Angiotensin II potentiates adrenocorticotrophic hormone-induced cAMP formation in bovine adrenal glomerulosa cells through a capacitative calcium influx

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Angiotensin II (AngII) plays a crucial role in the control of aldosterone biosynthesis in adrenal glomerulosa cells through the stimulation of two distinct Ca^{2+} entry pathways: (1) opening of voltage-operated calcium channels, and (2) activation of a capacitative Ca^{2+} entry that is dependent on calcium release from intracellular pools. Adrenocorticotrophic hormone (ACTH), on the other hand, a major hormonal regulator of steroidogenesis, induces an increase in intracellular cAMP through the activation of a G-protein-coupled adenylyl cyclase. Recent studies have demonstrated that the rise in cAMP induced by ACTH can be potentiated by AngII in bovine glomerulosa cells. The aim of the present study was to investigate the mechanism of AngII action on ACTH-induced cAMP production. In primary cultures of bovine glomerulosa cells, we found that AngII (100 nM), which had no effect by itself on cAMP production, significantly potentiated maximal ACTH-induced cAMP formation in the presence of extracellular calcium (1.2 mM). In contrast, in the absence of extracellular calcium, AngII did not affect ACTH-induced cAMP production. These results suggest that calcium entry into the cell plays an important role in the activation of the cyclase by AngII. The inhibition of voltage-operated calcium channels by nicardipine, a dihydropyridine calcium antagonist blocking both low-threshold (T-type) and high-threshold (L-type) Ca^{2+} channels, did not significantly affect the potentiating effect of AngII. Moreover, the cAMP response to ACTH was insensitive to activation of these Ca^{2+} channels induced by potassium ions and, even when cytosolic free-calcium concentration ($[Ca^{2+}]_c$) was kept elevated with the Ca^{2+} ionophore, ionomycin, no stimulation of adenylyl cyclase was observed at concentrations of $[Ca^{2+}]_c$ up to 640 nM. In contrast, thapsigargin, an activator of capacitative Ca^{2+} influx, mimicked the potentiating effect of AngII on ACTH-induced cAMP formation. In agreement with the characteristics of cAMP modulation by Ca^{2+} in these cells, the presence of type III adenylyl cyclase was observed by immunodetection in bovine glomerulosa cell membranes. In conclusion, these data suggest a tight coupling between the capacitative Ca^{2+} influx induced upon stimulation by either AngII or thapsigargin and a calcium-sensitive isoform of adenylyl cyclase, probably type III, in bovine glomerulosa cells.

INTRODUCTION

The importance of extracellular calcium in the steroidogenic action of potassium ions (K^+) and angiotensin II (AngII) in adrenal glomerulosa cells has been well established [1–4] and direct activation of aldosterone synthesis by cytosolic calcium has been demonstrated [5,6]. On one hand, potassium ions activate exclusively the voltage-operated calcium (VOC) channels by depolarizing the cell membrane. Indeed, the presence of both low threshold (T-type) and high threshold (L-type) calcium channels has been demonstrated in bovine glomerulosa cells [7,8].

On the other hand, AngII binding to its receptor is linked to the formation of diacylglycerol, an activator of protein kinase C (PKC) [9] and Ins(1,4,5)P_3, which is responsible for calcium release from intracellular pools [10,11]. Stimulation with AngII induces a biphasic response of cytosolic free calcium concentration ($[Ca^{2+}]_c$), the first phase being due to calcium release from intracellular pools, the second phase reflecting a sustained calcium entry. This second phase is principally attributable to the activation of a capacitative influx resulting from the depletion of intracellular calcium stores [12,13], but is also partially due to cell depolarization leading to the opening of VOC channels.

Recent studies have demonstrated a positive modulatory action of AngII on cAMP formation induced by adrenocorticotrophic hormone (ACTH), another hormonal regulator of aldosterone production. Indeed, AngII has been found to increase ACTH-induced cAMP production in bovine adrenocortical cells [14]. This result, however, is in contrast with the well-documented G_i-mediated inhibitory effect of AngII on adenylyl cyclase in rat glomerulosa cells [15,16].

In the present study, we have investigated the mechanism(s) of the potentiation by AngII of ACTH-induced cAMP formation. We show that AngII action necessitates calcium entry into the bovine glomerulosa cell and that this influx is insensitive to blockade of both T- and L-type VOC channels by nicardipine (Nic). In addition, activation of a capacitative influx of Ca^{2+} by thapsigargin (Tg), a blocker of microsomal Ca^{2+} pumps [17], induced an effect similar to that elicited by AngII on the ACTH-induced cAMP formation. These results strongly support the

Abbreviations used: AngII, angiotensin II; ACTH, adrenocorticotrophic hormone; $[Ca^{2+}]_c$, cytosolic free-calcium concentration; $[Ca^{2+}]_{ext}$, total extracellular calcium concentration; VOC, voltage-operated calcium (channels); IBMX, 3-isobutyl-1-methyl-xanthine; DMEM, Dulbecco's modified Eagle's medium; Nic, nicardipine; Tg, thapsigargin; Nif, nifedipine; BAG, bovine adrenal glomerulosa; PKC, protein kinase C; CRAC, calcium release-activated calcium (channel).

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hypothesis that the capacitative Ca^{2+} influx activated by AngII has a modulatory effect on bovine adrenal glomerulosa (BAG) cell adenylyl cyclases.

MATERIALS AND METHODS

Materials

Nic, 3-isobutyl-1-methyl-xanthine (IBMX), ACTH, Hepes, insulin, transferrin, sodium selenite, dithiothreitol, PMSF, aprotinin, Triton X-100 and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Tg was obtained from Anawa (Zurich, Switzerland), ionomycin from Calbiochem (Lucerne, Switzerland) and [Ile^5]AngII from Bachem AG (Bubendorf, Switzerland). Dispase (grade II) was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and Percoll from Pharmacia (Piscataway, NJ, U.S.A.). Horse serum, fetal calf serum, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, U.S.A.), glycerol and Bromophenol Blue from Fluka, and Tween-20 from Merck. Metyrapone was purchased from Aldrich (Milwaukee, WI, U.S.A.) and fura-2 was from Molecular Probes (Eugene, OR, U.S.A.). Rabbit polyclonal antibodies selectively raised against rat adenylyl cyclase types I–VI were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Isolation and culture of BAG cells

BAG cells were prepared by enzymic dispersion with dispase and purified on a Percoll density gradient, as described previously [18]. Cells were transferred to DMEM containing antibiotics [18], supplemented with 2 μg/ml insulin, 2 μg/ml transferrin, 2 ng/ml sodium selenite, 5 μM metyrapone, 2 mM glutamine, 6 units/ml nystatin, 2 % (v/v) fetal calf serum and 10 % (v/v) horse serum. The cells were plated on 24-well tissue culture plates (10^6 cells/well) and incubated overnight at 37 °C in 5 % CO_2. The next day, the medium was removed and replaced with serum-free DMEM.

Determination of cAMP secretion

Cells were cultured for 3 days, as described above, before being stimulated in multi-well plates. The serum-free DMEM was replaced with Krebs–Ringer buffer (containing 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.2 mM $CaCl_2$, and 5.5 mM D-glucose and buffered to pH 7.4 with 20 mM Hepes) and cells were preincubated at 37 °C for at least 1 h. Cells were then incubated for 30 min at 37 °C in the same medium containing IBMX (0.2 mM), an inhibitor of phosphodiesterases, and various agents (indicated in the Figure and Table legends). The presence of IBMX only slightly affected basal cAMP production, which amounted to 13.5 ± 9.6 and 10.8 ± 7.7 pmol/μg of protein ($n = 3$) with and without the phosphodiesterase inhibitor respectively.

In the experiments with clamped cytosolic calcium (see Figure 2), cells were preincubated for 10 min at 37 °C in Ca^{2+}-free (EGTA 0.2 mM) Krebs–Ringer buffer containing 2 μM ionomycin. The medium was then replaced with the same buffer (containing 2 μM ionomycin) and supplemented with 0.2 mM IBMX and increasing concentrations of Ca^{2+}; at this time, cells were incubated for 30 min in the presence or absence of ACTH (100 nM or 10 μM). Similar results were obtained at each ACTH concentration.

At the end of the incubation period, the media were collected and the cAMP content was determined using a commercially available kit (PerSeptive Diagnostics Inc., Framingham, MA, U.S.A.). Cellular protein was measured in each well using the Coomassie Blue method of Bradford [19].

Patch-clamp measurements

The effect of Nic on the activity of VOC channels was assessed under voltage clamp in the whole-cell configuration of the patch-clamp technique, essentially as previously described [20].

Measurement of $[Ca^{2+}]_c$

The determinations of $[Ca^{2+}]_c$ in glomerulosa cell populations were performed with the fluorescent probe fura-2. Freshly prepared cells, purified on Percoll density gradients, were washed twice and resuspended in a Krebs–Ringer buffer; cells were then incubated at 37 °C for 60 min before being washed, resuspended at a concentration of 10^7 cells/ml and incubated again for 45 min in the presence of 2 μM fura-2 acetoxymethyl ester. The excess dye was then washed away, and the cells were kept at ambient temperature in the same medium. Batches of 2×10^6 cells were sedimented just before use and resuspended in 2 ml of Krebs–Ringer buffer in a thermostated cuvette at 37 °C. For cytosolic calcium-clamp experiments (Figure 2), cells were resuspended in Ca^{2+}-free Krebs–Ringer buffer supplemented with EGTA (0.2 mM) in order to obtain very low $[Ca^{2+}]_c$ at the beginning of the experiment.

Fura-2 fluorescence (excitation at 340/380 nm and emission at 505 nm) was recorded with a Jasco CAF-110 fluorescence spectrometer (Hachioji City, Japan) and $[Ca^{2+}]_c$ was calibrated as previously described [21,22], using a K_d value of 224 nM for fura-2.

Extraction and SDS/PAGE of membrane proteins

BAG cells in primary culture, as well as Wistar rat cerebellum, heart and liver tissues, were homogenized with a glass Potter homogenizer, in a medium containing 5 mM Tris/HCl (pH 7.4), 2 mM EDTA, 10 mM EGTA, 0.25 mM sucrose, 10 mM dithiothreitol, 1 μg/ml leupeptin, 1 mM PMSF and 1 μg/ml aprotinin, at 4 °C. The homogenates were then centrifuged at 100000 ***g*** for 1 h (at 4 °C) to remove soluble (cytosolic) proteins and the pellets were incubated for 30 min at 4 °C in the same buffer supplemented with 1 % Triton X-100. The extracts were subjected to a second centrifugation (100000 ***g***, 1 h) and solubilized membrane proteins contained in the supernatants were quantified using a protein microassay (Bio-Rad, Munich, Germany).

SDS/PAGE was performed according to the method of Laemmli [23]. Extracts of membrane proteins (30 μg/lane) were solubilized in sample buffer [60 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol and 0.01 % (v/v) Bromophenol Blue] and loaded on to an SDS/8 %-polyacrylamide minigel (Mini-Protean II system, Bio-Rad). Electrophoresis was performed at 150 V for 1 h.

Blotting and immunodetection of adenylyl cyclase isoforms

SDS/PAGE-resolved proteins were electrophoretically transferred on to a nitrocellulose membrane (Schleicher & Schuell, Riehen, Switzerland). After transfer, the membrane was incubated in blocking buffer [PBS containing 0.1 % Tween-20 and 5 % (w/v) non-fat dried milk] for 2 h at room temperature and then incubated for 1 h in the same buffer (containing only 1 % non-fat dried milk) with rabbit polyclonal antibodies raised against various rat isoforms of adenylyl cyclase (no cross-reactivity with other adenylyl cyclase isoforms has been described). The membrane was washed with the same buffer without milk, and then incubated for 1 h with horseradish peroxidase-

labelled goat anti-rabbit IgG (CovalAb, Oullins, France). The membrane was washed (3×10 min) with PBS containing 0.1 % Tween-20, and the antigen–antibody complex was revealed by enhanced chemiluminescence using a Western blot detection kit and Hyper-ECL film (Amersham, Zürich, Switzerland).

Statistics

Statistical significance of differences was assessed by the Student's t test and indicated by the two-tail P value.

RESULTS

Extracellular calcium is required for the potentiation by AngII of ACTH-induced cAMP formation

The dependence on extracellular Ca^{2+} of the AngII effect on cAMP formation has been investigated in primary cultures of bovine glomerulosa cells. In the presence of extracellular calcium (1.2 mM in the medium; Table 1), AngII alone, at 100 nM, did not significantly affect basal cAMP production (4.60 ± 0.65 pmol/μg of protein in the absence and 4.31 ± 0.29 pmol/μg of protein in the presence of AngII, $n = 3$). However, AngII increased in a significant manner ($P < 0.05$) the maximal ACTH-induced formation of cAMP. Preliminary experiments allowed us to determine that AngII action was concentration-dependent and optimal at 100 nM (results not shown). At this concentration, AngII increased cAMP levels from 265 ± 28 % to 382 ± 3 % of basal values in the presence of 100 nM ACTH, and from 269 ± 44 % to 377 ± 85 % of basal values with 10 μM ACTH. These results demonstrate that AngII increases cAMP synthesis only when adenylyl cyclases have previously been activated by ACTH, even at supramaximal concentrations of the latter.

In contrast, in the absence of extracellular calcium (Table 1), no potentiation of cAMP formation by AngII was observed, in the presence of either 100 nM ACTH (103 ± 8 % versus 109 ± 4 %, $n = 3$) or 10 μM ACTH (270 ± 44 % versus 273 ± 28 %, $n = 3$). The low cAMP response to 100 nM ACTH in the absence of extracellular calcium was thought to result from a decrease in the affinity of the ACTH receptor for its ligand [1,24]. Indeed, this could be overcome by increasing the concentration of the hormone to 10 μM, a concentration which gave a maximal cAMP response in the absence of extracellular Ca^{2+} (results not shown).

Table 1 Effect of extracellular calcium on the potentiation by AngII of ACTH-induced cAMP production

Cultured bovine glomerulosa cells were incubated, as described in the Materials and methods section, in the presence of 1.2 mM Ca^{2+} or in the absence of extracellular calcium (0.4 mM EGTA in the medium). Cells were exposed for 30 min to various concentrations of ACTH (0, 100 nM or 10 μM) with or without 100 nM AngII. cAMP release in the medium (in the presence of 0.2 mM IBMX) was determined by RIA and expressed as a percentage of basal production (4.60 ± 0.65 and 5.40 ± 0.51 pmol cAMP/μg of protein in the presence and in the absence of Ca^{2+} respectively). Data are the means $\pm$ S.E.M. of three independent experiments performed in triplicate. *Significantly different from control (in the absence of AngII) according to Student's t-test, with $P < 0.05$.

	cAMP production (% of basal)			
	$[Ca^{2+}]_{ext} = 1.2$ mM		$[Ca^{2+}]_{ext} = 0$	
[ACTH]	Control	+ AngII	Control	+ AngII
Basal	100	94 ± 6	100	90 ± 8
100 nM	265 ± 28	382 ± 3*	109 ± 3	103 ± 8
10 μM	269 ± 44	377 ± 85*	273 ± 28	270 ± 44

Table 2 Lack of effect of Nic on cAMP production

Cultured glomerulosa cells were incubated in the presence of IBMX (0.2 mM), as described in the Materials and methods section, in the absence or presence of 2 μM Nic, in a medium containing either 100 nM ACTH alone or 100 nM ACTH plus 100 nM AngII. cAMP levels in the medium were determined and expressed as a percentage of the response to ACTH alone, which amounted approx. to 300% of basal values (2.2 ± 0.6 pmol cAMP/μg of protein) in this series of experiments. Data are the means $\pm$ S.E.M. of three independent experiments performed in triplicate.

	cAMP production (% of control)	
	ACTH alone	ACTH + AngII
Control	100	173 ± 11
Nic (2 μM)	97 ± 9	181 ± 13

These results strongly suggested a role for some calcium influx in the potentiation by AngII of ACTH-stimulated cAMP production. It has been previously demonstrated that AngII induces both cell depolarization, resulting in the opening of VOC channels, and depletion of intracellular calcium pools, leading to the activation of a capacitative calcium influx [4]. Each of these two Ca^{2+} influx pathways might be implicated in the potentiation of cAMP formation induced by AngII and we have investigated their relative importance.

Lack of action of VOC channels in the potentiation of cAMP production

Nic, a dihydropyridine antagonist blocking both T- and L-type (VOC) channels in bovine glomerulosa cells [12], was used to discriminate between the two types of Ca^{2+} influx induced by AngII, the capacitative influx and the calcium influx passing through the VOC channels. As shown in Table 2, we observed that Nic (2 μM) did not affect the potentiation by AngII of ACTH-induced cAMP formation (173 ± 11 % and 181 ± 13 % of the response to ACTH alone, in the absence and in the presence of Nic respectively; $n = 3$). Moreover, the presence of Nic had no effect on basal (results not shown) and on ACTH-induced cAMP production. The ability of Nic to block VOC channels was assessed for both Ba^{2+} currents, with the patch-clamp technique (Figure 1A), and for the $[Ca^{2+}]_c$ response to stimulation with K^+, determined with the fluorescent probe fura-2 (Figure 1B). Voltage-dependent Ba^{2+} currents were elicited by a linear ramp depolarization (from -90 mV to $+40$ mV in 1.8 s), as previously described [20]. Both T-type channels (activated between -40 and -20 mV) and L-type channels (responsible for most of the current measured above -30 mV) were markedly affected by 100 nM Nic and completely blocked when the drug concentration was raised to 1 μM (Figure 1A). The $[Ca^{2+}]_c$ response to 9 mM potassium, exclusively due to activation of VOC channels, was completely reversed after the addition of 1 μM Nic, and $[Ca^{2+}]_c$ even decreased below resting levels (Figure 1B), confirming the presence of a basal activity of Nic-sensitive channels in resting cells [12]. These results demonstrate that micromolar concentrations of Nic completely inhibit the opening of VOC channels in response to cell depolarization and therefore suggest that the activation of these channels upon stimulation by AngII is not implicated in the phenomenon of cAMP potentiation.

Moreover, K^+ was unable to mimic AngII action (Table 3). Indeed, although a 9 mM elevation of potassium concentration

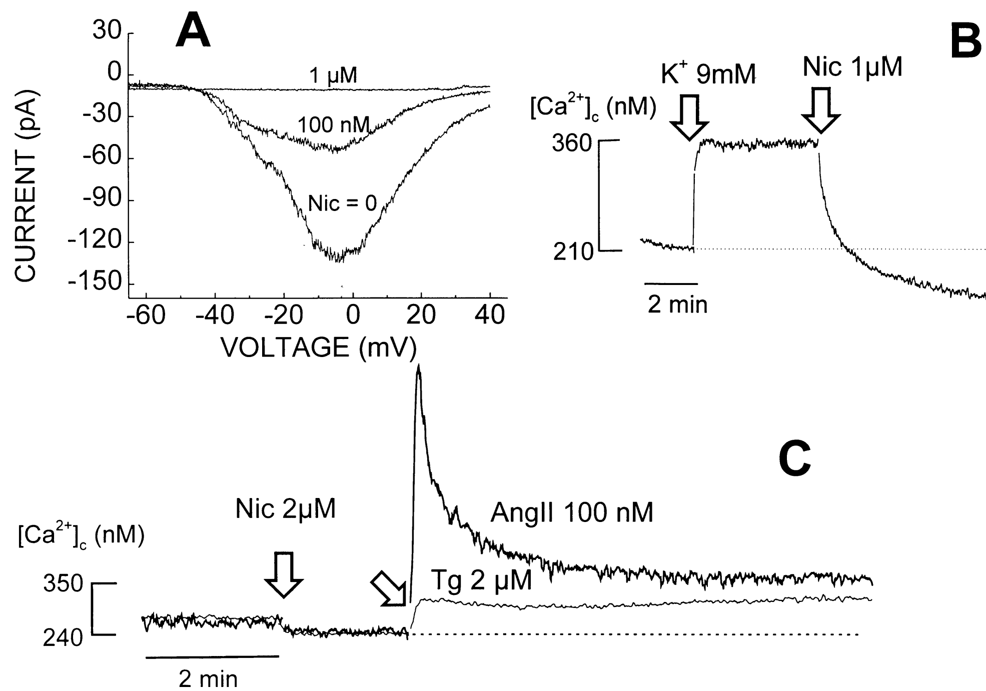

Figure 1 Cytosolic calcium increases mediated by VOC channels and capacitative influx

(**A**) Voltage-activated Ba^{2+} currents were recorded from an isolated bovine glomerulosa cell with the patch-clamp technique in the whole cell configuration, as indicated in the Materials and methods section. Superimposed traces of Ba^{2+} currents, elicited by a linear depolarization of the cell from a holding potential of −90 to +40 mV, before or after addition of various concentrations of Nic, are shown. Traces are representative of recordings from five cells giving similar results. (**B**) Fura-2-loaded cells were stimulated with 9 mM K^+, and 5 min later 1 μM Nic was added. (**C**) Fura-2-loaded cells were exposed to 2 μM Nic before being stimulated with 2 μM Tg (thin, lower trace) or 100 nM AngII (thick, upper trace). Traces shown in (**B**) and (**C**) are representative of three experiments from two cell preparations that gave similar results.

Table 3 Lack of effect of extracellular potassium on ACTH-induced cAMP formation

Cultured glomerulosa cells were incubated, as described in the Materials and methods section, in the presence of 3 mM (control) or 12 mM (9 mM added) K^+, with or without 100 nM ACTH. cAMP release in the medium was determined and expressed as a percentage of the basal production (2.2 ± 0.6 pmol cAMP/μg of protein). Data are the means ± S.E.M. of four independent experiments performed in triplicate.

	cAMP production (% of basal)	
	Basal	+ACTH
Control	100	346 ± 25
K^+ (12 mM)	110 ± 11	289 ± 38

Table 4 Implication of the capacitative influx in cAMP production

Cultured glomerulosa cells were incubated, as indicated in the Materials and methods section, in the presence or absence of 2 μM Tg or 100 nM AngII, with or without 100 nM ACTH. Release of cAMP in the medium was determined and expressed as a percentage of the basal production of control cells (3.5 ± 1.2 pmol cAMP/μg of protein). Data are the means ± S.E.M.of four independent experiments performed in triplicate. *Significantly different from control (ACTH alone) according to Student's *t*-test, with $P < 0.05$.

	cAMP production (% of basal)	
	Basal	+ACTH
Control	100	283 ± 17
Tg (2 μM)	106 ± 4	363 ± 21*
AngII (100 nM)	94 ± 6	403 ± 19*

induced a sustained increase in $[Ca^{2+}]_c$ of approximately 150 nM (Figure 1B), resulting from Ca^{2+} influx through VOC channels, it did not increase the cAMP formation stimulated by 100 nM ACTH (346 ± 25 % versus 289 ± 38 % of basal production in the presence of 3 mM K^+ and 12 mM K^+ respectively). These results confirm the fact that calcium influx through the VOC channels is unable to modulate the activity of adenylyl cyclases in bovine glomerulosa cells.

Role of the capacitative influx in the potentiation of cAMP formation

In order to determine whether the alternative Ca^{2+} entry pathway activated by AngII, namely the capacitative influx, plays a role in the phenomenon of potentiation of cAMP production, we have investigated the effect of Tg. Tg, a sesquiterpene lactone tumour promoter, is an inhibitor of the microsomal Ca^{2+} pumps necessary for Ca^{2+} sequestration in intracellular pools, and therefore induces store depletion followed by capacitative influx of Ca^{2+} [12,17,25]. The action of this agent on ACTH-induced cAMP formation was compared with that of AngII and the results are presented in Table 4. We observed that Tg, at 2 μM, did not affect basal cAMP formation (106 ± 4 % of basal, $n = 4$). However, in the presence of 100 nM ACTH, 2 μM Tg significantly ($P < 0.05$) increased cAMP formation from 283 ± 17 to 363 ± 21 % of basal values ($n = 4$). Tg action was slightly less pronounced

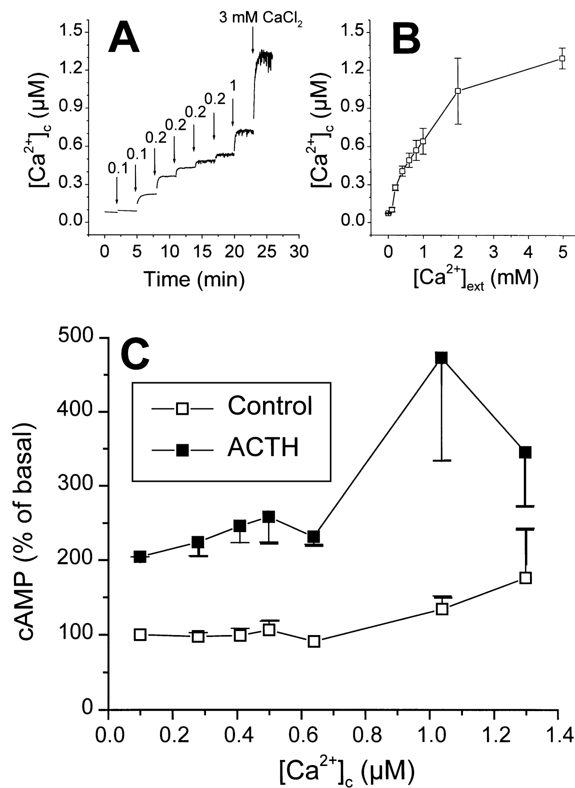

Figure 2 Control of cAMP production in cytosolic calcium-clamped cells

(**A**) Fura-2-loaded cells were incubated for 3–5 min in a Ca^{2+}-depleted medium containing 0.2 mM EGTA and in the presence of 2 μM ionomycin (results not shown), before being exposed to increasing concentrations of extracellular calcium (mM) at the times indicated by the arrows. (**B**) Similar results obtained in six experiments were averaged (means $\pm$ S.E.M.) and the measured $[Ca^{2+}]_c$ was expressed as a function of $[Ca^{2+}]_{ext}$. (**C**) In parallel experiments, cultured glomerulosa cells were incubated for 30 min as described in the Materials and methods section, in the presence of 2 μM ionomycin and various concentrations of extracellular calcium. Incubations were performed in the presence or in the absence of ACTH. No difference was observed between the results obtained with 100 nM or 10 μM ACTH and data were therefore averaged. cAMP production, in the presence of IBMX, was then determined in the medium and expressed as a percentage of the basal levels which amounted to 3.88 $\pm$ 0.47 pmol/μg of protein. Results are represented as a function of expected $[Ca^{2+}]_c$ (determined in **B**) and data are the means $\pm$ S.E.M. of four independent experiments performed in triplicate.

than that of 100 nM AngII, which, in the same experiments, raised cAMP production to 403 $\pm$ 19 %. This difference in potency was paralleled by the lower increase in $[Ca^{2+}]_c$ induced by 2 μM Tg as compared with that elicited by 100 nM AngII in the presence of Nic (Figure 1C). In this experiment, intended to selectively measure the capacitative component of the $[Ca^{2+}]_c$ response, the presence of Nic (2 μM) was required: (1) to prevent activation of VOC channels upon stimulation with AngII;, and (2) because micromolar concentrations of Tg affect (basal) VOC channel activity [26]. This result strongly suggests that the capacitative influx stimulated by AngII is implicated in the phenomenon of potentiation of cAMP production by the hormone.

Activation of cAMP production by ionomycin-induced elevations of cytosolic calcium

In order to investigate further the role of Ca^{2+} in the action of AngII on cAMP production, we examined the effect of modulating $[Ca^{2+}]_c$ with ionomycin. In preliminary experiments, a large increase in $[Ca^{2+}]_c$ (up to 2.5 μM), elicited by the addition of 2 μM ionomycin, did not significantly affect either basal or ACTH-induced cAMP production, which rose from 100 to 104 $\pm$ 12 % in the absence of ACTH and from 180 $\pm$ 18 to 196 $\pm$ 22 % of basal values in the presence of 100 nM ACTH (results not shown).

However, because such a large increase in $[Ca^{2+}]_c$ has been suggested to exert possible inhibitory effects on adenylyl cyclases normally activated by Ca^{2+} [27], we decided to 'clamp' the $[Ca^{2+}]_c$ at more physiological levels, as previously described [6], and to determine cAMP production under these conditions (Figure 2). For this purpose, we treated glomerulosa cells with ionomycin (2 μM) in the absence of extracellular Ca^{2+}, as described in the Materials and methods section, before adding various concentrations of Ca^{2+} to the medium. The effect of Ca^{2+} addition on $[Ca^{2+}]_c$ was monitored with fura-2 (Figure 2A) and was sustained for at least 1 h (not shown). The relationship between the total concentration of Ca^{2+} added to the medium ($[Ca^{2+}]_{ext}$) and $[Ca^{2+}]_c$ values determined in six independent experiments is shown in Figure 2(B). In spite of the presence of ionomycin in the plasma membrane, a large gradient of Ca^{2+} concentration between the cytosol and the extracellular medium was maintained, suggesting that Ca^{2+} pumps are active and tend to oppose Ca^{2+} influx, allowing the establishment of an equilibrium between Ca^{2+} fluxes, and therefore the clamping of $[Ca^{2+}]_c$. In separate experiments, cAMP production was determined in the presence and in the absence of ACTH and under various cytosolic Ca^{2+} clamp conditions (Figure 2C). We found that cAMP production was significantly increased at high $[Ca^{2+}]_c$, only at concentrations above 640 nM. Such values of cytosolic Ca^{2+} are never maintained upon stimulation of intact glomerulosa cells with AngII, Tg or K^+ (Figure 1). Interestingly, in the presence of ACTH, cAMP production appeared to be slightly reduced when $[Ca^{2+}]_c$ was increased above 1 μM, suggesting a bell-shaped dependence of cyclase activity on $[Ca^{2+}]_c$. It is therefore conceivable that no effect of ionomycin was observed in our preliminary experiments with ionomycin because the $[Ca^{2+}]_c$ values elicited under those conditions (2.5 μM) were largely above the active range of Ca^{2+} concentrations.

Identification of adenylyl cyclase isoforms expressed in bovine glomerulosa cells

The expression of various isoforms of adenylyl cyclase in BAG cells was analysed by immunoblotting of membrane proteins with specific antibodies, and results were compared with those obtained from various tissues (cerebellum, heart, liver) isolated from Wistar rats (Figure 3). Under our experimental conditions, only the antibody raised against the type III adenylyl cyclase, a Ca^{2+}/calmodulin-activated isoform, revealed a band of relative molecular mass around 128 kDa in BAG cells. This band at 128 kDa was not observed when the first antibody was omitted or when incubation with the first antibody was performed in the presence of an excess of a peptide corresponding to the C-terminus of rat type III adenylyl cyclase (not shown). A type III cyclase of slightly higher molecular mass was also detected in rat cerebellum and rat liver, but was absent from rat heart, a tissue in which cAMP formation is known to be inhibited by Ca^{2+}. In contrast, heart expressed particularly large amounts of type II cyclase, an isoform apparently absent in other tissues tested. The type I cyclase was essentially observed in heart and liver, probably in a highly glycosylated form, whereas the antibody raised against type V and VI cyclases (Ca^{2+}-inhibited isoforms) recognized a high-molecular-mass protein in the heart exclusively.

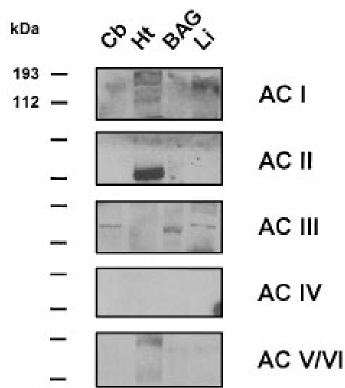

Figure 3 Tissue-specific expression of various types of adenylyl cyclase

Membrane proteins from cultured BAG cells and from rat cerebellum (Cb), rat heart (Ht) and rat liver (Li) were extracted and separated by SDS/PAGE as described in the Materials and methods section. Proteins (30 μg/lane) were analysed by immunoblotting for their adenylyl cyclase content with polyclonal antibodies raised against domains of the enzyme specific for each isoform. The type of isoform recognized by each antibody is indicated on the right (one antibody recognized both types V and VI). Proteins of molecular mass between approximately 100 and 200 kDa are shown. These results are representative of two (rat tissues) or seven (BAG cells) similar experiments. The positions of molecular-mass markers are shown on the left and are the same for each panel.

Finally, no protein of molecular mass between 100 and 200 kDa was detected by the antibody raised against the type IV cyclase in any of these tissue extracts.

These results therefore suggest that the antibodies used in this study are specific and sensitive enough to discriminate between the various cyclase isoforms expressed in different tissues, and that type III adenylyl cyclase is by far the main isoform expressed in BAG cells. This cyclase therefore appears to be responsible for functionally linking cAMP production to the capacitative Ca^{2+} influx in these cells.

DISCUSSION

This study has allowed us to demonstrate that the potentiation by AngII of ACTH-induced cAMP formation in BAG cells, previously described by Baukal et al. [14], is associated with a particular type of calcium influx. This calcium entry does not involve either the T- or the L-type of VOC channels, but has been identified as the capacitative calcium influx stimulated upon Ca^{2+} release from intracellular stores by Ins(1,4,5)P_3 [12,13]. Moreover, this modulation of cAMP by Ca^{2+} in bovine glomerulosa cells is probably due to the presence of a type III adenylyl cyclase, an isoform preferentially expressed in these cells.

In a recent report, Gallo-Payet et al. [28] showed a functional link between dihydropyridine-sensitive Ca^{2+} channels and adenylyl cyclase activity in human adrenal glomerulosa cells. Particularly, nifedipine (Nif) markedly reduced ACTH-induced cAMP production in human cells. This result contrasts with our observation that, in bovine glomerulosa cells, neither Nic (Table 2) nor Nif (results not shown) affected the cAMP production stimulated by ACTH alone or in combination with AngII. Moreover, opening of VOC channels with extracellular K^+ (Figure 1B, Table 3) or BayK 8644 (results not shown), an agonist of L-type Ca^{2+} channels, had no effect on adenylyl cyclase activity. This discrepancy could be due to differential regulation of cAMP production in glomerulosa cells among the various species. Indeed, AngII, which exerts a negative action on murine glomerulosa cell adenylyl cyclase, probably through a G_i protein [4,16], clearly potentiates the response to ACTH in bovine cells [14]; moreover, whereas this potentiation in bovine cells appears to be selectively due to the capacitative Ca^{2+} influx elicited by the hormone, in human cells, Ca^{2+} entering the cell through VOC channels appears to act as a direct activator of the adenylyl cyclases.

Different mechanisms of modulation of cAMP production by the Ca^{2+} messenger system among species probably reflect differential expression of the adenylyl cyclase isoforms in the cells. At the present time, nine different isoforms of adenylyl cyclase are known [29–31], which have been classified in three groups: (1) the Ca^{2+}/calmodulin-activated (types I, III and VIII) [32–35], (2) the Ca^{2+}-inhibited (types V, VI and possibly IX), and (3) the Ca^{2+}-insensitive (types II, IV and VII) adenylyl cyclases.

Because, in the present study, the effect of Ca^{2+} on cAMP production was examined in the presence of IBMX, an inhibitor of phosphodiesterases, our results suggest the presence in bovine glomerulosa cells of a Ca^{2+}/calmodulin-activated adenylyl cyclase isoform, specifically regulated by the capacitative Ca^{2+} influx. Fagan et al. [36] found that in transfected HEK293 cells, heterologously expressing different isoforms of adenylyl cyclase (types I, III and VIII), a $[Ca^{2+}]_c$ increase directly stimulates type I and VIII cyclases (even in the absence of a previous stimulation of the cyclase with forskolin), but that type III cyclase has to be primed to become sensitive to calcium. Moreover, the type III cyclase is insensitive to a large increase in $[Ca^{2+}]_c$ elicited by ionomycin in these cells. Because the type III adenylyl cyclase is the only cyclase isoform requiring pre-stimulation through a G_s protein (activated in glomerulosa cells by ACTH) in order to be positively modulated by Ca^{2+}, it appears to be the best candidate to explain our observations in bovine cells, such as the lack of activation by AngII or Tg alone. This hypothesis was confirmed by demonstrating, with selective antibodies, that the type III cyclase is the main isoform expressed in bovine glomerulosa cells.

This finding is also in agreement with the observation by Baukal et al. [14] that cAMP production in bovine cells is activated by PKC, since the type III cyclase is known to be sensitive to this kinase [37]. Additional activation of the type III cyclase by PKC could partially explain the slightly more pronounced effect of AngII on cAMP formation as compared with that of Tg (Table 4), which does not stimulate PKC.

A specific implication of the calcium capacitative influx in the regulation of Ca^{2+}/calmodulin-activated or Ca^{2+}-inhibited adenylyl cyclases has been previously demonstrated in other cell types, such as HEK293 [36,38] and C6-2B glioma [39] transfected cells expressing various isoforms of adenylyl cyclases.

In BAG cells, the observed functional relationship between the capacitative Ca^{2+} entry pathway and the activation of cAMP production, strongly suggests a close co-localization of the channels responsible for this influx and the type III cyclase expressed in these cells. This notion of topological organization is in agreement with the effect of ionomycin. Indeed, the ionophore is able to activate the cyclase through an increase in $[Ca^{2+}]_c$, but, because this Ca^{2+} elevation is diffuse in the cytosol, it requires on average much higher concentrations of Ca^{2+} than those elicited by Tg or AngII. An elegant approach to investigate the Ca^{2+} environment of Ca^{2+}-sensitive cyclases has recently been described by Nakahashi et al. [40]. They have constructed a chimaera containing a full-length type VI adenylyl cyclase fused with aequorin, a photoprotein sensitive to Ca^{2+} and currently used for measuring localized Ca^{2+} variations. As expected, the chimaera detected much higher Ca^{2+} concentrations than its cytosolic counterpart when capacitative influx was stimulated in HEK 293 cells. A similar approach, using the type III cyclase, could be used to compare local changes of $[Ca^{2+}]_c$ in glomerulosa

cells upon opening of VOC channels or activation of the capacitative influx.

Indeed, a close relationship between the formation of cAMP, a potent trigger of steroid biosynthesis, and a specific pathway of Ca^{2+} influx is particularly relevant in a cell endowed with multiple types of Ca^{2+} channels. We have recently demonstrated that T-type and L-type Ca^{2+} channels have distinct functions in glomerulosa cells, the former being more efficiently coupled to steroidogenesis [20]. Specific coupling between capacitative Ca^{2+} entry, exclusively activated upon Ca^{2+} release from intracellular stores by phospholipase C-activating hormones, and the activity of adenylyl cyclase, represents another example of functional specialization for a given type of Ca^{2+} channel (in this case the CRAC or **c**alcium **r**elease-**a**ctivated **c**alcium channels) and highlights the high degree of confinement of the cellular Ca^{2+} signal in glomerulosa cells [41]. This notion of compartmentalization of cross-talk between Ca^{2+} and cAMP in adrenal glomerulosa cells clearly helps our understanding of the different efficacies of steroidogenic agents like K^+ and AngII. Indeed, in spite of a larger increase in $[Ca^{2+}]_c$ induced by K^+, AngII is more efficient in stimulating aldosterone output from bovine glomerulosa cells, probably because, unlike K^+, the hormone is able to modulate the production of cAMP through the activation of the capacitative pathway. This hypothesis is in agreement with the observation that AngII increases aldosterone production from cells in which the cytosolic Ca^{2+} has been previously clamped with the Ca^{2+} ionophore ionomycin [6]. In the same study, hormone-induced potentiation of steroid synthesis was mimicked by addition of dibutyryl cAMP or forskolin, suggesting that AngII steroidogenic efficacy is related to its ability to activate cAMP formation.

In conclusion, the present results suggest a high degree of spatial organization in adrenal glomerulosa cells, allowing a colocalization of some Ca^{2+}/calmodulin-activated isoforms of adenylyl cyclase (most probably type III) and CRAC channels responsible for the capacitative influx of Ca^{2+}. This structural organization is apparently relevant for optimal steroidogenic function in the bovine glomerulosa cell.

We are grateful to Liliane Bockhorn, Walda Dimeck and Gisèle Dorenter for their excellent technical assistance. This work was supported by grants (#32-39277.93, #31-42178.94) from the Swiss National Science Foundation and from the Helmut Horten Foundation. M.F.R. is the recipient of a grant from the Professor Max Cloëtta Foundation.

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Received 27 June 1997/20 October 1997; accepted 4 November 1997