulat-
ed by independent Ca2+- and G0s-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 Ca2+-dependent inhibition
of PGE2 on AVP-stimulated cAMP synthesis is of very
low magnitude (1, 7, 9). In contrast, such a dual regu-
lation can be studied in OMCD intercalated cells,
where about one-half of glucagon-dependent AC activ-
ity is inhibited by either a Ca2+-dependent carbachol
pathway (6) or a G0s-sensitive PGE2 pathway (3). In
our study, the simultaneous addition of carbachol and
PGE2 induced a cumulative inhibition of glucagon-
dependent cAMP synthesis, demonstrating that these
agents inhibit the same AC catalytic units. The results
therefore localize Ca2+-inhibitable AC mRNAs in
OMCD and demonstrate the functional expression of
Ca2+-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
[a-32P]dCTP. A multiple rat tissue Northern blot (Clontech
Laboratories) was hybridized, as per the manufacturer's in-
structions, 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 corre-
sponding to nucleotides 1205–2285 (EcoRI, Pou II) of type 5
AC cDNA coding region was subcloned in BSSK+ (Strat-
agene). 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 [35S]UTP_S (>1,000 Ci/mmole, Amersham, Les
Ulis, France).

Rat kidneys were fixed in 4% paraformaldehyde in PBS,
flushed with 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 rehy-
drated by a graded series of ethanol (100–30%). After boiling
in a solution of 0.01 M citric acid in a microwave oven, slices
were treated with 0.1% H2O2 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- DNA, 2×
SSC, 70 mM dithiothreitol (DTT)) and 0.5–1.106 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 in 50% formamide-1× SSC-12.5 mM DTT. They
were further treated by RNAase A (20 μg/ml). For immuno-
staining, the slides were then incubated for 5 min in PBS
containing 1% BSA (PBS/BSA), followed by overnight incu-
Bation 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, Charles-
town, MA) antisera 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 anti-
odies (6 μg/ml) in PBS/BSA. The sections were washed 2×
10 min in PBS. Staining was revealed with diaminobenz-
idine, 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 VanOX).

Isolation of rat kidney OMCDs. The experimental proce-
dure used to microdissect intact segments from collagenase-
treated rat kidneys 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 me-
dium 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
composed of (in mM) 137 NaCl; 5 KCl; 0.8 MgSO4; 0.33
Na2HPO4; 0.44 KH2PO4; 1 MgCl2; 4 NaHCO3; 10
CH3COONa; 1.0 or 2.0 CaCl2 (see below); 5 glucose; and 20
HEPES, pH 7.4, and 0.1% (wt/vol) BSA (fraction V, Pentex,
Miles, Kankakee, IL).

Measurement of [Ca2+]i. [Ca2+]i was measured in single
OMCD samples by using the calcium-sensitive fluorescence
probe acetoxy-methyl ester of fura 2 (fura 2-AM, Molecular
Probes, Eugene, OR) as previously described (1, 10, 27). Brie-
ly, 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 μl/min, corresponding to ~10
changes/min. The superfusion medium (microdissection me-
dium without serum albumin) contained either 2 mM Ca2+ or
no Ca2+ (nominally Ca2+-free medium without CaCl2 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 alternately, 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 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 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\(^{-1}· \text{min}^{-1}\)). 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 PGE₂ on [Ca²⁺]ᵢ increases in rat OMCD. As underlined at the beginning of this study, [Ca²⁺]ᵢ increases induced by PGE₂ and carbachol in rat OMCD (1, 20) appear a prerequisite condition to observe the inhibition elicited by PGE₂ 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 PGE₂-mediated [Ca²⁺]ᵢ increase might be mainly effective in the vasopressin-sensitive cells, whereas a carbachol-mediated [Ca²⁺]ᵢ increase might be located in the glucagon-sensitive cells. This hypothesis was tested by comparing [Ca²⁺]ᵢ variations induced by the addition of both agents to the responses obtained with each agent added alone to the superfusion medium.

Carbachol and PGE₂ were used at concentrations inducing maximal [Ca²⁺]ᵢ increases and did not elicit homologous desensitization (20 and data not shown). In a same tubule, the superfusion of 0.3 μM PGE₂ 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 Ca\(^{2+}\) 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 [Ca\(^{2+}\)]\(_i\) than the response observed with the superfusion of only one agonist (Fig. 6A). The same observation was made...
whatsoever 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, G\(_\alpha_i\)-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\(^{-2}\)·min\(^{-1}\)) was not different from the theoretical value calculated assuming an hypothesis of cumulative inhibition (7.5 ± 0.6 fmol·mm\(^{-2}\)·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
**A**

![Representative traces of \([\text{Ca}^{2+}]_i\) variations elicited by different agonists added in 2 mM \(\text{Ca}^{2+}\)-free medium and 2 mM \(\text{Ca}^{2+}\) medium. The tubule was superfused with 100 \(\mu\text{M}\) carbachol, then the combination of both agonists, and finally 0.3 \(\mu\text{M}\) 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 \([\text{Ca}^{2+}]_i\) variations elicited by different agonists added in \(\text{Ca}^{2+}\)-free medium. The tubule was superfused with 2 mM \(\text{Ca}^{2+}\) and then with \(\text{Ca}^{2+}\)-free medium. Rapid superfusion of PGE2 (3 \(\mu\text{M}\)) and then carbachol (100 \(\mu\text{M}\)) elicited successive \([\text{Ca}^{2+}]_i\) increases (left). The magnitude of carbachol- or PGE2-mediated \([\text{Ca}^{2+}]_i\) 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 \(\text{Ca}^{2+}\)-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 \(\text{Ca}^{2+}\)-free medium to refill the intracellular \(\text{Ca}^{2+}\) stores. The magnitude of the different responses was independent from the order of superfusion (see Table 2).

**Table 2. Additive effect of PGE2 and carbachol on \([\text{Ca}^{2+}]_i\) increases**

| Experimental Conditions | \(2 \text{ mM Ca}^{2+}\) medium \((n = 10)\) | \(\text{Ca}^{2+}\)-free medium \((n = 14)\) |
|-------------------------|-----------------------------------------------|
| PGE2                    | 87 ± 10\(^\text{a}\)                         | 95 ± 8\(^\text{a}\) |
| Carbachol               | 106 ± 13\(^\text{a}\)                        | 85 ± 12\(^\text{a}\) |
| Exp (PGE2+carbachol)    | 202 ± 16\(^\text{b}\)                        | 165 ± 12\(^\text{b}\) |
| The (PGE2+carbachol)    | 193 ± 16                                     | 180 ± 14          |

Values are means ± SE obtained from different recordings (n), examples of which are given in Fig. 6. The intracellular \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}\)]\(_i\)) variations obtained at the peak in either 2 mM \(\text{Ca}^{2+}\) or \(\text{Ca}^{2+}\)-free medium (0 Ca\(^{2+}\) + 0.1 mM EGTA) are expressed as maximal increases over basal value. The mean basal value of [\(\text{Ca}^{2+}\)]\(_i\) 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 \(\text{Ca}^{2+}\) in the superfusion medium, respectively. PGE2 (0.3 \(\mu\text{M}\) in 2 mM \(\text{Ca}^{2+}\) and 3 \(\mu\text{M}\) in \(\text{Ca}^{2+}\)-free medium) and carbachol (100 \(\mu\text{M}\)) 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 [\(\text{Ca}^{2+}\)]\(_i\), increases. \(^\text{a}\)\(P < 0.01\) compared with the theoretical or experimental value of additivity. \(^\text{b}\)Not significant compared with the theoretical value of additivity.

**Ca\(^{2+}\)-mediated process that is insensitive to pertussis toxin (1, 3). In addition, PGE2 inhibits glucagon-dependent cAMP synthesis through a \(\text{Ca}^{2+}\)-independent, \(\text{G}_{\alpha}\)-mediated process (3). These mechanisms are summarized in Fig. 7. In the OMCD, carbachol and PGE2 increase \([\text{Ca}^{2+}]_i\) through an interaction with the m1 subtype of the**

**Fig. 6. Additive effect of PGE2 and carbachol on \([\text{Ca}^{2+}]_i\) increases in rat outer medullary collecting duct (OMCD). A: Representative traces of \([\text{Ca}^{2+}]_i\) variations elicited by different agonists added in 2 mM \(\text{Ca}^{2+}\) medium. The tubule was superfused with 100 \(\mu\text{M}\) carbachol, then the combination of both agonists, and finally 0.3 \(\mu\text{M}\) 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 \([\text{Ca}^{2+}]_i\) variations elicited by different agonists added in \(\text{Ca}^{2+}\)-free medium. The tubule was superfused with 2 mM \(\text{Ca}^{2+}\) and then with \(\text{Ca}^{2+}\)-free medium. Rapid superfusion of PGE2 (3 \(\mu\text{M}\)) and then carbachol (100 \(\mu\text{M}\)) elicited successive \([\text{Ca}^{2+}]_i\) increases (left). The magnitude of carbachol- or PGE2-mediated \([\text{Ca}^{2+}]_i\) 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 \(\text{Ca}^{2+}\)-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 \(\text{Ca}^{2+}\)-free medium to refill the intracellular \(\text{Ca}^{2+}\) stores. The magnitude of the different responses was independent from the order of superfusion (see Table 2).**

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

| Experimental Conditions | cAMP Synthesis, \(\text{fmol} \cdot \text{mm}^{-1} \cdot 4 \text{ min}^{-1}\) | %Inhibition of Response to Glucagon |
|-------------------------|---------------------------------------------------------------|
| Glucagon                | 43.7 ± 8.1                                                    |                                   |
| Glucagon + PGE2         | 14.9 ± 2.0\(^\text{a}\)                                       | 63.7 ± 4.6                        |
| Glucagon + carbachol    | 21.9 ± 3.3\(^\text{a}\)                                       | 48.6 ± 3.3                        |
| Glucagon + PGE2 + carbachol | 8.7 ± 0.9\(^{4,5}\)                                        | 78.2 ± 3.33                       |

Values are means ± SE calculated from 5 experiments. In each experiment, 0.3 \(\mu\text{M}\) PGE2 and 100 \(\mu\text{M}\) carbachol were used alone and in combination in the presence of 1 \(\mu\text{M}\) glucagon and 1 mM IBMX; the percentage of inhibition was calculated from the corresponding response obtained with glucagon. \(^4\)\(P < 0.05\) compared with the mean value obtained with glucagon. \(^5\)\(P < 0.05\) compared with the response obtained with either PGE2 or carbachol added alone. \(^4\)Not significant compared with the theoretical values calculated by assuming a cumulative inhibition hypothesis (7.5 ± 0.6 \(\text{fmol} \cdot \text{mm}^{-1} \cdot 4 \text{ min}^{-1}\) 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.
[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 G\(_{q}\)-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/or bicarbonate reabsorption (21).

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