Localization of mRNAs Encoding Ca\textsuperscript{2+}-inhibitable Adenylyl Cyclases along the Renal Tubule

FUNCTIONAL CONSEQUENCES FOR REGULATION OF THE AMP CONTENT*

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Expression of Ca\textsuperscript{2+}-inhibitable types V and VI adenylyl cyclases was studied by reverse transcription-polymerase chain reaction in rat renal glomeruli and nephron segments isolated by microdissection. Quantitation of each mRNA was achieved using a mutant cRNA which differed from the wild type by substituting two bases to create a new restriction site in the corresponding cDNA. Type VI mRNA was present all along the nephron but was more abundant in distal than in proximal segments. The expression of type V mRNA was restricted to the glomerulus and to the initial portions of the collecting duct. Expression of the Ca\textsuperscript{2+}-insensitive type IV AC mRNA studied on the same samples was evidenced only in the glomerulus. The functional relevance of the expression of Ca\textsuperscript{2+}-inhibitable isoforms was studied by measuring cAMP content in the microdissected outer medullary collecting duct which expressed both type V mRNA (2367 ± 178 molecules/mm tubular length; n = 8) and type VI mRNA (5658 ± 543 molecules/mm, n = 8). Agents known to increase intracellular Ca\textsuperscript{2+} in this segment induced a Ca\textsuperscript{2+}-dependent inhibition on either arginine vasopressin- or glucagon-stimulated cAMP level. The characteristics of these inhibitions suggest a functional and differential expression of types V and VI adenylyl cyclases in two different cell types of the rat outer medullary collecting duct.

In the past few years, the control of cAMP content in mammalian cells has become more intricate by the description of several types of adenylyl cyclase with different regulatory properties (1–3). Among the eight isoforms of adenylyl cyclase cloned up to date, the type V and the type VI are characterized by an activity negatively regulated by sub-micromolar concentrations of Ca\textsuperscript{2+}. This property, established in vitro on membrane preparations (4–7), has been observed also on the cAMP content measured on cultured cells from different tissues that express Ca\textsuperscript{2+}-inhibitable AC isoforms (4, 8–13). These results demonstrate therefore that type V and type VI adenylyl cyclases can be inhibited in intact cells in response to a rise in [Ca\textsuperscript{2+}].

Northern blot analyses have demonstrated that types V and VI AC mRNAs are expressed in the rat kidney (8, 14). The renal tissue is, however, structurally highly heterogeneous and includes, in addition to the nephron epithelial cells, other cell types such as interstitial and vascular cells (15). The main functions of the kidney are achieved by the glomerulus and the different segments of the nephron, and many of these physiological processes, including the maintenance of Ca\textsuperscript{2+} homeostasis (16), are regulated by the cAMP and/or the phosphodiesterase C pathway. In addition, recent data demonstrated the expression of an extracellular Ca\textsuperscript{2+} receptor in the rat kidney that might participate in Ca\textsuperscript{2+}-sensitive regulations in some segments of the nephron (17). In this context, the presence of Ca\textsuperscript{2+}-inhibitable AC mRNAs in the rat kidney (8, 14) suggests that these isoforms might contribute to the regulation of physiological processes sensitive to Ca\textsuperscript{2+} in some defined cell(s) of the nephron. The localization of Ca\textsuperscript{2+}-inhibitable AC activities in epithelial cells of the rat nephron is suggested by previous data obtained on the terminal segments of the collecting tubule microdissected from either the outer or the inner medulla. In these two segments, the AVP-dependent cAMP content is decreased by a Ca\textsuperscript{2+} ionophore. The mechanism of this inhibition involves modulation of both adenylyl cyclase and cAMP phosphodiesterase activities and depends on extracellular Ca\textsuperscript{2+} (18, 19).

The aim of the present study was to investigate the expression of type V and type VI AC mRNAs in the different structures of the rat nephron. Type IV AC mRNA, which is also expressed in the rat kidney and which is not sensitive to Ca\textsuperscript{2+} (20), was studied in parallel. A quantitative RT-PCR assay, similar to other such assays described by our laboratory (21), was developed. This assay was carried out using mutant cRNAs as internal standards and allowed us to measure steady state mRNA levels in glomeruli as well as in different nephron segments isolated by microdissection. The results demonstrate a structure-specific localization of Ca\textsuperscript{2+}-inhibitable AC mRNAs, expressed particularly in the terminal segments of the nephron. The functional relevance of the expression of these mRNAs was analyzed on the intact native outer medullary

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1 The abbreviations used are: AC, adenylyl cyclase; AVP, arginine vasopressin; [Ca\textsuperscript{2+}], intracellular free concentration of Ca\textsuperscript{2+}; IBMX, 3-isobutyl-1-methylxanthine; RT-PCR, reverse transcription-polymerase chain reaction; Ro20–1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; DTT, dithiothreitol; OMCD, outer medullary collecting tubule; bp, base pair(s); CCD, cortical collecting duct; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}.
collecting duct which contains two cell types (15, 22). The results establish a cell-specific and Ca2+-dependent inhibition on either the AVP- or the glucagon-dependent cAMP content.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unless otherwise specified, the enzymes were purchased from New England Biolabs (Beverly, MA) and the compounds from Sigma. Radioactive nucleotides and deoxynucleotides were from Amer sham Life Sciences (Little Chalfont, U.K.). Oligonucleotide primers were purchased from Bioprobe Systems (Montreal, 1, 1, N. France). RT-PCR reactions were carried out using a Hybaid TR1 thermal reactor (Hybaid, Teddington, U.K.).

Isolation of Nephron Segments and RNA Extraction—Experiments were performed on male Sprague-Dawley rats (120–200 g of body weight, Charles River Laboratory) that were maintained on a standard diet with free access to water. After anesthesia (sodium pentobarbital, 5 mg/100 g of body weight), the left kidney was perfused with microdissection medium containing 0.2% collagenase (Type A, Boehringer Mannheim). The microdissection medium was prepared from Hank's balanced salt solution containing 1 g/liter glucose and supplemented with 1 M HEPES (pH 7.4). Thin pyramids were cut from the kidney and was adjusted to pH 7.4. Thin pyramids were cut from the kidney and incubated for 25 min at 30 °C (60 min at 37 °C for the inner medulla) in 0.15% collagenase solution and then thoroughly rinsed in microdissection solution. Glomeruli and different nephron segments (see legends of Table II and Fig. 7) were microdissected at 4 °C according to anatomical and morphological criteria (29). Several pieces isolated from identical segments were carefully rinsed to avoid contaminating cells or debris, transferred on a glass slide, and photographed for subsequent measurement of the total tubular length.

RNAs were extracted from glomeruli or microdissected segments using a micromethod (21) adapted from the guanidinium thiocyanate-phenol/chloroform method developed by Chomczynski and Sacchi (23). Briefly, pools of glomeruli (10–50 glomeruli) or of identical segments (4–20 mm) were transferred with 5–10 μl of microdissection medium into 400 μl of denaturing solution (4% guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol, and 20 mM of yeast RNA). After phenol/chloroform extraction and isopropyl alcohol precipitation, the final pellet was vacuum-dried and resuspended in RNA dilution buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 2 mM dithiotreitol, 0.1 mg/ml RNAse-free bovine serum albumin) and 200 or 100 μl of reverse transcription buffer (200 μM dATP, dGTP, dCTP, dTTP, 5 mM dithiotreitol, 0.1 g/ml yeast RNA, 200 units/ml ribonuclease inhibitor (RNAse-free RNasin, Promega Corp., Madison, WI)). It was determined previously that the yield of this RNA extraction procedure was ≥90% (21).

**Oligonucleotide Primers Selection—**For RT-PCR and PCR experiments, the primers were selected from rat sequences of type IV (20), type V (14), and type VI (8) cDNAs by using Oligo Primer Analysis Software (Medprobe, Oslo, Norway). Sense and antisense primers, specific for each AC type, were chosen in divergent cDNA portions. Each pair of primers had similar Tm, close to 62 °C.

**PCR Cloning of Type V and Type VI Adenylyl Cyclase Isoforms—**For type V AC, an 1865-bp cDNA fragment from the coding region was obtained by RT-PCR from total RNAs extracted from the rat kidney medulla. The sense (5'-GGAGGTCCGCTGCTGGAGTT) and antisense (5'-GGAGGTCCGCTGCTGGAGTT) primers corresponded to positions 574–595 and 2415–2438, respectively, from the beginning of the published sequence (14). Reverse transcription (45 min at 41 °C) was carried out in a 50-μl reaction volume containing 5 μl of 10× PCR buffer (200 mM Tris-HCl (pH 8.3), 15 mM MgCl2, 500 μM KCl, 1 mg/ml gelatin) supplemented with 100 nM antisense primer, 400 μM 2'-deoxyribonucleotide 5'-triphosphate (Pharmacia Biotech Inc.), 2.0 mM MgCl2, 8.2 mM DTT, 0.03 mM acetylated bovine serum albumin, 600 units/ml of RNAse, 100 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies), and 1 μl of RNA. Then PCR was performed in the same reaction tube by adding 50 μl of a mix containing 5 μl of 10× PCR buffer, 100 nM sense primer, and 1.25 units of Taq DNA Polymerase (Eurobio, Les Ulis, France). Amplification was performed during 31 cycles (95 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min), followed by an additional cycle with an elongation time of 10 min. The amplification products from seven different RT-PCR reactions were pooled, digested by ApoI and PvuII, and fractionated on a 1% low melting point agarose (Life Technologies). The cDNA fragment was recovered from agarose with β-Agarase I. The ApaI site was blunt-ended using T4 DNA polymerase, and the cDNA fragment was cloned into the EcoRV site of pBluescript II SK+ (BSSK+, Stratagene, La Jolla, CA).

With regard to type VI AC, a 1623-bp cDNA fragment corresponding to the 3′ end coding and untranslated region was obtained by PCR from 0.2 or 0.3 μg of rat genomic DNA. The sense (5'-AAGGTACCAGGGAATAGGATG-3') and antisense (5'-TCAGGCAGAATTCCG-3') primers corresponded to positions 3442–3465 and 5041–5064 from the ATG initiation codon, respectively (8). These primers amplified a genomic DNA fragment of the size predicted from the cDNA sequence, indicating the lack of intron in this portion of the type VI AC gene. PCR reaction was carried out in a 100-μl reaction volume containing 10 μl of 10× PCR buffer supplemented with 1.0 mM MgCl2, 4 mM DTT, 200 μM 2'-deoxyribonucleotide 5'-triphosphate, 62.5 nM of each primer, and 1.25 units of Taq DNA polymerase. PCR cycles were similar to those performed for type V AC. The DNA fragments obtained from seven different PCR reactions were pooled, digested with ApoI and EcoRI, and cloned into ApoI-EcoRI-digested BSSK+ vector.

Type V and type VI AC cDNA fragments had the predicted size and restriction profiles (8, 14). With regard to type IV AC, a full-length cDNA cloned into BSSK+ vector was kindly provided by A. G. Gilman and W. J. Tang.

Site-directed Mutagenesis and Synthesis of Mutant cRNAs—A transformant directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA) was used to introduce a new restriction site (HindIII from positions 1547, 2143, and 3942 for types IV, V, and VI, respectively) in cloned types IV, V, and VI AC cDNAs. Mutated sense (for type V) or antisense (for types IV and VI) primers containing two mismatches were annealed to BSSK+ vector simultaneously to a selection primer. The selection primer contained two mismatches that suppressed SpeI and XbaI unique restriction sites of BSSK+ vector.

Mutated sense cRNAs were synthesized from 1 μg of either BamHI-(type IV), XhoI-(type V), or SalI (type VI)-linearized DNA templates. Transcription was performed as described (21, 24) using tracer amounts of [32P]UTP and T3 (type V) or T7 (types IV and VI) RNA polymerase (Stratagene). The amount of each cRNA synthesized (2–5 pmol/reaction) was measured by liquid scintillation counting of the trichloroacetic acid-precipitated material. The size and homogeneity of the cRNAs were checked by agarose gel electrophoresis and autoradiography.

Quantitative RT-PCR Analysis of Types IV, V, and VI AC mRNAs—The amount of mRNA coding for each of the three adenylyl cyclases was quantitated by RT-PCR using the corresponding mutant cDNA as an internal standard.

Primers specific for each type of adenylyl cyclase were chosen (Table I). Primers for type IV AC were located in the coding region, predicting
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the amplification of a 467-bp cDNA fragment. Primers for type V AC were chosen in the coding region with a low homology with type VI DNA sequence; the fragment expected was of 338 bp. With regard to the type VI AC, a first pair of primers was selected in the untranslated region (Table I) from the DNA sequence published by Krtupinski et al. (8). This pair of primers gave a cDNA fragment of 419 bp and was used in quantitative RT-PCR experiments. A second pair of primers, chosen in the coding region published by two laboratories (8, 14), was used in some qualitative RT-PCR experiments to check the distribution observed with the first set of primers. The sense (5'-CTGCTTATGTCGCTGACC-3') and antisense (5'-ACAGGAAGCAGCACCATG-TAGCAC-3') primers of this second set corresponded to positions 527-549 and 863-886, respectively, of the coding region. The expected size of the cDNA fragment was of 360 bp.

The experimental procedure for quantitative RT-PCR was similar to that previously developed in our laboratory (21, 24). The following compounds were added to a final volume of 25 μl, 2.5 μl of 10× PCR buffer, RNAs extracted from a defined renal structure, a known amount of mutated cRNA, and 100 μl of the antisense primer. After amplification at 45 °C, 25 μl of RT mix (2.5 μl of 10× PCR buffer, 400 μM of each 2'-deoxynucleotide 5'-triphosphate, 4 mM MgCl\textsubscript{2}, 16 mM DTT, and the addtion or not of 200 units of Moloney murine leukemia virus reverse transcription) was added to each tube. Reverse transcription was performed at 45 °C for 45 min. The amplification reaction was carried out in the same reaction tube by adding to each sample 50 μl of a mix containing 5 μl of 10× PCR buffer, 100 μl of the sense primer, 5 μl of [\textsuperscript{32}P]dCTP (6000 Ci/mmol, Amersham Life Sciences), and 1.25 units of Taq polymerase. The samples were submitted to sequential steps (94 °C, 30 s; 62 °C, 30 s; 72 °C, 1 min) during 27 cycles. An additional cycle was performed with an elongation time of 10 min. In all experiments, the presence of possible contaminants was checked by control RT-PCR reactions carried out on samples in which (i) RNA was excluded (blank) and (ii) reverse transcriptase was omitted from the reverse transcription mixture (RT-1).

Cyclic AMP fragments formed from the wild type and mutant RNAs were discriminated by digestion with HindIII as described (24). The samples were then electrophoresed through a 2% agarose slab gel (1% low melting point). After fixation in acetic acid and drying, the gel was submitted to autoradiography. Several exposures were performed to obtain nonsaturated bands that were analyzed by densitometry or, in a few experiments, by using a PhosphorImager (Molecular Dynamics, Evry, France). In each experiment, serial dilutions of mutant RNAs were studied, and RNAs extracted from different renal structures were analyzed in parallel.

For each AC type, the number of mRNA copies per sample was calculated from the ratio of the signals obtained for wild type and mutant RNAs. This calculation was made using dilutions of biological samples that gave amounts of DNA close to those obtained from the corresponding mutant. The results are expressed as the number of mRNA molecules per millimeter of tubular length or per glomerulus. For one AC type (type VI), it was checked that wild type mRNA and mutated cRNA had similar characteristics of RT-PCR amplification (see “Results”).

Measurement of Hormone-dependent Cyclic AMP Accumulation in Rat Outer Medullary Collecting Duct (OMCD)—The experimental conditions used to measure cyclic AMP accumulation on an intact single microdissected segment have been detailed previously (25, 26) and will be recalled briefly here. The composition of the medium was close to that used for the microdissection step described above except that it contained 1 μM indomethacin and 0.5 units/ml adenosine deaminase (Boehringer Mannheim, Germany) to prevent the synthesis of prostaglandins and the release of adenosine, two agents known to inhibit cyclic AMP accumulation in the rat OMCD (26). The effect of extracellular Ca\textsuperscript{2+} on cAMP accumulation was investigated in OMCD samples microdissected and incubated in the same experiments either in 2 mM Ca\textsuperscript{2+} (control) or in 0 Ca\textsuperscript{2+}--free medium containing 0.1 mM EGTA (0 Ca\textsuperscript{2+}).

Pieces of OMCD were isolated from collagenase-treated kidneys (see above), transferred on glass slides (1 or 2 pieces per slide), and photographed in order to measure their length. After a preincubation step (10 min at 30 °C), each sample was incubated during 4 min at 35 °C in the presence of the agonists to be tested. Due to the small number of cells per tubular sample (from about 100 to 400 cells), hormone-dependent cyclic AMP accumulation can be measured only in the presence of a phosphodiesterase inhibitor. Either 50 μM Ro 20–1724, a specific inhibitor of the low Km cyclic AMP phosphodiesterase (27), or 1 mM IBMX, inhibitor of all phosphodiesterase types, was added to the incubation medium. The amounts of cAMP were measured by radioimmunoassay (ERIA diagnostic Pasteur, France). The micromethod used allows the determination of 2–80 fmol of cAMP per reaction tube. In our conditions, the basal level of cAMP present in one single piece of tubule is close to, or below, the sensitivity threshold of the assay (25, 26). The results were calculated in femtomoles of cyclic AMP accumulated per mm of segment per 4 min of incubation time (fmol-mm\textsuperscript{-1}*4 min\textsuperscript{-1}). In each experiment, different experimental conditions were tested on replicate OMCD samples microdissected from the same rat kidney (6–8 samples per condition). Results are expressed as the mean value ± S.E. calculated from the mean data obtained in different experiments. The statistical evaluation of the results was assessed by unpaired Student’s t-test.

**RESULTS**

Specificity of the Oligonucleotide Primers—Initial RT-PCR experiments performed with primers selected from type V and type VI cDNA sequences showed that both target mRNAs are expressed in the rat nephron and most particularly in the OMCD. The amplified products had the expected size, and the DNA fragments obtained after digestion with appropriate restriction enzymes corresponded to those predicted from the restriction map (Fig. 1). Similarly, on RNAs extracted from the whole kidney, oligonucleotide primers selected from type IV AC sequences allowed the amplification of a single fragment of the predicted size, and the restriction map carried out with XbaI and AccI demonstrated the specificity of the primers (data not shown).

Characteristics of the Quantitative RT-PCR Assay—Serial dilutions of RNA extracted from 0 Ca\textsuperscript{2+} OMCD were co-amplified with a known amount of either type V or type VI AC mutant cDNA during 28 cycles (Fig. 2). The number of type V mRNA molecules calculated per mm was of 2423, 2486, and 2220 for 9-, 3-, and 1-fold dilutions of wild type RNA, respectively. The corresponding values for type VI mRNA were 6848, 6828, and 5296 molecules, this last value being likely underestimated due to the large difference between wild type and mutant signals. Subsequent determinations were made by comparing wild type and mutant signals of similar magnitude. Fig. 2 also shows that the intensity of the mutant signal was constant whatever
the amount of wild type RNA. These data therefore suggest that there was no competition between mutant and wild type mRNAs.

The validity of the above determinations requires similar RT-PCR amplification efficiency for the wild type and mutant RNAs. This was checked with type VI AC templates by measuring the amounts of DNA formed as a function of the number of PCR cycles. Fig. 3 shows constant and similar amplification rates for both targets up to 29 cycles, a result that validates the number of 28 cycles used in our experiments. Between 23 and 29 cycles, the mean amplification factor per cycle was 1.89 and 1.91 for wild type and mutant targets, respectively. Between 29 and 32 cycles, the corresponding values were 1.78 and 1.77, and they were reduced to 1.40 between 32 and 35 cycles. Note that the amplification efficiency had already decreased when DNA formation could be detected by ethidium bromide staining (i.e. for \( \geq 32 \) cycles). These results demonstrate (i) a similar amplification efficiency for wild type and mutant targets and (ii) that increasing the sensitivity of the detection method by labeling the product allows the PCR reaction to be stopped in its exponential phase, therefore avoiding the formation of wild type DNA fragments (bp): type V, 338; type VI, 419; mutant DNA fragments (bp): type V, 294 + 44; type VI, 252 + 167. The reactions were performed in the absence (−) of reverse transcriptase (RT). The control reaction tube where RNAs were omitted gave no signal.

Different RT-PCR experiments demonstrated that types V and VI AC mRNAs are expressed in different structures of the rat nephron (see below). To increase the accuracy of the analysis, experiments were performed to test the possibility of the simultaneous quantitation of types V and VI mRNAs in a same sample. First, on RNAs extracted from OMCD or pars recta, it was checked that the quantitation of types V or VI AC mRNAs was similar when they were amplified either with their respective mutant alone or with the two mutant cRNA species added simultaneously (data not shown). Second, a comparison was made between separate and co-amplification of types V and VI AC mRNAs (Fig. 4). For separate amplifications, the amounts calculated were 3226 (type V) and 5398 (type VI) molecules per mm of OMCD. For co-amplification the corresponding values were 2773 (type V) and 5988 (type VI). These results show that co-amplification can be achieved without interferences between the two targets. Consequently, type V and type VI AC mRNAs were analyzed simultaneously in most experiments performed; as with separate amplification (Figs. 2 and 5), the co-amplification allowed the observation of a good relationship between the intensity of the signal and the amount of wild type RNA introduced in the assay (Fig. 6).

Distribution of types IV, V, and VI AC mRNAs along the Rat Nephron—RT-PCR experiments performed on RNAs extracted from glomeruli and different segments indicated that (i) in the glomerulus, all three types of AC mRNA are expressed, albeit to different levels (Fig. 5, Table II); (ii) type VI AC mRNA was also expressed in several segments and was predominant as compared with types IV and V AC mRNAs (Fig. 6).

The distribution of types V and VI AC mRNAs in all struc-
In several segments, types IV and V AC mRNAs were not or barely detectable (<100 molecules per mm, Table II). Since the number of cells per mm of segment ranges from about 200 to 500 (28), these faint levels correspond to less than one copy per cell. Conversely, the expression of type VI AC mRNA in most nephron segments and that of type V AC mRNA in the collecting duct are likely to be higher than one copy per cell.

![FIG. 5. Differential expression of types IV, V, and VI AC mRNAs in the glomerulus. For each AC type, wild type RNAs (WT), corresponding to ¼ or ½ glomerulus were analyzed with the corresponding mutant cRNA (Mut) used as internal standard. The amounts of mutant cRNAs were type IV and VI, 2000 molecules and type V, 1000 molecules, respectively. RT-PCR assay and analysis of the products were carried out as described in Fig. 2. The size of each cDNA fragment corresponded to those expected. The reaction was performed in the absence (RT−) or presence of reverse transcriptase; blank (B), reaction performed without RNAs.](image)

### Table II

| Structures | Adenylyl cyclase mRNA isoforms |
|------------|-----------------------------|
|            | Type IV | Type V | Type VI |
| Glomerulus | 1655 ± 252 (4) | 1063 ± 249 (5) | 3112 ± 357 (5) |
| PCT        | <100 (4) | <100 (6) | 409 ± 48 (8) |
| PR         | <100 (4) | <100 (6) | 506 ± 88 (6) |
| DTL        | <100 (3) | <100 (4) | 595 ± 108 (5) |
| ATL        | <100 (6) | <100 (6) | 834 ± 108 (7) |
| MTAL       | <100 (2) | <100 (5) | 2058 ± 307 (7) |
| CTAL       | <100 (3) | <100 (4) | 2715 ± 382 (7) |
| CDD        | <100 (4) | 915 ± 216 (6) | 4225 ± 505 (7) |
| OMCD       | <100 (4) | 2367 ± 178 (8) | 5658 ± 543 (8) |
| IMCD       | <100 (2) | <100 (4) | 4053 ± 623 (4) |

- **FIG. 6. Differential expression of adenylyl cyclase isoforms in different segments of the nephron.** Expression of type V and VI AC mRNAs was analyzed simultaneously in the same reaction tube as described in Fig. 4. Serial dilutions of wild type RNAs corresponding to different lengths of cortical thick ascending limb (CTAL), cortical collecting tubule (CCD), or proximal convoluted tubule (PCT) were co-amplified (28 cycles) with mutant cRNAs. The amounts of mutant cRNA introduced per reaction tube were type IV, 200 molecules; type V, either 2000 molecules (PCT) or 1000 molecules (CTAL and CCD); type VI, either 2000 molecules (PCT) or 2000 molecules (CTAL and CCD). Control reactions carried out either without RNA or without reverse transcriptase gave no signals. The cDNA fragments amplified from wild type (WT) and mutant (M) RNAs were discriminated by digestion with HindII.

- **TABLE I**  
  Quantitation of types IV, V, and VI AC mRNAs in different structures of the rat nephron

The data are expressed as molecules of mRNA per glomerulus or per mm of segment and are given as mean values ± S.E. calculated from the number of samples indicated in parentheses. Depending on the structure studied, most of the data were obtained on samples microdissected from 4 to 7 kidneys. Signals not detectable or below 100 molecules per mm obtained from different samples of a given segment are indicated as <100. The abbreviations used to define the segments are: PCT, proximal convoluted tubule; PR, pars recta; DTL and ATL, descending and ascending portions of the thin limb of Henle's loop; MTAL and CTAL, medullary and cortical portions of the thick ascending limb of Henle's loop; CCD and OMCD, cortical and outer medullary portions of the collecting duct; IMCD, terminal portion of the inner medullary collecting duct.
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(31), and the Ca\(^{2+}\) ionophore, ionomycin (18). A few experiments were also conducted with prostaglandin E\(_2\) (PGE\(_2\)) which inhibits AVP-dependent cAMP content by a Ca\(^{2+}\)-dependent process (30) mainly linked to the metabolism of cAMP (26). In 2 mM Ca\(^{2+}\) medium, 0.1 mM carbachol and 0.3 μM PGE\(_2\) induce similar biphasic increases of [Ca\(^{2+}\)]\(_i\), with peak and plateau phase values of 70–150 and 30–50 nM above basal levels, respectively (30–32).

Table III gives the results obtained with 0.1 mM carbachol on the cAMP content produced by either 1 mM AVP or 0.1 mM glucagon in the presence of 1 mM IBMX. These concentrations of AVP and glucagon and the use of 1 mM IBMX induce a maximal production of hormone-dependent cAMP content in the rat OMCD (25, 29). Carbachol elicited a clear-cut inhibition of glucagon-dependent cAMP generation, and this inhibitory effect was abolished in the absence of extracellular Ca\(^{2+}\) (0 Ca\(^{2+}\)). By contrast, carbachol did not modify the response to AVP studied in the same experiments (Table III). It should be noted that the glucagon-sensitive cAMP generation was enhanced in Ca\(^{2+}\)-depleted medium whereas the response to AVP was not modified (Table III).

The possible regulation of the response to AVP by Ca\(^{2+}\) was further tested by the use of ionomycin and PGE\(_2\). Ionomycin was used at 10 nM or 1 μM concentrations which increase [Ca\(^{2+}\)]\(_i\) to a level comparable with the plateau phase obtained with PGE\(_2\) or carbachol (30–32) or to 10 times higher values, respectively (data not shown). When all phosphodiesterase activities were blocked with 1 mM IBMX, 1 μM ionomycin elicited an inhibition of 65.9 ± 10.5% (n = 3) on AVP-stimulated cAMP synthesis (Table IV). A lower ionomycin concentration (10 nM) had either no effect (n = 2) or induced a slight and nonsignificant inhibition (17%, n = 1). In the presence of 50 μM Ro 20–1724, which clamps partially the phosphodiesterase activity in the rat OMCD (26), this low concentration of ionomycin nevertheless decreased by 75.1 ± 11.1% the response to AVP (Table IV). The action of PGE\(_2\) (0.3 μM) inhibited by 42.6% the AVP-dependent cAMP accumulation in the presence of Ro 20–1724 but had no effect in the presence of IBMX. These results confirmed those previously obtained in a large experimental series (26).

**DISCUSSION**

This study demonstrates that types IV, V, and VI AC mRNAs, previously detected by Northern hybridization on whole rat kidney extracts (8, 14, 20), are expressed in different structures of the nephron. Both the relative and quantitative expression of these AC isoforms vary from one nephron portion to another. Types V and VI AC mRNAs coding for Ca\(^{2+}\)-inhibitable adenylyl cyclases are especially expressed in the outer medullary collecting duct. The role of Ca\(^{2+}\) observed on the regulation of either AVP- or glucagon-dependent cAMP accumulation in this segment supports the hypothesis that Ca\(^{2+}\)-inhibitable adenylyl cyclase mRNAs are functionally expressed in this segment.

**FIG. 7. Comparative expression of types V and VI AC mRNAs along the rat nephron.** Type V and type VI AC mRNAs were analyzed simultaneously in the same reaction tube as described in Fig. 4. Wild type RNAs corresponding to 1/2 glomerulus or 0.5 mm of each segment studied were co-amplified (28 cycles) with mutant cRNAs (type V, 500 molecules; type VI, 2000 molecules). The cDNA fragments obtained from wild type (WT) and mutant (Mut) RNAs were discriminated by digestion with HindIII. The reaction was carried out in the absence (−) or the presence (+) of reverse transcriptase (RT); blank, reaction performed without RNAs. The renal structures studied were Gl, glomerulus; PCT, proximal convoluted tubule; PR, pars recta; DTL, descending thin limb; ATL, ascending thin limb; MTAL, medullary thick ascending limb; CTAL, cortical thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, terminal portion of the inner medullary collecting duct.

**TABLE III**

| Experimental conditions | cAMP production |
|-------------------------|-----------------|
|                         | (2 mM Ca\(^{2+}\)) | (0 Ca\(^{2+}\)) |
| n = 5                   | 19.5 ± 2.1      | 40.6 ± 3.6\(^a\) |
| Glucagon                | 9.6 ± 1.1\(^b\) | 37.6 ± 3.2 |
| Glucagon + carbachol    | 130.2 ± 8.8     | 115.4 ± 13.8\(^c\) |
| AVP                     | 119.3 ± 10.6\(^c\) |  |
| AVP + carbachol         |  |

\(^a\) Comparison of the mean values obtained with glucagon in 2 mM Ca\(^{2+}\) and 0 Ca\(^{2+}\) medium, p < 0.005.

\(^b\) p < 0.005 when compared with the corresponding mean value in the presence of glucagon.

\(^c\) Not different from the corresponding mean values.

**TABLE IV**

| Experimental conditions | cAMP accumulation |
|-------------------------|--------------------|
|                         | (2 mM Ca\(^{2+}\)) | (0 Ca\(^{2+}\)) |
| IBMX                    | 140.0 ± 18.5 |
| AVP 1 nM                | 135.1 ± 21.2 |
| AVP + 10 nM ionomycin   | 49.9 ± 14.4\(^a\) |
| AVP + 1 μM ionomycin    |  |
| Ro 20-1724              | 66.6 ± 17.0 |
| AVP 1 nM                | 18.7 ± 7.8\(^b\) |

\(^a\) p < 0.001 when compared to the response obtained with AVP.

\(^b\) p < 0.01 when compared to the response obtained with AVP.
Ca\textsuperscript{2+}-inhibitable Adenylyl Cyclases along the Rat Nephron

in Different Structures of the Rat Nephron—Each of the segments that constitute the renal tubule represents a small number of cells (200–500 cells per mm) (28) and of total proteins (0.10–0.25 μg per mm) (33). We used therefore the method of RT-PCR to localize AC mRNAs along the nephron. Accurate conditions to perform quantitative RT-PCR on small amounts of RNA extracted from isolated segments were previously developed in our laboratory, i.e. (i) amplified sequences corresponding to mutant and wild type mRNAs have the same length and only differ from each other by two base pairs to introduce a new restriction site, and (ii) both templates were co-amplified in the same reaction tube by using the same set of primers (21). The validity of these conditions to quantitate AC mRNAs is sustained by different results of this study. 1) The relatively low number of PCR cycles corresponds to an exponential phase of amplification for both mutant and wild type cDNAs, and the amplification efficiencies are very close for both targets. 2) There is no evidence for competition between different mutant and/or wild type targets when they are introduced in the same reaction tube. 3) The intensity of the signal obtained is proportional to the amount of RNAs introduced in the assay.

The characteristics of the primers used allowed the study of the amplification of different AC types in the same RT-PCR assay and thus allowed us to investigate more accurately the differential expression of the corresponding mRNAs. The pattern of distribution is characterized by the widespread presence of type VI AC mRNA along the whole renal tubule, whereas the localization of the two other mRNAs studied is much more restricted either to the glomerulus (type IV and type V) or to the initial segments (CCD and OMCD) of the collecting duct (type V).

As noted under the "Results," several AC mRNAs are present in the glomerulus, the CCD and the OMCD. The expression of several AC types in these structures might be due to the presence of different cell types (15, 22). A localization at the cellular level of these AC mRNAs cannot be accurately studied by using in situ hybridization due to the small size of the renal tubule cells (about 100 μm\textsuperscript{2} or less, Ref. 15).

Functional Expression of Ca\textsuperscript{2+}-inhibitable Adenylyl Cyclases in the Rat Outer Medullary Collecting Tubule—At the present time, there is no specific antibody that allows us to discriminate type V from type VI AC protein. Consequently, the expression of Ca\textsuperscript{2+}-inhibitable AC proteins was investigated by studying the regulation of hormone-dependent cAMP content.

Several lines of evidence support the conclusion that AVP and glucagon stimulate AC activity in different cells of the rat OMCD. (i) Morphological studies have described two cell types in this segment, the principal cell and the type A intercalated cell (15, 22). (ii) Biochemical studies have established that α\textsubscript{2}-adrenergic agonists inhibit AVP- but not glucagon-stimulated cAMP synthesis (25). (iii) Numerous results have firmly demonstrated that AVP mediates its antidiuretic action via the formation of cAMP in the principal cells, whereas glucagon has no antidiuretic effect but is implicated in bicarbonate transport, a process present in intercalated cells (29, 34).

Measurement of cAMP content in native cells integrates factors and pathways prevailing in vivo and is therefore a suitable technique to study the physiological regulation of adenylyl cyclase activity. However, the study of the synthesis of cAMP per se implies that hydrolysis of the cyclic nucleotide must be fully blocked. This condition is fulfilled in the experiments performed in the presence of 1 mM IBMX since the AVP- and glucagon-stimulated values obtained on intact segments correspond to those which can be expected from adenylyl cyclase values obtained on permeabilized structures (25, 26, 29). Since we used maximal hormonal concentrations (see "Results"), the cAMP levels measured in the presence of IBMX therefore correspond to the maximal cAMP production due to the functional expression of the particular AC protein(s) present in either the AVP- or the glucagon-sensitive cells. In contrast, the use of Ro 20–1724, a specific inhibitor of the low K\textsubscript{m} cAMP phosphodiesterase (27), allows the measurement of cAMP accumulation that integrates regulations on both the synthesis and a partial catabolism of the cyclic nucleotide in the rat OMCD (26).

Our studies demonstrate that the muscarinic agonist carbachol inhibits glucagon-dependent cAMP production and that this effect depends on the presence of Ca\textsuperscript{2+} in the incubation medium. This regulation therefore appears linked to the ability of carbachol to increase [Ca\textsuperscript{2+}]. It has to be noted that although carbachol-induced [Ca\textsuperscript{2+}], is of relatively low magnitude (31), this agent elicited a marked Ca\textsuperscript{2+}-dependent inhibition of glucagon-dependent cAMP production. Moreover, the cAMP production stimulated by glucagon was lower in 2 mM Ca\textsuperscript{2+} than in Ca\textsuperscript{2+}-depleted medium. These data and the presence of types V and VI AC mRNAs in the OMCD suggest that a Ca\textsuperscript{2+}-inhibitable AC is functionally expressed in the glucagon-sensitive cells. Type V AC has been described to be more sensitive to low Ca\textsuperscript{2+} concentrations than type VI (7) and therefore might be a suitable enzyme to be present in the glucagon-sensitive cells of the rat OMCD.

In contrast to its inhibitory effect on the response elicited by glucagon, carbachol did not modulate AVP-dependent cAMP production. Similar results were obtained in the presence of Ro 20–1724 (32). These data therefore indicate that carbachol does not regulate cAMP pathway in the AVP-sensitive cells of the rat OMCD. With regard to the regulation of the response to AVP by ionomycin, a high concentration (1 μM) inhibited AVP-stimulated cAMP production. A lower concentration of ionomycin (10 nM) had no reproducible inhibitory effect on cAMP production but decreased AVP-dependent cAMP content if phosphodiesterase activity was not fully blocked. Similar results were obtained with different concentrations of A23187 (data not shown), an observation in agreement with the results of Kusano et al. (18). The regulation elicited by PGE\textsubscript{2} was similar to that induced by the low concentration of Ca\textsuperscript{2+} ionophores and confirmed the results observed in a previous study: in the presence of IBMX a slight (12%) inhibition by PGE\textsubscript{2} was observed that, probably because of the biological scatter of cAMP contents from one segment to another, did not reach statistical significance; by contrast a marked 53% inhibitory effect of PGE\textsubscript{2} was obtained in the presence of Ro 20–1724 (26). All the data obtained on AVP-sensitive cells demonstrate that the mechanism of action of low doses of Ca\textsuperscript{2+} ionophores and PGE\textsubscript{2} is linked mainly to the catabolism of the cyclic nucleotide, whereas the effect of high concentrations of Ca\textsuperscript{2+} ionophores is due to an interaction with a Ca\textsuperscript{2+}-inhibitable AC.

The adenylyl cyclase type present in AVP-sensitive cells of the OMCD can therefore be negatively modulated provided a high rise in [Ca\textsuperscript{2+}], is achieved. This observation suggests that type VI adenylyl cyclase mRNA is functionally expressed in the principal cells, an hypothesis supported by the following arguments. (i) As we observed with Ca\textsuperscript{2+} ionophores, the level of type VI AC inhibition in different cells is correlated with the rise of [Ca\textsuperscript{2+}], achieved with different concentrations of agonists or ionophores (13, 35). (ii) The extent of inhibition by Ca\textsuperscript{2+} is smaller for type VI AC than for type V (4, 5, 7); this observation might account for the lack of a clear-cut inhibition by PGE\textsubscript{2} and low concentrations of Ca\textsuperscript{2+} ionophores that induce small increases of [Ca\textsuperscript{2+}]. (iii) The AVP-stimulated cAMP production is not modulated by protein kinase C in the rat OMCD.
(32), a result in agreement with the lack of regulation of this kinase on type VI AC (36, 37). The presence of a Ca\(^{2+}\)-inhibitable AC in the principal cells is also supported by the comparison between the properties of regulation of the CAMP content known in the rat OMCD and those that result from the presence of the Ca\(^{2+}\)-inhibitable type VI AC in different cell lines. Indeed, different studies allowed the definition of several properties of PGE\(_2\)-induced inhibition of AVP-dependent CAMP accumulation. This effect is insensitive to pertussis toxin, dependent on extracellular Ca\(^{2+}\), and is linked to the increase of [Ca\(^{2+}\)]\(_i\) (13). Such properties are similar to those observed for the effects of phosphodiesterase inhibitors and by 34% in their absence (30, 32). It is noteworthy that these properties of regulation of CAMP levels are identical to those described in different cells that express type VI AC mRNA (4, 5, 9, 35).

In conclusion, our study demonstrates a differential expression of type V and type VI AC mRNAs in the epithelial cells of the renal tubule. Functional studies performed on microdissected OMCD establish that increasing [Ca\(^{2+}\)]\(_i\) negatively modulates the hormone-dependent CAMP levels in this segment. These results obtained on intact native cells suggest that physiological increases of [Ca\(^{2+}\)]\(_i\), such as those induced by PGE\(_2\) or acetylcholine, very likely play a crucial role in the regulation of the CAMP pathway, including the phosphodiesterase activity, in conditions prevailing in vivo. Physiological studies performed on microperfused rabbit cortical collecting duct have established that an increase of [Ca\(^{2+}\)]\(_i\) inhibits transport functions (38). To date, such experiments have not been performed in the rat OMCD. The expression of types V and VI AC mRNAs, the different patterns of regulation induced by a rise of [Ca\(^{2+}\)]\(_i\) on either the glucagon- or AVP-dependent CAMP level, and the properties of Ca\(^{2+}\)-inhibitable adenyl cyclase isoforms described in the literature lead us to propose that type V AC is mainly, if not exclusively, expressed in the glucagon-sensitive cells and that type VI AC is present in the AVP-sensitive cells of the outer medullary collecting duct of the rat kidney.

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