Co-expression of a Ca\(^{2+}\)-inhibitable Adenylyl Cyclase and of a Ca\(^{2+}\)-sensing Receptor in the Cortical Thick Ascending Limb Cell of the Rat Kidney

INHIBITION OF HORMONE-DEPENDENT cAMP ACCUMULATION BY EXTRACELLULAR Ca\(^{2+}\)

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Marie Céleste de Jesus Ferreira, Cécile Héliès-Toussaint, Martine Imbert-Teboul, Claire Bailly, Jean-Marc Verhavatz, Anne-Christine Bellanger, and Danielle Chabardès

From the Service de Biologie Cellulaire, Département de Biologie Cellulaire et Moléculaire, CEA Saclay, France

The Ca\(^{2+}\)-sensing receptor protein and the Ca\(^{2+}\)-inhibitable type 6 adenylyl cyclase mRNA are present in a defined segment of the rat renal tubule leading to the hypothesis of their possible functional co-expression in a same cell and thus to a possible inhibition of cAMP content by extracellular Ca\(^{2+}\). By using microdissected segments, we compared the properties of regulation of extracellular Ca\(^{2+}\)-mediated activation of Ca\(^{2+}\) receptor to those elicited by prostaglandin \(E_2\) and angiotensin II. The three agents inhibited a common pool of hormone-stimulated cAMP content by different mechanisms as follows. (i) Extracellular Ca\(^{2+}\), coupled to phospholipase C activation via a pertussis toxin-insensitive G protein, induced a dose-dependent inhibition of cAMP content (1.25 mM Ca\(^{2+}\) eliciting 50% inhibition) resulting from both stimulation of cAMP hydrolysis and inhibition of cAMP synthesis; this latter effect was mediated by ca-capacitive Ca\(^{2+}\) influx as well as release of intracellular Ca\(^{2+}\). (ii) Angiotensin II, coupled to the same transduction pathway, also decreased cAMP content; however, its inhibitory effect on cAMP was mainly accounted for by an increase of cAMP hydrolysis, although angiotensin II and extracellular Ca\(^{2+}\) can induce comparable release of intracellular Ca\(^{2+}\). (iii) Prostaglandin \(E_2\), coupled to pertussis toxin-sensitive G protein, inhibited the same pool of adenylyl cyclase units as extracellular Ca\(^{2+}\) but by a different mechanism. The functional properties of the adenylyl cyclase were similar to those described for type 6. The results establish that the co-expression of a Ca\(^{2+}\)-inhibitable adenylyl cyclase and of a Ca\(^{2+}\)-sensing receptor in a same cell allows an inhibition of cAMP accumulation by physiological concentrations of extracellular Ca\(^{2+}\).

A new type of G protein-coupled membrane receptor that is activated by increasing concentrations of extracellular ionized calcium ([Ca\(^{2+}\)]\(_i\)) and polyvalent cations has been cloned recently from bovine parathyroid cells (BoPCaR1, Ref. 1) and rat kidney (RaKCaR, Ref. 2). When expressed in Xenopus oocytes, these Ca\(^{2+}\)-sensing receptors are coupled to phospholipase C stimulation (1, 2). Increasing [Ca\(^{2+}\)]\(_i\) also stimulates phospholipase C activity in parathyroid cells (3, 4) and inhibits hormone-dependent cAMP accumulation (5), but, so far, no interaction between these two transduction pathways has been established. The presence of BoPCaR1 in parathyroid cells explains the crucial role of [Ca\(^{2+}\)]\(_i\) to elicit a negative feedback on parathyroid hormone secretion (6, 7). In rat kidney, a predominant expression of RaKCaR mRNA has been localized in the cortical portion of the thick ascending limb (8, 9), a segment which ensures cAMP-stimulated paracellular Ca\(^{2+}\) reabsorption, from the lumen of the renal tubule to the extracellular fluid compartments (10). A functional Ca\(^{2+}\) receptor is expressed in the plasma membrane of the rat and mouse cortical thick ascending limb (CTAL) as evidenced by the properties of the dose-dependent increase in the concentration of intracellular calcium ([Ca\(^{2+}\)]\(_i\)) as a function of pertibular [Ca\(^{2+}\)]\(_o\) (11, 12). High [Ca\(^{2+}\)]\(_o\) (5 mM) in the mouse CTAL decreases hormone-dependent cAMP accumulation, an effect which has been ascribed to a direct inhibition of adenylyl cyclase (AC) activity (13).

Experiments using quantitative reverse transcription-polymerase chain reaction (RT-PCR) have shown that the rat CTAL also expresses the Ca\(^{2+}\)-inhibitable type 6 AC mRNA (14). All the Go-coupled receptors studied so far in this segment activate a single pool of AC catalytic units (15, 16), and in addition, electron microscopy studies describe a single cell type in this epithelium (17). These observations lead to the hypothesis that the functional expression of the type 6 AC mRNA accounts for the hormone-dependent cAMP synthesis in the rat CTAL.

The aim of the present study was therefore to investigate the functional expression of the AC present in the rat CTAL and the consequences of the possible co-localization in a same cell of a Ca\(^{2+}\)-inhibitable AC and of a Ca\(^{2+}\)-sensing receptor on the regulation of cAMP synthesis and/or hydrolysis. In order to study the regulation of cAMP levels elicited by potentially similar or different mechanisms of action, we compared the effect of extracellular Ca\(^{2+}\) to those of two agents also active in this segment. The first agent, angiotensin II, induces [Ca\(^{2+}\)]\(_i\) increases in the rat CTAL (11, 18). The pattern of the responses of Ca\(^{2+}\); AC, adenylyl cyclase; AVP, arginine vasopressin; [Ca\(^{2+}\)]\(_i\), intracellular free concentration of Ca\(^{2+}\); CTAL, cortical portion of the thick ascending limb; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphates; PDE, phosphodiesterase; PGE\(_2\), prostaglandin \(E_2\); PTX, Bordetella pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction; Ro 20-1724, 4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone.
observed demonstrates that a same intracellular Ca\(^{2+}\) pool is released by angiotensin II and extracellular Ca\(^{2+}\) (11). The second agent, prostaglandin E\(_2\) (PGE\(_2\)), inhibits hormone-dependent cAMP synthesis (19) likely as a result of the interaction of the PGE\(_2\) receptor with a GTP-dependent, pertussis toxin-sensitive G\(_0\) protein as demonstrated in the medullary portion of the rat thick ascending limb (20).

The experiments were performed on rat CTAL isolated by microdissection, and the results establish that PGE\(_2\) (coupled to pertussis toxin-sensitive G\(_0\) protein), angiotensin II, and extracellular Ca\(^{2+}\) (both coupled to phospholipase C pathway) are effective in a same cell to decrease arginine vasopressin-dependent CAMP accumulation. [Ca\(^{2+}\)]\(_i\), in the physiological range, decreases hormone-dependent CAMP accumulation by more than 50%, an effect which results from both an inhibition of CAMP synthesis and an increase of CAMP hydrolysis. Angiotensin II also regulates both mechanisms, but its ability to inhibit CAMP synthesis is much smaller. The adenylyl cyclase present in this segment has the functional properties previously described for the Ca\(^{2+}\)-inhibitable type 6 AC (21, 22); in particular, AC activity is inhibited by both G\(_0\) and phospholipase C pathways by different mechanisms. Accordingly, in situ hybridization shows a homogeneous distribution of type 6 AC mRNA in CTAL cells. Taken together, the results establish that the co-expression of a Ca\(^{2+}\)-sensing receptor and of a Ca\(^{2+}\)-inhibitable AC in the rat CTAL cell allows a specific inhibition by physiological [Ca\(^{2+}\)]\(_o\) of hormone-stimulated CAMP intracellular content.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise specified, the compounds were from Merck (Darmstadt, Germany), Sigma, and Calbiochem.

*In Situ Hybridization*—A probe specific of type 6 AC was chosen in the most divergent region of AC cDNA sequences. A 376-bp fragment (PvuII-SphI, nucleotides 3766–4143) of the type 6 AC cDNA, located in the 3' untranslated region, was subcloned in pGEM3Zf(+) in the corresponding sites (SmaI-SphI). This sequence was included in the region previously used for RT-PCR experiments (14). A 1080-bp fragment corresponding to nucleotides 1205 (EcoRI) to 2285 (PvuII) of the type 5 AC cDNA coding region was subcloned in pGEM3Zf(+), introduced in the corresponding site of the broad host-range plasmid pBluescript KS+. Sonden-labeled cRNA probes were transcribed in vitro with T7, T3, or SP6 RNA polymerase (Promega Biotech, Madison, WI) according to the manufacturer’s instructions, in the presence of e-35S-UTP (>3,000 Ci/ml, Amersham Pharmacia Biotech, Les Ulis, France).

Adult rat kidneys were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline. Kidney slices were post-fixed overnight in the same fixative and then dehydrated in a series of ethanol and butanol. Tissues were paraffin-embedded, and sections of 5–7 μm were mounted on silane-coated slides. In *in situ* hybridization was performed as described by Shammugam et al. (23). Briefly, slides were deparaffinized in toluene and then rehydrated by a graded series of ethanol (100 to 30%). Pretreatment was done by boiling in 0.1% citric acid in a microwave oven and then treated with 0.1% H\(_2\)O\(_2\) in phosphate-buffered saline. These modifications have been shown to increase specific signal. After a second run of fixation in 4% paraformaldehyde-phosphate-buffered saline, and a proteinase K treatment, slides were covered with hybridization buffer (50% formamide, 12% dextran sulfate (50%), 11% salmon sperm DNA, 11% 20% ethanol (100 to 30%). Pretreatment was continued by boiling in a 2% solution of proteinase K in phosphate-buffered saline, and a proteinase K treatment (Promega Biotech, Madison, WI) according to the manufacturer’s instructions, in the presence of e-35S-UTP (>3,000 Ci/ml, Amersham Pharmacia Biotech, Les Ulis, France).

Measurement of Hormone-dependent CAMP Accumulation—The experimental conditions used to measure hormone-dependent CAMP accumulation on an intact single segment (20, 24) will be recalled briefly. Microdissected pieces of CTAL were transferred to 2 μl of incubation solution (550 mM NaCl, 127 mM KCl, 5 mM MgSO\(_4\), 0.8 mM KH\(_2\)PO\(_4\), 4 mM CaCl\(_2\), 5.6 mM glucose, 20 mM HEPES, pH 7.4, and 0.1% (w/v) bovine serum albumin fraction V, protease-free, minimal fatty acid content, Pentex, Miles Inc., Kankakee, IL). This medium was supplemented with 10 μM ibuprofen and 0.5 unit/ml adenosine deaminase (Boehringer Mannheim) in order to measure their length. Unless otherwise specified, each sample was preincubated for 10 min at 30 °C in 0.5 mM [Ca\(^{2+}\)]\(_o\) and, after addition of 2 μl of incubation medium containing the agonists to be tested, incubated for a further 4 min at 35 °C. Adenylyl cyclase activity was stimulated by arginine vasopressin (AVP) which binds to V2 receptor in the rat CTAL, the only AVP receptor expressed in this segment (26). Due to the small number of cells per tubular sample (from about 100 to 600 cells), hormone-dependent CAMP accumulation can be measured only in the presence of a phosphodiesterase inhibitor. Either 50 μM Ro 20-1724, a specific inhibitor of the low K\(_m\) cyclic AMP phosphodiesterase (27, 28), or 1 mM IBMX, inhibitor of all phosphodiesterases in the rat kidney (29), was added to the incubation medium. The concentrations of the different agents given in the results are those present during the incubation step. For longer preincubation periods, in experiments performed with Bordetella pertussis toxin and bisindolylmaleimide I, all media were supplemented with essential and nonessential amino acids as well as vitamins (minimum Eagle’s medium, Eurobio, Les Ulis, France).

Co-expression of Ca\(^{2+}\)-inhibitable AC and Ca\(^{2+}\)-sensing Receptor—In the experiments performed with parathyroid cells (3–5) in each experiment, different experimental conditions were tested on replicate CTAL samples microdissected from a same rat kidney (6–9 samples per condition). The mean of the cAMP values obtained in each condition was taken as one single data point, and the results were expressed in absolute value or in percentage of the response to AVP or in percentage of inhibition calculated from the corresponding AVP value. Results are given as the mean values ± S.E. calculated from n different experiments. Unless otherwise indicated, the statistical evaluation of the data was assessed by unpaired Student’s t test.

Measurement of Inositol Phosphate Production—Assays were performed by the microtechnique developed for proximal tubule fragments (29) with slight modifications. Briefly, in each experiment, CTAL (150–200 mm of total length) were microdissected from a collagenase-treated kidney. The medium used was supplemented with 10 mM HEPES, 1 mM MgSO\(_4\), 10 μM CaCl\(_2\) and, after the incubation was performed, CTAL pieces were radioiodinated in 50 μl of this medium containing 

\[\text{myo-}^{14}\text{H}\]inositol (1 μCi/ml, Amersham Pharmacia Biotech) for 2 h at 30 °C. After this labeling period, tubules were extensively rinsed in 0.5 mM [Ca\(^{2+}\)]\(_o\), medium, and tubule samples (4–7 mm length each) were incubated at 37 °C during 15 min in 0.5 mM [Ca\(^{2+}\)]\(_o\), medium supplemented with lithium. The reaction was stopped, and the radioactivity
associated with phosphoinositides, free inositol, inositol phosphates (IP), and glycerophosphoinositol was separated and counted as described previously (29).

In several experiments, it was checked that there was a good correlation between inositol phosphate formation expressed either per unit of tubule length or as a percentage of the total radioactivity counted \( r = 0.93 \pm 0.03, n = 4 \), an observation in agreement with that previously observed with proximal tubule fragments (29). Consequently, IP production was expressed as percentage of the total radioactivity counted. In each experiment, different experimental conditions (5 replicate samples each) were tested, and the mean of the IP values measured in each condition was taken as one single data point. The results are given as the mean values \( \pm \) S.E. calculated from \( n \) different experiments. The statistical evaluation of the results was assessed by paired Student's \( t \) test.

**Measurement of Intracellular Ca\(^{2+}\) Concentration**—Intracellular \( \text{Ca}^{2+} \) concentration was measured in single CTAL samples microdissected from collagenase-treated kidneys by using the calcium-sensitive fluorescence probe fura-2 as described previously (11). Briefly, CTAL were loaded for 60 min with 10 \( \mu \)M fura-2 AM. Each CTAL was then transferred to a superfusion chamber fixed on the stage of an inverted fluorescence microscope (Zeiss IM 35, Oberkochen, Germany). The tubule was superfused at 37 °C at a rate of 10–12 ml/min, corresponding to about 10 exchanges per min. The composition of the superfusion medium was similar to that used in cAMP experiments, except that serum albumin, ibuprofen, and adenosine deaminase were not added since the superfusion medium was flushed continuously. After a 5- to 10-min equilibration period, agonists were added to either the 0.5 mM \([\text{Ca}^{2+}]_e\) medium or to the \( \text{Ca}^{2+} \)-free medium and superfused over tubule. Due to the dead space of the superfusion setup, the time necessary to achieve a full equilibration of agonist concentration in the chamber was of 15–20 s. The tubular portion selected for fluorescence measurement included about 30 cells. Double wavelength measurements of fura-2 fluorescence were recorded every 2 s. Calculations of \([\text{Ca}^{2+}]_i\) were performed as described previously (11). The results obtained from different tubules microdissected from several rats were expressed as mean values \( \pm \) S.E.

**RESULTS**

**In Situ Hybridization**

The localization of type 6 AC mRNA in the rat kidney was examined by *in situ* hybridization at the light microscope. The most intense labeling in the kidney cortex was found in thick ascending limb, including CTAL (Fig. 1), collecting duct, and glomeruli (not shown). Fig. 1 shows strong labeling of CTALs (\( a, c, \) and *arrowheads*), whereas proximal tubules (\( a, c, \) and *)

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**Fig. 1. In situ hybridization of the Ca\(^{2+}\)-inhibitable type 6 AC mRNA.** \( a \) and \( b \), in indirect fluorescence on Evans blue counterstained kidney sections, CTALs (white arrowheads) are clearly distinguishable from proximal tubules (*). \( c \) and \( d \), on the same field in light microscopy, the antisense type 6 AC cRNA (\( c \)) strongly labels CTALs (black arrowheads), whereas proximal tubules are weakly labeled (*). No labeling was observed when the sense probe was used (\( d \)). \( \text{Bar} = 20 \mu \text{m} \).
FIG. 2. Effect of [Ca$^{2+}$]$_i$ on AVP-dependent cAMP accumulation in the cortical thick ascending limb. The results are the mean values ± S.E. calculated from different experiments performed in the presence of either 1 mM IBMX (n = 4 experiments) or 50 μM Ro 20-1724 (n = 3). Left panel, mean absolute values of cAMP accumulation induced by 10 nM AVP in CTAL incubated in 0.5 mM [Ca$^{2+}$]$_i$. [Ca$^{2+}$]$_i$, indicated on the figure are the final concentrations present during the incubation. From 1.5 to 2.5 mM [Ca$^{2+}$]$_i$, the percentages of responses to AVP obtained in the presence of Ro 20-1724 were significantly lower than the corresponding percentages obtained in the presence of IBMX (p < 0.005 to 0.001).

are weakly labeled. In CTAL, labeling was equally intense in all cells. No labeling was observed when the sense cRNA probe was used (Fig. 1, b and d). In contrast to type 6 AC, no significant labeling for type 5 AC was found in CTAL (not shown). This is consistent with previous quantitative RT-PCR results showing that the type 6 AC mRNA was more abundant in CTAL than in proximal tubule and that type 5 AC mRNA was not detected in CTAL (14).

Effect of Extracellular Ca$^{2+}$ on AVP-dependent cAMP Content and on the Activation of Phospholipase C

AVP was used at 10 nM, a concentration that induces a maximal stimulation of cAMP accumulation in intact cells (Ref. 19 and data not shown) and thus allows us to study the maximal amount of functional AC proteins present in the npt CTAL.

AVP-dependent cAMP accumulation was about 2-fold higher in the presence of 1 mM IBMX, which reflects AVP-stimulated cAMP synthesis only, than in the presence of 50 μM Ro 20-1724, which allows the measurement of cAMP accumulation that integrates both the synthesis and a partial catabolism of cAMP (Fig. 2, left panel).

In the presence of increasing [Ca$^{2+}$]$_i$, there was a dose-dependent inhibition of AVP-dependent cAMP levels with half-maximal inhibition of about 1.2 and 2.0 mM [Ca$^{2+}$]$_i$ in the presence of Ro 20-1724 and IBMX, respectively (Fig. 2, right panel). With Ro 20-1724, a steep inverse relationship was observed, and thus a small variation of [Ca$^{2+}$]$_i$, induced a high variation of hormone-dependent cAMP accumulation. The shape of the curve obtained with either IBMX or Ro 20-1724 shows that [Ca$^{2+}$]$_i$, below 1.5 mM had a small effect on cAMP synthesis but increased cAMP hydrolysis; conversely, at higher [Ca$^{2+}$]$_i$, the inhibition was mediated mainly by a decrease of cAMP synthesis.

Altogether in this study, the inhibition induced by 1.25 mM [Ca$^{2+}$]$_i$ in the presence of Ro 20-1724 was of 63.3 ± 3.9% (p < 0.001 versus 10 nM AVP, n = 11) and that induced by 1.5 mM [Ca$^{2+}$]$_i$ in the presence of IBMX was of 30.5 ± 3.5% (p < 0.01 versus 10 nM AVP, n = 8).

The implication of the Ca$^{2+}$ receptor RaKCaR to explain the inhibitory effect of [Ca$^{2+}$]$_i$, was supported by the results obtained with an agonist of this receptor, neomycin (1, 2). In 0.5 mM [Ca$^{2+}$]$_i$, 100 μM neomycin inhibited by 82.2 ± 5.8% (n = 3) and 43.5 ± 5.7% (n = 5) cAMP accumulation by 10 nM AVP in the presence of Ro 20-1724 and IBMX, respectively.

In the presence of IBMX, 2.5 mM [Ca$^{2+}$]$_i$, inhibited cAMP synthesis stimulated by either 10 μM forskolin or 10 nM AVP with a comparable efficiency (62.3 ± 7.2% of inhibition and 73.5 ± 2.5% in forskolin and AVP experimental groups, respectively, n = 3). These results suggest that [Ca$^{2+}$]$_i$, inhibited AC activity at post-receptor sites.

In our experimental conditions, no detectable [Ca$^{2+}$]$_i$, variations were obtained with 1.25 mM [Ca$^{2+}$]$_i$, (Table I), and the response to 1.5 mM [Ca$^{2+}$]$_i$, was characterized in most tubules by a low but sustained [Ca$^{2+}$]$_i$, increase without a clear-cut peak phase (Fig. 3 and Table I). With 2.5 mM [Ca$^{2+}$]$_i$, the response was a transient peak followed by a lower sustained plateau (Fig. 3) that reflects Ca$^{2+}$ entry (11). The simultaneous superfusion of 10 nM AVP and [Ca$^{2+}$]$_i$, did not modify [Ca$^{2+}$]$_i$, variations (data not shown). Unlike the peak, the plateau was blocked by nonspecific Ca$^{2+}$ channel blockers such as La$^{3+}$ and Ni$^{2+}$, whereas addition of voltage-sensitive channel blockers, i.e. verapamil and nifedipine, did not modify [Ca$^{2+}$]$_i$, (Ref. 11 and data not shown).

These [Ca$^{2+}$]$_i$, variations were due, at least in part, to phospholipase C activation since increasing [Ca$^{2+}$]$_i$, elicited a dose-dependent production of IPs (Table I). A significant stimulation was observed with 1.25 mM [Ca$^{2+}$]$_i$, although this concentration had no detectable effect on [Ca$^{2+}$]$_i$. This apparent discrepancy is probably linked to the following two methodological reasons: (i) discrete and local [Ca$^{2+}$]$_i$, variations may not have been detected in fura-2-loaded CTAL; (ii) a small stimulation of IP production is amplified by the 15-min duration of the incubation performed in the presence of lithium, which results in IP accumulation, whereas [Ca$^{2+}$]$_i$, changes are transient.

Comparison of the Effects of the Ca$^{2+}$ Receptor and Others Agonists to Decrease AVP-dependent cAMP Accumulation

Angiotensin II and PGE$_2$ were used at concentrations inducing maximal effects (18, 19), and in most experiments, their regulatory properties were studied in parallel to those of [Ca$^{2+}$]$_i$.

As expected from previous observations (11, 18), 0.1 μM angiotensin II increased IP production and [Ca$^{2+}$]$_i$, (Fig. 3 and Table I). The pattern of [Ca$^{2+}$]$_i$, variations was characterized by

| Experimental condition | IPs production (stimulation factor) | Δ[Ca$^{2+}$]$_i$, Increases (nM) |
|------------------------|-----------------------------------|----------------------------------|
| Peak                   | Plateau                           |                                  |
| 1.25 mM [Ca$^{2+}$]$_i$ | 1.52 ± 0.05% (4)                  | 1.3 ± 1.3                        |
| 1.5 mM [Ca$^{2+}$]$_i$ | 2.06 ± 0.09% (4)                  | 54 ± 10.2                        |
| 2.5 mM [Ca$^{2+}$]$_i$ | 8.97 ± 1.16% (7)                  | 42.0 ± 37.0                      |
| 0.1 mM angiotensin II  | 3.46 ± 0.27% (5)                  | 436.5 ± 48.0                     |
| 0.3 mM PGE$_2$         | 1.17 ± 0.04% (5)                  | 0                               |

* Mean value statistically different from the corresponding control. [Ca$^{2+}$]$_i$, variations (Δ[Ca$^{2+}$]$_i$) are expressed as increases over basal observed during the peak and plateau phases, respectively. Basal value of [Ca$^{2+}$]$_i$, calculated from 28 tubules was of 99.2 ± 7.2 nM. Data are the mean values ± S.E. calculated from 5 to 16 tubules microdissected from 3 to 8 kidneys, depending on the agent tested.
either Ro 20-1724 or IBMX. In these two conditions, 0.3 mM angiotensin II- and 2.5 mM Ca$^{2+}$, respectively, affected neither AVP-dependent cAMP accumulation induced by different agents. A, in each experiment (N), tubule samples microdissected in 0.5 mM [Ca$^{2+}$], control medium were incubated in the presence of either 50 μM Ro 20-1724 (open columns) or 1 mM IBMX (hatched columns); the mean cAMP content obtained with the addition of each agent was calculated as the percentage of inhibition of the corresponding response to 10 nM AVP. Data are the mean values ± S.E. calculated from the different experiments. *, p < 0.01; **, p < 0.001 refer to the absolute mean value obtained with each agent compared with the corresponding mean value measured with AVP alone. PGE$_2$ was tested at 0.3 μM and angiotensin II at 0.1 μM; 2.5 mM [Ca$^{2+}$]$_i$ was the final concentration present during the incubation step. B, in each experiment presented in A, the camp hydrolysis elicited by Ro 20-1724-insensitive PDEs was calculated by the difference of inhibition between the data obtained in Ro 20-1724- and IBMX-incubated tubules. The same value was calculated from the paired data obtained with 1.5 mM [Ca$^{2+}$]$_i$ in an additional experimental series (see "Results"). Data are the mean values ± S.E. calculated from the different experiments.

Action on cAMP Synthesis and/or on cAMP Hydrolysis—Fig. 4A shows the mean inhibitions obtained in the presence of either Ro 20-1724 or IBMX. In these two conditions, 0.3 mM PGE$_2$ elicited an inhibition of about 60%, which suggests that PGE$_2$-mediated inhibition was due only to a decrease of cAMP synthesis in the CTAL. In the presence of Ro 20-1724, the inhibition induced by angiotensin II was of smaller extent than that obtained with 2.5 mM [Ca$^{2+}$]$_i$ (Fig. 4A). The use of IBMX instead of Ro 20-1724 decreased angiotensin II-mediated inhibition by more than 50% (p < 0.001 versus Ro 20-1724) and also reduced, but more slightly, the inhibitory effect of 2.5 mM Ca$^{2+}$, (p < 0.005 versus Ro 20-1724). Therefore, although both 2.5 mM Ca$^{2+}$, and angiotensin II induced high [Ca$^{2+}$]$_i$ peak values (Fig. 3), the inhibition of AVP-dependent cAMP synthesis was much more pronounced with the addition of [Ca$^{2+}$]$_i$.

The difference between the observations obtained with Ro 20-1724 and IBMX reflected the cAMP hydrolysis due to PDE families insensitive to Ro 20-1724 (Fig. 4B). For comparison, the same cAMP hydrolysis value was calculated from the paired data obtained with 1.5 mM [Ca$^{2+}$]$_i$ in an additional experimental series. When compared with AVP, 1.5 mM [Ca$^{2+}$]$_i$ induced 73.8 ± 1.7% of inhibition, p < 0.001, and 35.0 ± 3.9%, p < 0.005, in the presence of Ro 20-1724 and IBMX, respectively. n = 5. PDEs insensitive to Ro 20-1724 had a similar efficiency to decrease AVP-dependent cAMP accumulation in angiotensin II- and 2.5 mM [Ca$^{2+}$]$_i$-incubated CTAL, and this effect was higher with 1.5 mM [Ca$^{2+}$]$_i$ (Fig. 4B). However, this hydrolysis of cAMP due to PDEs insensitive to Ro 20-1724 represented 59.1, 52.2, and 32% with angiotensin II, 1.5 and 2.5 mM [Ca$^{2+}$]$_i$, respectively, of the total inhibition observed in the presence of Ro 20-1724. Therefore the proportion of inhibition of cAMP accumulation due to cAMP hydrolysis alone was more pronounced with angiotensin II and 1.5 mM [Ca$^{2+}$]$_i$, in agreement with the data presented in Fig. 2. The addition to the media of 8-methoxymethyl-IBM, an inhibitor of the Ca$^{2+}$/calmodulin-dependent PDEs (27), decreased angiotensin II- and extracellular Ca$^{2+}$-mediated inhibitions (Table II) leading to values comparable to those observed with the use of IBMX (Figs. 2 and 4A). The Ca$^{2+}$/calmodulin-dependent family there-
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TABLE II

Effect of an inhibitor of the Ca\(^{2+}\)/calmodulin-dependent PDEs on [Ca\(^{2+}\)]\(_j\) or angiotensin II-mediated inhibition of AVP-dependent cAMP accumulation

In each experiment, tubule samples were preincubated and incubated in 0.5 mM [Ca\(^{2+}\)]\(_j\) and in the presence of either 50 μM Ro 20–1724 (control) or Ro 20–1724 + 75 μM 8-methoxymethyl-IBMX. The effect of [Ca\(^{2+}\)]\(_j\) or angiotensin II was expressed in percentage of inhibition calculated from the corresponding response to 10 nM AVP; 8-methoxymethyl-IBMX increased AVP-dependent cAMP accumulation by about 14%. The data are the mean values calculated from different experiments (n).

| Experimental condition | % inhibition of the response to 10 nM AVP |
|------------------------|------------------------------------------|
| Control                | -                                        |
| 1.25 mM [Ca\(^{2+}\)]\(_j\) (4) | 53.3 ± 7.9                              |
| 2.5 mM [Ca\(^{2+}\)]\(_j\) (3) | 95.6 ± 1.2                              |
| 0.1 mM angiotensin II (4) | 43.0 ± 7.4                              |

\(\ast \ p < 0.01; \ \ast \ast \ p < 0.001 \) when compared with the values obtained in control conditions.

TABLE III

Effect of inhibitors of protein kinase C or phospholipase A2 pathways on [Ca\(^{2+}\)]\(_j\) or angiotensin II-mediated inhibition of AVP-dependent cAMP accumulation

In each experiment, tubule samples were microdissected from the same kidney, preincubated (4 h at 35 °C), and incubated (4 min at 35 °C) with or without pertussis toxin (PTX, 500 ng/ml); the effect of each agent was calculated as the percentage of inhibition of the corresponding response to 10 nM AVP. Data are the mean values ± S.E. calculated from 4 experiments performed in the presence of 50 μM Ro 20-1724. *p < 0.001 refers to the effect of PTX on PGE\(_2\)-mediated inhibition; NS indicates that the difference was not significant. *p < 0.05; **p < 0.01 or 0.001 when compared with the corresponding mean value obtained with AVP. AVP-stimulated cAMP accumulation was increased in PTX-treated groups (94.2 ± 8.0 fmol-mm\(^{-1}\)-4 min\(^{-1}\) and 51.4 ± 2.7 with or without PTX, respectively, p < 0.005).

| Experimental condition | % inhibition of the response to 10 nM AVP |
|------------------------|------------------------------------------|
| Control                | -                                        |
| -4 μM 4-bromophenacyl bromide | -                                        |
| 1.25 mM [Ca\(^{2+}\)]\(_j\) (5) | 65.7 ± 3.1                              |
| 2.5 mM [Ca\(^{2+}\)]\(_j\) (4) | 94.5 ± 0.7                              |
| 0.1 mM angiotensin II (5) | 40.3 ± 2.2                              |
| -7.8 μM 17-octadecynoic acid | -                                        |
| 1.25 mM [Ca\(^{2+}\)]\(_j\) (3) | 60.8 ± 3.7                              |
| 0.1 mM angiotensin II (3) | 46.9 ± 6.2                              |
| -1.0 μM bisindolylmaleimide I | -                                        |
| 1.25 mM [Ca\(^{2+}\)]\(_j\) (2) | 73.6 ± 7.4                              |
| 2.5 mM [Ca\(^{2+}\)]\(_j\) (4) | 92.8 ± 0.9                              |
| 0.1 mM angiotensin II (7) | 49.7 ± 4.4                              |

![Fig. 5. Effect of pertussis toxin on PGE\(_2\), angiotensin II, and 2.5 mM [Ca\(^{2+}\)]\(_j\) -mediated inhibition of AVP-dependent cAMP accumulation.](image)

The two agents inhibit cAMP synthesis by two different mechanisms. In order to confirm this hypothesis, and to examine whether these two effects occurred or not in the same cell, we performed experiments of multiple combined inhibition in the presence of IBMX. Experimental conditions and criteria previously defined (24) were used to test a possible summation of inhibitions (if in different cells) or cumulative inhibition (if different mechanisms in a same cell) of AC activity. The residual cAMP content observed with the simultaneous addition of PGE\(_2\) and [Ca\(^{2+}\)]\(_j\) was lower than the value obtained with each inhibitor alone, but the response to AVP was not fully abolished although inhibition higher than 50% were obtained with each agent alone (Table IV). This result indicates that PGE\(_2\) and extracellular Ca\(^{2+}\) were active in a same cell. Analysis of the results showed that the measured cAMP value (11.5 ± 3.3 fmol-mm\(^{-1}\)-4 min\(^{-1}\), Table IV) was not different from the theoretical cAMP content calculated by assuming a hypothesis of different mechanisms leading to a cumulative inhibition (13.1 ± 4.0 fmol-mm\(^{-1}\)-4 min\(^{-1}\)). These results and those obtained in pertussis toxin-treated tubules (Fig. 5) demonstrate that PGE\(_2\) and [Ca\(^{2+}\)]\(_j\) inhibited AVP-dependent AC activity by different and independent mechanisms effective on a same pool of catalytic units.

Combined inhibition experiments were also performed with PGE\(_2\) and angiotensin II but in the presence of Ro 20-1724 to compare inhibitions of about the same range. The inhibition obtained with the simultaneous addition of both agents (58.6 ± 8.4% of inhibition, n = 4) was higher than the inhibition induced by angiotensin II added alone (37.7 ± 4.3%) but was in a range comparable to that obtained with PGE\(_2\) (48.8 ± 3.3%) and did not represent a full summation of inhibitions. PGE\(_2\) and angiotensin II therefore inhibited a same pool of intracellular cAMP. A more thorough analysis of the results was not possible because angiotensin II-induced inhibition resulted from an effect on both cAMP synthesis and cAMP hydrolysis, and the relative part of each process was not known in these experiments.

Stimulates IP production by a process sensitive to pertussis toxin (PTX). The possible role of a GTP-dependent pertussis toxin-sensitive G protein in the inhibitions observed was studied in CTAL preincubated with 500 ng/ml PTX. When compared with the corresponding responses obtained with 10 nM AVP alone, PGE\(_2\)-induced inhibition was of 63.4 ± 2.3% (p < 0.001) in control CTAL and was decreased to 28.4 ± 3.4% (p < 0.05 when compared with AVP) in PTX-treated CTAL (Fig. 5). A preincubation with PTX therefore suppressed by more than 50% the inhibition elicited by PGE\(_2\), a result identical to that observed in the medullary portion of this segment and due very likely to the interaction of the PGE\(_2\) receptor with Go, proteins (20). In contrast, angiotensin II- and 2.5 mM [Ca\(^{2+}\)]\(_j\)-mediated inhibition measured in the same experiments were not modified by PTX (Fig. 5).

Experiments of Multiple Combined Inhibition—The results presented above indicate that only PGE\(_2\)-mediated inhibition was sensitive to pertussis toxin (Fig. 5), whereas both PGE\(_2\) and 2.5 mM [Ca\(^{2+}\)]\(_j\)-inhibited AVP-dependent cAMP synthesis with the same efficiency (Fig. 4A). These results suggest that
Table IV
Cumulative inhibition of AVP-stimulated cAMP production by 2.5 mM [Ca\(^{2+}\)] \(_i\) and PGE\(_2\)

| Experimental conditions | cAMP content | % inhibition of the response to AVP |
|------------------------|--------------|-----------------------------------|
| 10 nM AVP              | 101.2 ± 19.1 |                                    |
| AVP + 0.3 mM PGE\(_2\) | 49.8 ± 12.1  | 52.6 ± 6.9                         |
| AVP + 2.5 mM [Ca\(^{2+}\)] \(_i\) | 25.8 ± 6.4  | 74.9 ± 2.8                         |
| AVP + PGE\(_2\) + [Ca\(^{2+}\)] \(_i\) | 11.5 ± 3.3   | 89.0 ± 1.8                         |

*Statistically different from the cAMP value obtained with either PGE\(_2\) or 2.5 mM [Ca\(^{2+}\)] \(_i\) added alone (one-way analysis of variance on weighted means followed by LSD Fisher’s t test).

Role of Intracellular Ca\(^{2+}\) Release and/or Capacitive Ca\(^{2+}\) Influx to Inhibit cAMP Synthesis

The results presented above establish that the decrease of AVP-dependent cAMP accumulation induced by [Ca\(^{2+}\)] \(_i\) or angiotensin II results partly from an inhibition of cAMP synthesis. [Ca\(^{2+}\)] \(_i\) induced both cytosolic Ca\(^{2+}\) release from intracellular stores and extracellular Ca\(^{2+}\) influx (Ref. 11, Table I). Different approaches were used in the presence of IBMX to assess the relative importance of these respective [Ca\(^{2+}\)] \(_i\) variations in the inhibition of cAMP synthesis.

The potential inhibitory effect of the capacitive Ca\(^{2+}\) influx was investigated by the use of thapsigargin. This inhibitor of the endoplasmic reticular Ca\(^{2+}\)-ATPase (31) depletes intracellular Ca\(^{2+}\) stores which was shown to produce capacitive Ca\(^{2+}\) entry (32). Thapsigargin added into the Ca\(^{2+}\)-free medium evoked a transient [Ca\(^{2+}\)] \(_i\), which declined slowly to basal value reflecting Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) extrusion from the CTAL cell (Fig. 6A, lower part). On thapsigargin-treated CTAL, the addition of 200 mM neomycin did not evoke further [Ca\(^{2+}\)] \(_i\), increase indicating that 1 mM thapsigargin had actually emptied intracellular Ca\(^{2+}\) stores sensitive to Ca\(^{2+}\)-sensing receptor in the CTAL (data not shown). By contrast, the addition of 1.25 mM [Ca\(^{2+}\)] \(_i\), elicited high [Ca\(^{2+}\)] \(_i\) increases (Fig. 6A, lower part) which reflect a capacitive Ca\(^{2+}\) influx associated to the large emptying of intracellular Ca\(^{2+}\) pools induced by thapsigargin. Without thapsigargin, the addition of 1.25 mM [Ca\(^{2+}\)] \(_i\), to Ca\(^{2+}\)-free medium induced only a small and progressive elevation of [Ca\(^{2+}\)] \(_i\) (Fig. 6A, upper part). This discrete increase likely reflects a slow Ca\(^{2+}\) entry consequent to partial depletion of intracellular Ca\(^{2+}\) stores induced by superfusion in the Ca\(^{2+}\)-free medium.

The consequences on cAMP synthesis of the changes in [Ca\(^{2+}\)] \(_i\), elicited by the addition of 1.25 mM [Ca\(^{2+}\)] \(_i\), were studied in parallel experiments on CTAL preincubated in the Ca\(^{2+}\)-free medium with or without thapsigargin. AVP-stimulated cAMP synthesis was of the same magnitude in both cases (139.6 ± 12.9 fmol·mm\(^{-1}\)·4 min\(^{-1}\) and 157.9 ± 13.5, n = 4, without and with thapsigargin, respectively), which indicates that thapsigargin-induced intracellular Ca\(^{2+}\) release during the preincubation step had no inhibitory effect on cAMP synthesized during the following incubation step. AVP-dependent cAMP synthesis was significantly inhibited by 1.25 mM [Ca\(^{2+}\)] \(_i\), in CTAL treated with thapsigargin (mean value of 24.1 ± 1.9% of inhibition, p < 0.05, n = 4), whereas there was no inhibition in the absence of thapsigargin (Fig. 6B). In three of these experiments, the addition of 2.5 mM [Ca\(^{2+}\)] \(_i\) on CTAL treated with thapsigargin inhibited cAMP synthesis by 69.1 ± 2.0%, a value similar to that observed in control CTAL (see Figs. 2 and 4).

The potential inhibition of cAMP synthesis induced only by Ca\(^{2+}\) release from intracellular pools was studied in the Ca\(^{2+}\)-free medium with the Ca\(^{2+}\)-sensing receptor agonist neomycin to avoid Ca\(^{2+}\) influx associated with [Ca\(^{2+}\)] \(_i\). Neomycin was added after 10 min superfusion or preincubation of CTAL samples in either the 0.5 mM [Ca\(^{2+}\)] \(_i\), control medium or the Ca\(^{2+}\)-free medium. In control medium, neomycin induced [Ca\(^{2+}\)] \(_i\) responses similar to those observed with [Ca\(^{2+}\)] \(_i\), but with a low plateau phase (Fig. 7A compared with Fig. 3). The mean [Ca\(^{2+}\)] \(_i\) peak values were dose-dependent (Fig. 7D), an observation in agreement with the effect of neomycin on IP production; the mean stimulation factor of phospholipase C activity was 2.1 ± 0.3 (n = 4 experiments), 4.7 ± 0.3 (n = 3), and 10.0 ± 2.0 (n = 3) with 100, 200, and 400 μM neomycin, respectively. In Ca\(^{2+}\)-free medium, neomycin-induced [Ca\(^{2+}\)] \(_i\) peak values were decreased at all the concentrations tested (Fig. 7B), and the
plateau phase was not observed (Fig. 7A).

Fig. 7C shows the consequence on AVP-dependent cAMP synthesis of neomycin-evoked intracellular Ca$^{2+}$ release. In the Ca$^{2+}$-free medium, 100 μM neomycin did not impair cAMP synthesis in contrast to 200 and 400 μM. The mean data (Fig. 7C) indicate that the levels of inhibition obtained in the Ca$^{2+}$-free medium (37.7 ± 3.4% of inhibition and 67.3 ± 4.7% with 200 and 400 μM neomycin, respectively) were decreased when compared with the corresponding values obtained in the 0.5 mM [Ca$^{2+}$], (64.9 ± 3.4% of inhibition and 79.5 ± 1.9% with 200 and 400 μM, respectively).

The lower inhibitions of cAMP obtained in the Ca$^{2+}$-free medium could be due to the absence of Ca$^{2+}$ influx and/or to the decrease of intracellular Ca$^{2+}$ release (Fig. 7). In the absence of extracellular Ca$^{2+}$, this last effect may be secondary to partial emptying of intracellular Ca$^{2+}$ stores or to an alteration of the binding of neomycin to the Ca$^{2+}$ receptor. Additional experiments were performed in CTAL superfused or preincubated in a Ca$^{2+}$-free medium for only 2 min to prevent the emptying of intracellular Ca$^{2+}$ stores. In such conditions, 200 μM neomycin evoked intracellular [Ca$^{2+}$], peaks of magnitude (418 ± 52 nM, Fig. 8) similar to that observed in 0.5 mM [Ca$^{2+}$], medium (436 ± 47 nM, Fig. 7B). With a same experimental timing, the inhibition of cAMP production was 59.9 ± 3.7% (Fig. 8), a value close to that obtained in control medium (64.9 ± 3.4%, Fig. 7C). The similarity of the responses observed in Ca$^{2+}$-free medium and control medium indicates that the binding of neomycin is not affected by the absence of extracellular Ca$^{2+}$. Moreover, these results establish that neomycin-evoked intracellular Ca$^{2+}$ release alone can induce an inhibition of cAMP synthesis comparable to that obtained in 0.5 mM [Ca$^{2+}$], medium, a condition in which neomycin induces only a small Ca$^{2+}$ entry (Fig. 7A). By comparison, angiotensin II elicited high [Ca$^{2+}$], peak values in CTAL superfused for 2 min in the Ca$^{2+}$-free medium (603 ± 104 nM, Fig. 8), and its inhibitory effect on cAMP synthesis was in the range of that obtained with angiotensin II in the 0.5 mM [Ca$^{2+}$], medium (Fig. 4) with a mean value of only 14.4 ± 2.8% (Fig. 8). Altogether these results therefore support the hypothesis of a specific role played by the activation of the Ca$^{2+}$ receptor in the inhibition of AVP-dependent cAMP synthesis.

**DISCUSSION**

This study demonstrates that in a same epithelial cell of the rat renal tubule PGE$_2$, angiotensin II and the activation of the Ca$^{2+}$ receptor inhibit the same pool of AVP-dependent intracellular cAMP content by different mechanisms, i.e. G$_{i}$-mediated or Ca$^{2+}$-mediated inhibition of AC activity and PDE-mediated hydrolysis of cAMP. In particular, the strong and specific inhibition of hormone-dependent cAMP synthesis by [Ca$^{2+}$], in the CTAL likely results from the co-expression of the
Ca\(^{2+}\) receptor RaKCaR (8, 9, 11) and of the Ca\(^{2+}\)-inhibitable type 6 AC (Ref. 14 and this study).

**Transduction Pathways Involved in PGE\(_2\), Angiotensin II, and [Ca\(^{2+}\)]\(_i\)-mediated Inhibition of cAMP Content—**Previous studies have established that PGE\(_2\) decreases AVP-mediated CAMP synthesis in the rat thick ascending limb (19, 20). The preincubation of isolated segments with PTX reverses, at least partly, this inhibition in medullary (20) and cortical (Fig. 5) segments. The PGE\(_2\) receptor is therefore coupled to G\(_i\) proteins in the thick ascending limb, in agreement with the presence of the PGE\(_2\) receptor EP\(_2\) subtype (33) usually associated with the inhibition of AC activity (34). Multiple isoforms of the EP\(_2\) subtype have been described, some of which coupled to both G\(_i\)-mediated inhibition of AC activity and activation of phospholipase C (35). In our experiments, PGE\(_2\) did not increase IP production or [Ca\(^{2+}\)]\(_i\) (Table I). In addition, the magnitude of PGE\(_2\)-mediated inhibition did not depend on the type of phosphodiesterase inhibitor added to the incubation medium. Thus, very likely, the coupling of PGE\(_2\) receptor to G\(_i\)-mediated inhibition of AC activity alone accounts for the effect of PGE\(_2\) observed on CAMP synthesis in the rat CTAL.

Angiotensin II and [Ca\(^{2+}\)]\(_i\), stimulate phospholipase C activity (Table I and Fig. 3), in agreement with the expected signaling pathway. Indeed, type 1 angiotensin II receptor mRNA is present in the rat CTAL (18, 36) and, when expressed in different cell types, it activates phospholipase C through the PTX-insensitive G\(_i\) protein (37, 38). [Ca\(^{2+}\)]\(_i\) increases the production of IPs in parathyroid cells (3, 4). This production is insensitive to PTX (3) and appears to be the direct transduction pathway in parathyroid and human parathyroid Ca\(^{2+}\)-receptor-transfected cells (4). A similar transduction pathway has been observed in oocytes expressing the rat renal Ca\(^{2+}\)-sensing receptor or the bovine parathyroid Ca\(^{2+}\)-sensing receptor (1, 2), but, in these experiments, the IP production was impaired by PTX (1). However, as observed with other receptors expressed in oocytes (39), this sensitivity to PTX may be due to the coupling of the Ca\(^{2+}\) receptor to G proteins other than those expressed in native cells. In CTAL, [Ca\(^{2+}\)]\(_i\) and angiotensin II decrease AVP-dependent CAMP accumulation by a process insensitive to pertussis toxin (Fig. 5) and independent from activation of phospholipase A\(_2\), cytochrome P-450 monooxygenase, or protein kinase C (Table III). These results suggest that the inhibition of CAMP accumulation that we observed is a direct consequence of the activation of phospholipase C through a PTX-insensitive G protein. In contrast to our results, PTX reverses the [Ca\(^{2+}\)]\(_i\)-mediated inhibition of hormone-dependent CAMP synthesis in parathyroid cells (5) or mouse medullary thick ascending limbs (40). Several observations may help understanding these different results. First, as underlined by Brown (6), regulation of hormone-dependent CAMP synthesis in parathyroid cells has pharmacological properties that are different from those observed on IP production, thus suggesting the presence of different isoforms of Ca\(^{2+}\) receptor coupled to different G proteins. Second, in some cell types, including bovine parathyroid cells (4), [Ca\(^{2+}\)]\(_i\) increases result in an additional coupling to another pathway such as phospholipase A\(_2\)-induced arachidonic acid formation which in turn may inhibit AC activity through a PTX-sensitive process as was observed in the rat thick ascending limb (20). Finally, the cell present in the cortical portion of the thick ascending limb is morphologically different from the one described in the medullary portion (17) and exhibits specific physiological functions (10); thus, different cellular properties and/or species differences may explain the discrepant result observed with PTX in the mouse medullary thick ascending limb (40) and the rat cortical thick ascending limb (Fig. 5).

Taken together, our results lead to the conclusion that the negative regulation of the AVP-dependent CAMP accumulation in the rat CTAL involves two different transduction pathways, a PTX-sensitive G\(_i\)-mediated inhibition of AC activity induced by PGE\(_2\) and a PTX-insensitive phospholipase C activation induced by angiotensin II and the activation of the Ca\(^{2+}\) receptor. In addition, results obtained in experiments of multiple combined inhibition demonstrate that the three agents studied are active in a same cell and that PGE\(_2\) and [Ca\(^{2+}\)]\(_i\), decrease the same pool of AC catalytic units. These results are in agreement with the observations that only one pool of hormone-stimulable catalytic units is present in the CTAL (15, 16) and that a same intracellular pool of Ca\(^{2+}\) is released by angiotensin II and extracellular Ca\(^{2+}\) (11). Since type 6 AC mRNA is observed in all cells along CTAL (Fig. 1), the functional results obtained suggest that these regulations are effective in a cell which expresses the type 6 AC mRNA.

**Mechanisms of Inhibition Induced by Angiotensin II and [Ca\(^{2+}\)]\(_i\) on AVP-dependent CAMP Accumulation—**Different mechanisms account for the inhibition of AVP-dependent CAMP accumulation induced by [Ca\(^{2+}\)]\(_i\), depending on the Ca\(^{2+}\) concentration studied. Low [Ca\(^{2+}\)]\(_i\), below 1.5 mM, and angiotensin II used at maximal concentration elicit a modest inhibition of CAMP synthesis (Figs. 2 and 4). These inhibitions are in the range of those previously observed with agonist-evoked phospholipase C activation in cell lines that express type 6 AC (41, 42). Angiotensin II and low [Ca\(^{2+}\)]\(_i\), also induce a hydrolysis of CAMP likely mediated by Ca\(^{2+}\)/calmodulin-dependent PDEs. The data from Fig. 2 suggest that regulation of CAMP hydrolysis has a higher sensitivity to Ca\(^{2+}\) than regulation of CAMP synthesis, and thus the hydrolysis of CAMP appears to be the major process that accounts for the decrease of AVP-dependent CAMP accumulation (Figs. 2 and 4 and Table II). Therefore, the small inhibitory effect on CAMP synthesis induced by 1.25 mM [Ca\(^{2+}\)]\(_i\), or angiotensin II is amplified by the CAMP hydrolysis leading to a total inhibition of AVP-dependent CAMP accumulation of about 50% (Figs. 2 and 4 and Table II).

From about 1.5 mM, [Ca\(^{2+}\)]\(_i\), induces a higher inhibition of AVP-dependent CAMP accumulation due to an additional decrease of CAMP synthesis, an effect becoming dominant with high [Ca\(^{2+}\)]\(_i\), (Fig. 2). Previous experiments in the mouse medullary thick ascending limb have established that the inhibitory effect of 5.0 mM [Ca\(^{2+}\)]\(_i\), on hormone-dependent CAMP synthesis is not due to metabolic or cellular damages (43). These observations support the hypothesis that, even at high concentrations, the effect of [Ca\(^{2+}\)]\(_i\), is actually a regulating process.

Although both angiotensin II and [Ca\(^{2+}\)]\(_i\), stimulate phospholipase C pathway in the CTAL, the relative effects of these agents on CAMP synthesis suggest that [Ca\(^{2+}\)]\(_i\), elicits an additional and specific mechanism of action. As will be discussed in the following sections, this specific action of [Ca\(^{2+}\)]\(_i\), might be linked to the co-expression of a functional Ca\(^{2+}\)-inhibitable AC and of a Ca\(^{2+}\)-sensing receptor in the CTAL cell.

**Functional Characteristics of the Adenylyl Cyclase Expressed in the Cortical Thick Ascending Limb—**Among the different isoforms of adenylyl cyclase known up to date, types 4–6 sequences have been cloned from rat tissues and are expressed in the kidney (21). The type 9 AC cloned from mouse cell lines or tissues (44, 45) appears also expressed in the rat kidney (46). Experiments of quantitative RT-PCR have demonstrated the expression of the Ca\(^{2+}\)-inhibitable type 6 AC mRNA in the CTAL but not types 4 or 5 AC mRNA (14). Our in situ hybridization results establish that type 6 AC mRNA was expressed in all cells along CTAL (Fig. 1), whereas type 5 AC mRNA was not detected. If present in epithelial cells, the localization of
Several properties of regulation of AVP-dependent cAMP accumulation were observed in this study as follows. (i) [Ca\textsuperscript{2+}]	extsubscript{i} has an inhibitory effect on either hormone- or forskolin-stimulated cAMP production. (ii) cAMP synthesis is inhibited by agents coupled either to an activation of phospholipase C or to a Gq, protein-mediated process. (iii) These two pathways induce a cumulative inhibition of cAMP synthesis and therefore are effective by independent and different processes on the same pool of catalytic units. (iv) Experimental conditions inducing capacitive Ca\textsuperscript{2+} entry elicit an inhibition of cAMP synthesis with [Ca\textsuperscript{2+}]\textsubscript{i} as low as 1.25 mm. (v) There is no evidence of regulation by protein kinase C of either hormone-dependent cAMP synthesis or angiotensin II- and [Ca\textsuperscript{2+}]\textsubscript{i}-mediated inhibitions. (vi) Finally, the inhibition of cAMP accumulation observed with angiotensin II and low concentrations of [Ca\textsuperscript{2+}]\textsubscript{i} results from both a modest inhibition of cAMP synthesis and an increase of cAMP hydrolysis.

All these functional properties are in close agreement with those described in different cell lines that express type 6 AC (22), in particular the inhibitory effect of a capacitive Ca\textsuperscript{2+} entry on cAMP synthesis (42), a [Ca\textsuperscript{2+}]\textsubscript{i}-mediated inhibition of cAMP synthesis that is cumulative to a Gq-mediated pathway (47), and a combined effect of Ca\textsuperscript{2+} on both AC inhibition and PDE activation in the regulation of cAMP content (41, 42). The type 9 AC is not decreased directly by Ca\textsuperscript{2+} (45), but its activity is inhibited by [Ca\textsuperscript{2+}], increases through the activation of the Ca\textsuperscript{2+}/calmodulin-regulated protein phosphatase calcineurin (44). This isoform therefore may be involved in the regulations observed. However, the following two observations suggest that this isoform is unlikely expressed in the rat CTAL. (i) In the AtT20 cell line, from which type 9 AC has been cloned and in which it represents the majority (>90%) of the adenyl cyclase-related sequences (44), the blockade of calcineurin activity regulates hormone- but not forskolin-stimulated cAMP synthesis (44, 48); by contrast, Ca\textsuperscript{2+}-induced inhibition is observed on both responses in the CTAL (see "Results") and in cell lines that express type 6 AC (47, 49). (ii) In the AtT20 cell line, the activation of protein kinase C inhibits the synthesis of cAMP (50); by contrast, there is no evidence of interaction with protein kinase C in the rat CTAL (Table III) in agreement with the properties observed with type 6 AC (22).

The different results obtained and their comparison with the data from the literature allow us to conclude that type 6 AC is functionally expressed in the CTAL cell. This Ca\textsuperscript{2+}-inhibitable isofrom therefore is the best candidate to account for the inhibitory effect of [Ca\textsuperscript{2+}]\textsubscript{i} observed in this study.

Specific Inhibition of cAMP Synthesis by Extracellular Ca\textsuperscript{2+}—As already underlined, both [Ca\textsuperscript{2+}]\textsubscript{i} and angiotensin II activate the phospholipase C pathway in the same cell but have different consequences on the parameters studied. Indeed, for a comparable magnitude of inhibition of cAMP synthesis (15–20%, Fig. 2 and 4), a high [Ca\textsuperscript{2+}]\textsubscript{i} peak was observed with angiotensin II, whereas no detectable [Ca\textsuperscript{2+}]-variations were obtained with 1.25 mm [Ca\textsuperscript{2+}]\textsubscript{i} in our experimental conditions (Table I). Conversely, for comparable [Ca\textsuperscript{2+}]\textsubscript{i} peak values, activation of the Ca\textsuperscript{2+} receptor, but not angiotensin II, induced about 60% of inhibition of cAMP synthesis (Fig. 8). We also observed in the rat CTAL that a maximal concentration of bradykinin has properties similar to those of angiotensin II on [Ca\textsuperscript{2+}], variations and inhibition of cAMP accumulation (data not shown). These results support the hypothesis of a specific role of Ca\textsuperscript{2+} receptor activation in the inhibition of cAMP synthesis.

By contrast to angiotensin II, [Ca\textsuperscript{2+}]\textsubscript{i} and the Ca\textsuperscript{2+} receptor agonist, neomycin, elicit a sustained increase of [Ca\textsuperscript{2+}], which reflects an entry of Ca\textsuperscript{2+} (Ref. 11 and Figs. 3 and 7). An activation of cation channels by neomycin has been observed in Ca\textsuperscript{2+} receptor transfected cells (51), and such an effect in CTAL cell could account, at least in part, for this Ca\textsuperscript{2+} influx. The entry of Ca\textsuperscript{2+} might partly explain the properties of [Ca\textsuperscript{2+}]\textsubscript{i} in the inhibition of cAMP synthesis since (i) previous experiments have demonstrated that capacitive Ca\textsuperscript{2+} influx inhibits type 6 AC activity in a gliona cell line (42), and (ii) in the present study, a high inhibition of cAMP synthesis can be obtained in thapsigargin-treated CTAL. However, the experiments with neomycin in Ca\textsuperscript{2+}-free medium (Figs. 7 and 8) establish that a release of Ca\textsuperscript{2+} from intracellular stores can also induce a high inhibition of cAMP synthesis. Therefore, the presence of a Ca\textsuperscript{2+} influx, on its own, does not totally explain the specific inhibition of cAMP synthesis induced by the activation of the Ca\textsuperscript{2+} receptor.

The differential inhibitory effect observed with angiotensin II and [Ca\textsuperscript{2+}]\textsubscript{i} might be linked to the activation of another transduction pathway by one of these agents, and we have no formal argument to support or exclude such an hypothesis. The specific properties observed with the activation of the Ca\textsuperscript{2+} receptor recalled above lead, however, to another hypothesis supported by observations made in other polarized epithelial cells (52, 53). Indeed in the renal line of Madin-Darby canine kidney cells, inositol 1,4,5-trisphosphate receptors have multiple cellular localization including sites close to the basolateral plasma membrane and can thus generate localized [Ca\textsuperscript{2+}]\textsubscript{i} increases (52). In nasal epithelial cell preparations, phospholipase C activation results in [Ca\textsuperscript{2+}]\textsubscript{i}, release and Ca\textsuperscript{2+} influx in close proximity to the stimulated receptor (53). These data might explain the high efficiency of cAMP synthesis inhibition by [Ca\textsuperscript{2+}], if the Ca\textsuperscript{2+} receptor was more closely associated to type 6 AC than the angiotensin II receptor in the CTAL plasma membrane.

An intimate co-localization in a subdomain of the basal membrane of the Ca\textsuperscript{2+} receptor and of the Ca\textsuperscript{2+}-inhibitable AC might also explain the similar inhibition of cAMP synthesis observed with either a local [Ca\textsuperscript{2+}], increase (Ca\textsuperscript{2+} influx and/or Ca\textsuperscript{2+} release) elicited by low [Ca\textsuperscript{2+}], or a high intracellular Ca\textsuperscript{2+} release induced by angiotensin II. In addition, the cAMP hydrolysis observed with low [Ca\textsuperscript{2+}], or angiotensin II was similar (Table II), suggesting that a local [Ca\textsuperscript{2+}], increase must be effective to activate Ca\textsuperscript{2+}/calmodulin-dependent PDEs. These hypotheses cannot be tested on isolated tubule samples at the present time.

Whatever the precise mechanism involved, the co-expression of the Ca\textsuperscript{2+}-inhibitable type 6 AC and of the Ca\textsuperscript{2+}-sensing receptor RaKCaR in the plasma membrane very likely allows the specific inhibition of AC activity observed with [Ca\textsuperscript{2+}]. This conclusion is supported by previous results obtained in the vasopressin-sensitive cell of the rat outer medullary collecting tubule (14). In this cell type, different data support the functional expression of the type 6 AC, but [Ca\textsuperscript{2+}], increases do not inhibit cAMP synthesis (14). In contrast to the CTAL, there is no evidence for the presence of the Ca\textsuperscript{2+} receptor RaKCaR in the plasma membrane of the medullary collecting tubule (8, 9). The co-expression (CTAL) or the absence of co-expression (vasopressin-sensitive cell of the outer medullary collecting tubule) of the type 6 AC and of the Ca\textsuperscript{2+} receptor RaKCaR may explain, therefore, the different effect of [Ca\textsuperscript{2+}], on AC activity observed in these segments.

From a physiological point of view, the inhibition of AC activity in the CTAL cell, together with an effect on cAMP hydrolysis, confers a high sensitivity to [Ca\textsuperscript{2+}], on the inhibition of hormone-stimulated cAMP accumulation. The steep slope (Fig. 2) obtained shows that a large variation of cAMP
content is elicited by a small change in [Ca\(^{2+}\)]. It is noticeable that the same slope has been observed in parathyroid cells, and 1.0–1.2 mM [Ca\(^{2+}\)]\(_{i}\) defined as the “set point” of parathyroid hormone secretion, i.e., the concentration at which hormonal secretion is inhibited by 50% (6). Similarly in the kidney, the present data show that inhibition of hormone-stimulated cAMP accumulation is exquisitely sensitive to variations in [Ca\(^{2+}\)]\(_{i}\), that are physiologically relevant thus allowing the modulation of cAMP-mediated luminal Ca\(^{2+}\) reabsorption usually present in the CTAL (10). At higher [Ca\(^{2+}\)]\(_{i}\), like in hypercalcemia (6, 7), the strong inhibition of cAMP synthesis may be a potent factor to decrease sharply hormone-dependent cAMP content and thus to block Ca\(^{2+}\) reabsorption. Our functional data support that the co-expression of a Ca\(^{2+}\)-inhibitable AC and of a Ca\(^{2+}\)-sensing receptor in the plasma membrane of the CTAL cell contributes to the regulation of one of its main physiological functions.

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