Role of Ca^{2+} in the Action of Adrenocorticotropin in Cultured Human Adrenal Glomerulosa Cells

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Abstract

The present report details the role of Ca^{2+} in the early events of ACTH action in human adrenal glomerulosa cells. Threshold stimulations of both aldosterone and cAMP production were obtained with a concentration of 10 pM ACTH, an ED_{50} of 0.1 nM, and maximal aldosterone stimulation (5.5-fold increase over control) at 10 nM ACTH. ACTH also induced a sustained increase of intracellular calcium ($[Ca^{2+}]_i$) with maximal stimulation of 1.6±0.1-fold over control values. This increase does not involve mobilization of calcium from intracellular pools since no response was observed in Ca^{2+}-free medium or in the presence of nifedipine, suggesting the involvement of Ca^{2+} influx by L-type Ca^{2+} channels. This was confirmed by patch clamp studies that demonstrated that ACTH stimulates L-type Ca^{2+} channels. Moreover, the Ca^{2+} ion is not required for ACTH binding to its receptor, but is essential for sustained cAMP production and aldosterone secretion after ACTH stimulation. These results indicate that, in human adrenal glomerulosa cells, a positive feedback loop between adenylyl cyclase–protein kinase A–Ca^{2+} channels ensures a slow but sustained $[Ca^{2+}]_i$ increase that is responsible for sustained cAMP production and aldosterone secretion. (*J. Clin. Invest.* 1996. 98:460–466.) Key words: adrenocorticotropin • aldosterone • human adrenal cells

Introduction

In mammals, including humans, aldosterone secretion by adrenal zona glomerulosa is under multifactorial regulation. Potassium ion, angiotensin II (Ang II), and adrenocorticotropin hormone, as well as epinephrine, serotonin, endothelin, α-MSH, and vasopressin are all known to be potent secretagogues (1–7). Preliminary in vitro studies, conducted either in human adrenocortical cells (5, 8, 9) or with NCI-H295 adrenocortical carcinoma cells (10), indicate that ACTH (5, 8, 9) or dibutyryl cAMP (10) are as potent as Ang II in stimulating aldosterone secretion; however, there are no studies concerning the early events of ACTH action.

ACTH has both trophic and steroidogenic effects on the adrenal gland. It is well established that the stimulatory effect of ACTH can be divided into two phases. In its long term action, ACTH stimulates biosynthesis of the enzymes involved in steroidogenesis by increasing their respective mRNA levels (11). In its short term action, ACTH activates conversion of cholesterol to pregnenolone, and then to cortisol in fasciculata cells and to 18-hydroxy-corticosterone and aldosterone in glomerulosa cells (12). Binding of ACTH to its specific receptor leads to an increase in intracellular cAMP and an activation of protein kinase A (PKA).[1] In spite of several studies using both rat and bovine glomerulosa or fasciculata cells (1, 2, 7), the precise molecular mechanisms by which this hormone stimulates steroid synthesis and secretion is still poorly understood. In particular, it is still unknown whether the enhancement of cAMP-induced increase in aldosterone secretion is mediated by PKA alone (11). Aside from enhancing cAMP production, ACTH also stimulates Ca^{2+} influx (13, 14). However, a number of controversies still persist as to the exact locus of Ca^{2+} action. For example, it is not clear if Ca^{2+} is important for binding of ACTH to its receptor. In some studies, extracellular Ca^{2+} does not significantly alter ACTH binding (15, 16), although it has been shown to be essential in others (17). The role of Ca^{2+} in the production of cAMP is also subject to debate (18, 19). Even its role in hormone secretion is questioned since, in some studies, the addition of verapamil markedly inhibited corticosteroidogenesis in response to ACTH (20), while in others these Ca^{2+} channel blockers inhibited the effect of Ang II and potassium, with little effect on ACTH response (21). Recent data from our laboratory have shown that K^+ and Ca^{2+} channels are present in human glomerulosa cells (22). In rat cells, similar ionic channels were previously described to be modulated by ACTH (23, 24). To date, the mechanism of action of ACTH in humans has been poorly documented. Studies conducted in unpurified adrenocortical cells (26), and in the NCI-H295 cell line (10), have shown that the ionophore A23187 or K^+ depolarization were able to stimulate both aldosterone and cortisol secretion. However, the exact role of the Ca^{2+} ion in the ACTH action has not been investigated.

In the present study, we attempted to analyze in detail the molecular mechanisms by which ACTH stimulates steroid secretion in an enriched population of human glomerulosa cells in primary culture with particular attention to the role of calcium.

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1. *Abbreviations used in this paper:* Ang II, angiotensin II; HBS, Hanks' buffered saline; PKA, protein kinase A.

Methods

Chemicals. The chemicals used in the present study were obtained from the following sources: aldosterone and corticosterone antiserum from ICN Biomedicals, Inc. (Costa Mesa, CA); [^{3}H]adenine (24 Ci/mmol) and, ^{125}I-labeled [Tyr^{23}] ACTH-(1-39) (2,000 Ci/mmol) from Amersham International (Oakville, Ontario, Canada); [^{3}H]aldosterone (72 Ci/mmol) from New England Nuclear (Boston, MA); ATP, cAMP, and DNase from Sigma Chemical Co. (St. Louis, MO) arginine vasopressin and angiotensin II from Bachem California (Torrance, CA); ACTH 1-24 peptide (Cortrosyn) from Organon (Toronto, Canada); collagenase and MEM-Eagle Medium from GIBCO (Burlington, Ontario); and H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline-sulfonanide) from Seikagaku America Inc. (St. Petersburg, FL); All other chemicals were of A-grade purity.

Cell preparation. Adrenal glands were obtained from renal transplant donors, 16–22 yr old, through a collaboration with Quebec Transplant. This project was approved by the Human Subject Review Committee of the University of Sherbrooke. After removal, glands were kept on ice in McCoy's medium and transported within 4 h to the laboratory. Glands were processed as previously described (5). Briefly, adrenal glands were cleansed of fat and cut into four or five flat sections. Thin tissue slices were then obtained using a Stadie-Riggs tissue slicer (Thomas Science, St. Laurent, Quebec, Canada). Only the first slice containing mainly adrenal capsule and zona glomerulosa was used to prepare glomerulosa cells. These slices were finely chopped into 1–2-mm fragments, followed by four incubation periods of 30 min in Eagle's Minimum Essential Medium containing collagenase (2 mg/ml) and DNase (25 μg/ml). Cells were then disrupted by gentle aspiration with a sterile 10 ml pipette before being filtered and centrifuged for 10 min at 100 *g*. The cell pellet was resuspended in Eagle's Minimum Essential Medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at a density of about 10^5 in 35-mm Petri dishes or 2–4 × 10^4 on glass coverslips and grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The culture medium was changed 24 h after seeding and cells were used after 3 d. For studies using freshly isolated cells, red blood cells and broken cells were removed by centrifugation at 100 *g* over a 60% Percoll barrier (Pharmacia Biotech Inc., Baie D'Urfé, Quebec, Canada). The cells were then suspended in culture medium and preincubated for 2 h at 37°C in a humidified atmosphere (95% air, 5% CO_2). After this resting period, cells were centrifuged at 100 *g* for 10 min, washed and resuspended in Hanks' Buffered Saline (HBS buffer (mM): 130 NaCl, 3.5 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 2.5 $NaHCO_3$, 5 HEPES, supplemented with 1 g/liter sucrose and 0.5% BSA, pH 7.4). Cell number ranged from 1 to 2 × 10^5 cells/tube. Cell purity was determined by both electron microscopy (typical mitochondria), phase contrast examination (zona fasciculata cells are larger), and steroid secretion (aldosterone vs cortisol secretion, reference 5).

Membrane preparation. After isolation as described previously, glomerulosa cell suspensions were suspended in 5 ml of 10 mM ice-cold Tris–HCl buffer (containing (mM): 0.5 EDTA, 1 $MgCl_2$, 1.8 PMSF, 0.04 u/ml Aprotinine, 1 mM Benzamidin, pH 8.0) and homogenized in a Dounce glass Potter homogenizer (Kontes Glass Co., Vineland, NJ). Cell extracts were first centrifuged 5 min at 700 *g* followed by a second centrifugation of the supernatant 15 min at 30,000 *g* to obtain the membrane fraction. This membrane extract was suspended in 2 ml of 50 mM Tris–HCl buffer (containing (mM): 2 EDTA, 5 $MgCl_2$, and 250 sucrose) and stored at −20°C until binding experiments.

Incubations for aldosterone measurement. Before each experiment, the medium of cultured glomerulosa cells was aspirated and the cells washed twice with cold HBS buffer. The cells were incubated in 1 ml of 0.9 ml HBS-glucose supplemented with 0.5% BSA + 0.1 mg/ml bacitracin and 0.1 ml of stimuli. After a 2-h incubation at 37°C in a 95% air: 5% CO_2 atmosphere, the incubation medium was removed by aspiration and kept at −20°C until steroid measurement was performed. Isolated cell suspensions were also washed in HBS-glucose, centrifuged at 100 *g* for 10 min, and resuspended at a concentration of 5 × 10^4 cells/ml in HBS buffer and incubated for 2 h as described above. After incubation, the cells were centrifuged at 800 *g* and the supernatants stored at −20°C. Aldosterone was determined by radioimmunoassay directly from the medium, using specific antisera and tritiated steroid as tracers. When necessary, cells were incubated in a Ca^{2+}-free medium, corresponding to 100 nM free Ca^{2+}, obtained by adding 0.5 mM EGTA in an HBS buffer containing 0.28 mM $CaCl_2$, as determined by computer software designed by Fabiato (27).

Cyclic AMP determination. Intracellular cyclic AMP production was determined by measuring the conversion of [^{3}H]ATP into cyclic [^{3}H]AMP, as described previously (14). In short, cultured cells were incubated at 37°C in the same MEM Eagle culture containing 2 μCi/ml [^{3}H]-adenine. After 1 hour, cultured cells were washed while isolated cells were diluted and centrifuged. Cells were further incubated in HBS glucose containing 1 mM isobutyl methylxanthine for 15 min at 37°C. Hormones or drugs were then added to the incubation medium for an additional 15-min incubation period at 37°C. The reaction was stopped by aspiration of the medium and addition of 1 ml TCA 5%. Cells were scraped with a rubber policeman followed by the addition of 100 μl of cold 5 mM ATP-cyclic AMP. Cell membranes were pelleted at 5,000 *g* for 15 min and the supernatants were sequentially chromatographed on Dowex and alumina columns according to the method of Salomon et al. (28), allowing the separation of [^{3}H]ATP nucleotide (primarily [^{3}H]adenine from cyclic [^{3}H]AMP. Cyclic AMP formation was expressed as: percent conversion = (cyclic [^{3}H]AMP/cyclic [^{3}H]AMP + [^{3}H]ATP) × 100 per 15 min.

Measurement of intracellular calcium concentration. Intracellular calcium measurements were performed as described previously (29). Briefly, glomerulosa cells were loaded with 3.3 μM of fluorescent calcium chelator Fura 2 AM for 30 min at 37°C, in serum-free culture medium supplemented with Hepes (20 mM, pH 7.4). After the incubation, cells were washed 3× in HBS buffer. The coverslips containing loaded cells were placed in the perfusion chamber and mounted on an inverted microscope (Axiovert 110; Carl Zeiss, Inc., Thornwood N.Y.). Cells were then incubated at room temperature in HBS buffer with or without the agents to be tested. The cells were alternatively excited at 340 and 380 nm light (with a 150 W xenon lamp) and the emission measured at 510 nm through an MSP 21 microprocessor responsible for the filter changes. Images were taken by a low light level SIT LH 4036 camera from Lhésa (Paris, France) and digitized on line by a Quantel "Crystal" Imaging System. During the 2-s excitation period, eight videoframes were averaged per digitized image for each wavelength. Each image was recorded at 512 × 512 × 8 bits. Camera dark noise was subtracted from the recorded crude images. The resulting image was divided by its respective 340- or 380-nm homogenization image. The homogenization images were obtained at each excitation wavelength by illumination of a Fura-2–free acid standard solution containing 170 nM free Ca^{2+}. 1,024 frames were averaged, camera dark noise subtracted, and the resulting standard solution image divided by the mean of all pixel values having an intensity higher than 20 gray levels. $[Ca^{2+}]_i$ levels were estimated from the images obtained after division with the homogenization image by using the Turbo-Pascal software and equation of Grynkiewicz et al. (30).

Binding studies. Binding assays were performed on glomerulosa cell membranes incubated in 50 mM Tris-HCl buffer containing (mM): 3 $MgCl_2$, 0.5 EGTA, 0.1 PMSF, 1.8 or 0.1 μM free $CaCl_2$, (as described above for aldosterone measurements), and 0.1% BSA. Binding experiments were initiated by the addition of 50 μl (50 μg of membranes) into polypropylene tubes containing 50 μl buffer, 50 μl of increasing concentrations of iodinated ^{125}I-labeled[Tyr^{23}] ACTH-(1–39) or 10 μM unlabeled ACTH for determination of nonspecific binding. Incubations were conducted in triplicate for 30 min at 37°C. After incubation, cell-bound radioactivity was separated from unbound radioactivity by centrifugation with a microfuge (Beckman Instruments, Inc., Fullerton, CA) containing 200 μl Tris-buffer plus 1% BSA at 15,000 *g* for 1 min. Pellets were rinsed without resuspension with Tris-HCl buffer containing 10% sucrose. The tips of the mi-

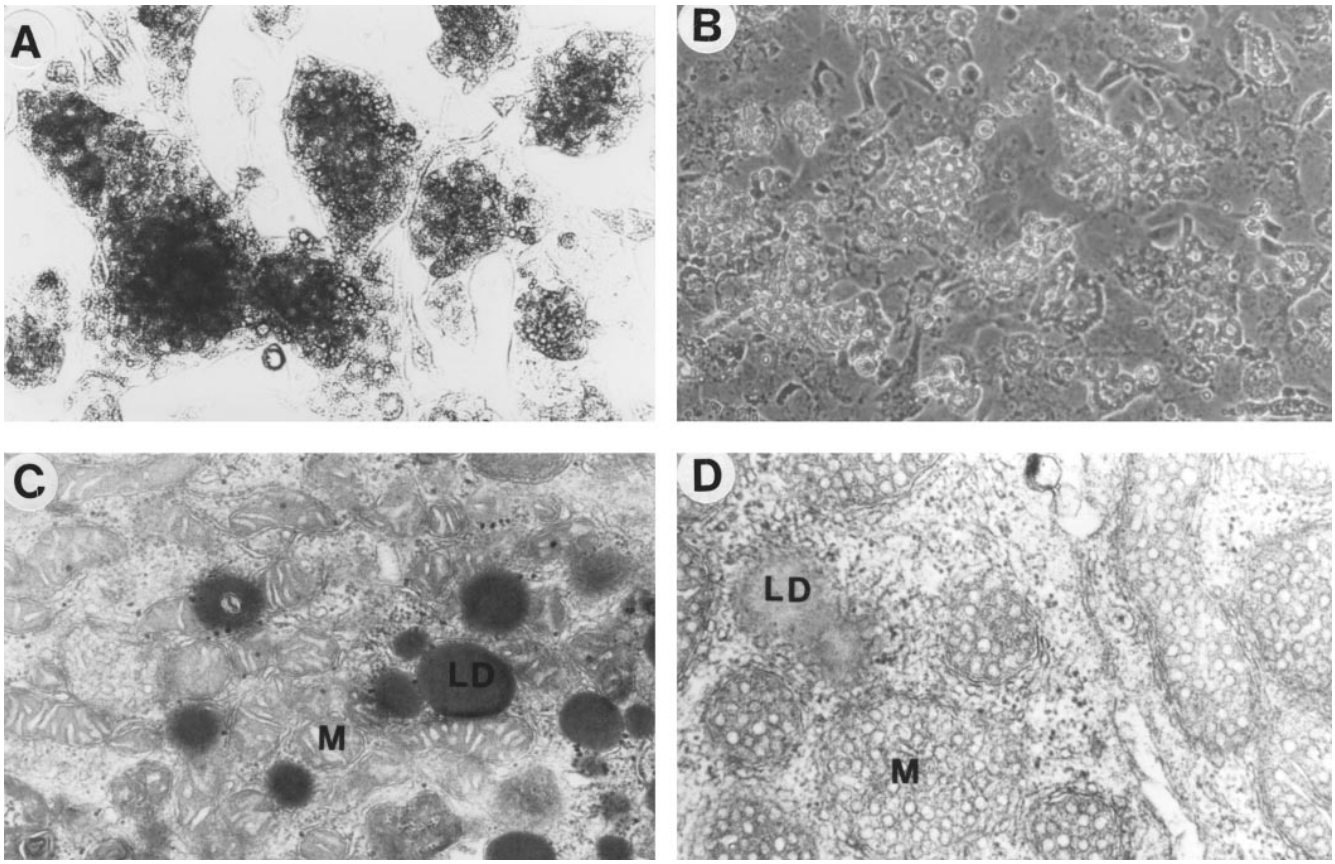

Figure 1. Morphological appearance of human adrenal cells. Fixed toluidine blue–stained cells enriched glomerulosa cell cultures 24 h after seeding (*A*). Phase-contrast microscopy of cultured cells after 3 d in culture (*B*) (×432). Electron micrograph of a glomerulosa cell after 3 d in culture, with typical elongated mitochondria (*M*) containing tubular cristae (*C*) (×14,808). Electron micrograph of a fasciculata cell after 3 d in culture, with ovoid mitochondria containing vesicular cristae and lipid droplets (*LD*) (*D*) (×14,808).

crofuge tubes were then cut and counted in a gamma counter (Beckman Instruments, Inc.).

Recording of calcium currents. Ionic currents recordings were obtained using the whole-cell configuration of the patch clamp method (22–24) in the voltage clamp configuration. Patch electrodes were made from Pyrex glass (7740; Corning Glass Works, Corning, NY) with a resistance ranging between 4–8 MΩ. The following solutions were used during the experiments: solution B in the bath (mM): 100 NaCl, 10 $CaCl_2$, 35 TEA, 1 $MgCl_2$, 5.4 CsCl, 5 HEPES, and 1 g/liter glucose, pH 7.4; and solution P in the pipette (mM): 126 CsCl, 18 NaCl, 1 $CaCl_2$, 11 EGTA, 2 $MgCl_2$, 5 HEPES, and 3 ATP, pH 7.3. These solutions were filtered through 0.2-μm Millipore filters (Millipore Corp., Bedford, MA) before use. Experiments were performed at room temperature.

The petri dish (1 ml solution) was mounted on the stage of an inverted microscope and the cells were observed at a magnification of 300. Currents were recorded with an axopatch 1 B amplifier (Axon Instruments Inc., Burlingame, CA); pulse stimulation and data acquisition were performed with an A/D interface DAS 16F (Metrabyte, Taunton, MA) and an IBM-compatible computer under the control of a custom-built program. Linear leak and capacitive currents were substracted. Currents were low pass filtered at 2 kHz and sampled at 5 kHz. Analysis was performed with a custom-made program.

Morphological investigation. Cultured cells were observed and photographed using an inverted phase-contrast Zeiss microscope. For electron microscopy studies, cells in suspension or in petri dishes were fixed with 2.5% glutaraldehyde, postfixed with 2% osmium, dehydrated, and embedded in Epon 812. Gold to silver-gray sections were then stained with uranyl acetate and lead citrate and examined in a Phillips 300 electron microscope.

Data analysis. The data are presented as means±SEM. ED_{50}s were obtained directly from dose-response curves. Statistical analysis of the data was performed using the one-way ANOVA test. Homogeneity of variance was assessed by Bartlett's test and *P* values were obtained from Dunnett's tables.

Results

Morphological appearence of human adrenal cells. When plated on Linbro culture dishes, 80% of cells adhered to the substratum in less than 1 h. Proliferating cells were observed after 24 h in culture, forming cell agregates as shown in Fig. 1 *A*. Fig. 1 *B* shows the appearance of cells under phase contrast after 3 d in culture. Cells exhibited polygonal morphology and an abundant content of refringent lipid droplets, characteristic of steroidogenic cells. Glomerulosa cells retained their ultrastructural properties characterized by typical mitochondria with tubular cristae (Fig. 1 *C*), while fasciculata cells exhibit mitochondria with vesicular cristae (Fig. 1 *D*). From these observations, we can estimate the purity of our zona glomerulosa cell cultures to 73±8% ($n = 5$).

Involvement of Ca^{2+} influx and protein kinase A in ACTH-stimulated aldosterone secretion. In 3-d cultured human glomerulosa-enriched cell populations, ACTH induced a dose-dependent increase in aldosterone secretion (Fig. 2 *A*). The ED_{50} was 0.08±0.05 nM ($n = 3$). Threshold was detected at a concentration of 10 pM. The maximal steroidogenic effect was

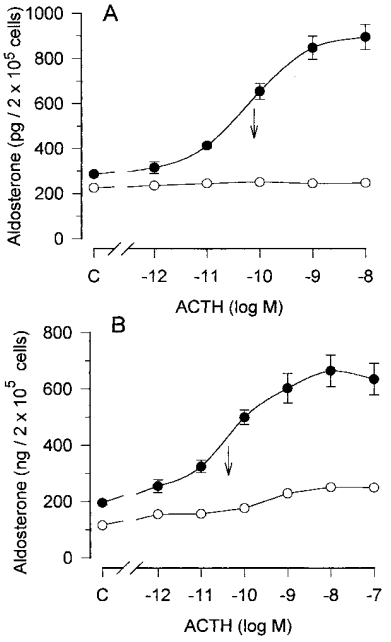

Figure 2. Dose-dependent effect of ACTH on aldosterone secretion in human glomerulosa cells. 3-d cultures of human glomerulosa (*A*) or freshly isolated cells (*B*) were incubated at 37°C for 2 h in 95% O_2, 5% CO_2 atmosphere in HBS medium containing either 1.8 mM $CaCl_2$ (●) or 100 nM Ca^{2+} (○) with or without (Control) increasing concentrations of ACTH. Accumulation of aldosterone secretion was measured by specific RIA. Results are the mean±SEM of triplicate determinations of one experiment representative of three. Arrow on the graph indicates ED_{50}.

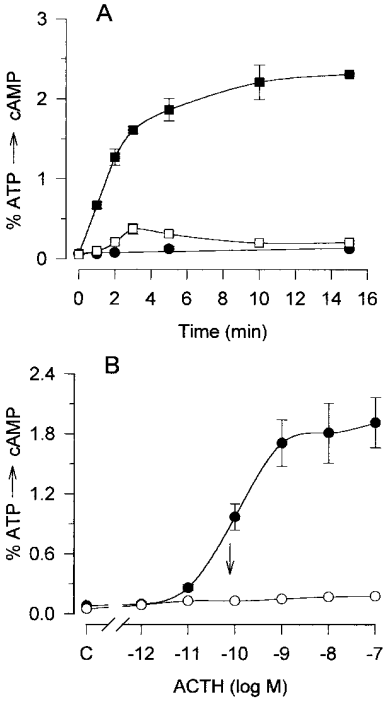

Figure 4. Time- and dose-dependent effect of ACTH on cAMP production in human glomerulosa cells. 3-d cultures of human glomerulosa were labeled with [^{3}H]adenine as described in Methods. (*A*) The time-course of cAMP accumulation stimulated with 10 nM ACTH in the absence (□) or in the presence (■) of external Ca^{2+}; (●) control, without ACTH and without external Ca^{2+}. (*B*) The dose-response curve of cAMP accumulation after a 15-min incubation period at 37°C with or without increasing concentrations of ACTH. Experiments were performed either in HBS buffer containing 1.8 mM $CaCl_2$ (●) or 100 nM $CaCl_2$ (○). Conversion of ATP into cAMP was calculated as described in Methods. Results are the mean±SEM of triplicate determinations of one experiment representative of three. Arrow on the graph indicates ED_{50}.

reached at 10 nM with an increase of 5.5±1.5 fold (n = 3) over controls for aldosterone secretion (Fig. 2 *A*). By comparison, in freshly isolated cells, similar dose responses were observed. Maximal stimulation ratios were similar (4.1-fold increase over control) (Fig. 2 *B*). Moreover, in the absence of extracellular calcium, the stimulating effects of ACTH on aldosterone secretion was abolished (Fig. 2, *A* and *B*).

The participation of Ca^{2+} channels in the stimulation of aldosterone secretion was investigated by incubating cells in the presence of known Ca^{2+} channel blockers. We have previously shown that, in human glomerulosa cells, $NiCl_2$ and nifedipine specifically blocked Ca^{2+} channels (22). Results from Fig. 3 *A* show that the calcium blockers did not affect basal steroid secretion. However, a 10-min preincubation with 200 nM $NiCl_2$, or 1 μM nifedipine significantly decreased maximal (10 nM) stimulating concentrations of ACTH by 52±12% and 47±6%, respectively.

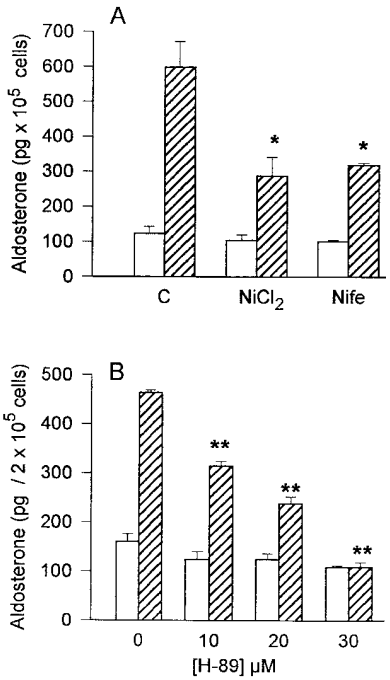

Figure 3. Effect of calcium channel blockers and protein kinase inhibitor on ACTH-induced aldosterone secretion in human glomerulosa cells. 3-d cultured human glomerulosa cells were washed with HBS buffer and preincubated either for 10 min with or without selective calcium channel blockers (1 μM nifedipine and 200 nM $NiCl_2$) (*A*) or 30 min at 37°C with or without increasing concentrations of H-89 (*B*). Cells were then further incubated for 2 h at 37°C as described in Fig. 2 in HBS medium supplemented with (▬) or without (▭) 10 nM ACTH. Aldosterone secretion was measured by RIA. Results are the mean±SEM of three distinct experiments, each conducted in triplicate. *$P < 0.01$; **$P < 0.001$ compared to corresponding ACTH-stimulated values.

The role of the PKA pathway was investigated by adding H-89, a potent inhibitor of protein kinase A (31). H-89 induced a dose-dependent inhibition of aldosterone secretion (Fig. 3 *B*). This inhibition was time dependent and maximal (100%) after a 30-min preincubation at 37°C with 20 μM H-89 (data not shown). When used in similar experimental conditions, preincubation with H-89 did not affect the Ang II- or the arginine vasopressin (AVP)-stimulation of aldosterone secretion (data not shown). This demonstrates good specificity of H-89 for cAMP-dependent phosphorylation.

Involvement of Ca^{2+} influx and protein kinase A in ACTH-stimulated cAMP production. At maximal concentration (10 nM), ACTH induced a time-dependent increase in the conversion of ATP into cAMP. This stimulation occurred rapidly with a 19-fold increase after a 1-min application and maximal simulation (38-fold increase) after 10 min. Moreover, ACTH-induced cAMP production was highly Ca^{2+}-dependent since, in a Ca^{2+}-free medium, a small and transient rise is observed (6-fold increase after 3 min) (Fig. 4 *A*). The effect of ACTH was dose-dependent (Fig. 4 *B*) with half maximal effective concentrations observed at 0.1±0.05 nM. Threshold concentration was about 10 pM.

Sensitivity of ACTH-stimulated cAMP production was dif-

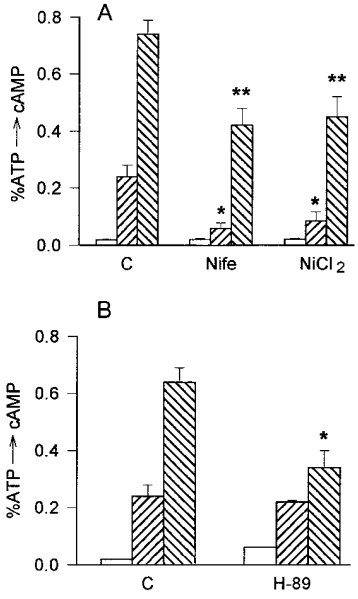

Figure 5. Effect of calcium channel blockers and protein kinase A inhibitor on ACTH-induced cAMP production in human glomerulosa cells. 3-d cultures of glomerulosa cells were labeled with [^{3}H]adenine and further incubated 15 min at 37°C as in Fig. 4 without effector (▭, *C*) or with 0.1 nM ACTH (▨) or 10 nM ACTH (▨). Cells were preincubated 10 min with selective calcium channel blockers (1 μM nifedipine or 200 nM $NiCl_2$) *A* or 10 min with 20 μM H-89, a specific protein kinase A inhibitor. Conversion of ATP into cAMP was calculated as described in Methods. Results are the mean±SEM of three distinct experiments, each conducted in triplicate. *$P < 0.01$; **$P < 0.001$ compared to corresponding ACTH-stimulated values.

ferently affected by Ca^{2+} channel blockers. 10-min preincubations with nifedipine (1 μM) or $NiCl_2$ (200 nM) abolished cAMP production induced by low concentrations of ACTH (0.1 nM), while stimulation induced by 10 nM ACTH was decreased by 50±5% (Fig. 5 *A*).

We also investigated the possible involvement of PKA in the control of ACTH-stimulated cAMP production. In glomerulosa cells preincubated 10 min with 20 μM H-89, cAMP production induced by low concentrations of ACTH was not affected, although stimulation induced by 10 nM ACTH was decreased by 58±7% (Fig. 5 *B*).

Effect of ACTH on intracellular calcium concentrations in human glomerulosa cells. Primary cultures of human glomerulosa cells loaded with Fura 2 were stimulated with either 100 nM ACTH or, as a control, with 100 nM AVP, a potent activator of phospholipase C in these cells (5). Fig. 6 *A* illustrates the time course of $[Ca^{2+}]_i$ changes induced by 100 nM ACTH in glomerulosa cells. The increase in intracellular Ca^{2+} response developed very slowly compared to AVP (Fig. 6 *B*). Latency periods averaged 2±1 min ($n = 6$). Once the plateau was reached (178±5 nM, 1.5±0.1-fold stimulation over basal $[Ca^{2+}]_i$, $n = 6$), stimulation was similar to that observed during the plateau phase of AVP (Fig. 6, *B* and *D*). This increase in intracellular Ca^{2+} by ACTH was completely blocked in Ca^{2+}-free medium (Fig. 6 *A*). Although the amplitude was lower, AVP still induced a transient spike of $[Ca^{2+}]_i$ in similar experimental conditions (Fig. 6 *B*).

When preincubated with 20 μM H-89 for 10 min at 37°C, glomerulosa cells did not exhibit any increase in $[Ca^{2+}]_i$ upon addition of ACTH (Fig. 6 *C*). However, under identical conditions, AVP was still able to mobilize intracellular calcium (Fig. 6 *D*). Similarly, when glomerulosa cells were preincubated with 1 μM nifedipine, ACTH was no longer able to increase $[Ca^{2+}]_i$ (Fig. 6 *E*). Under these conditions, nifedipine reduced the plateau response induced by AVP without affecting the transient calcium spike (Fig. 6 *F*).

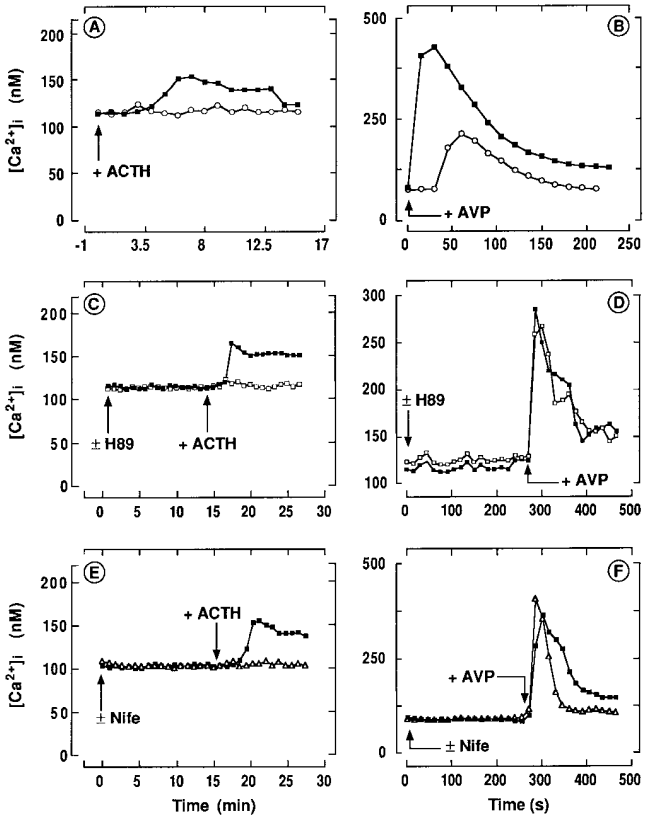

Figure 6. Influence of external calcium and protein kinase A blocker on intracellular $[Ca^{2+}]_i$ increase induced by ACTH in human glomerulosa cells. Glomerulosa cells were cultured for 3 d on glass coverslips and loaded with Fura 2 as described in Methods. (*A* and *B*) $[Ca^{2+}]_i$ in single glomerulosa cells were measured every minute after addition of 100 nM ACTH (*A*) or every 15 s after addition of 100 nM AVP (*B*), in medium containing either 1.8 mM $CaCl_2$ (●) or 100 nM $CaCl_2$ (○). (*C* and *D*) Cells were incubated in HBS medium containing 1.8 mM $CaCl_2$ in the presence (■) or absence (□) of 20 μM H-89. $[Ca^{2+}]_i$ was measured each minute (*C*) or each 15 s (*D*) after addition of H-89 or vehicle only. (*E* and *F*) Cells were pre-incubated 10 min in HBS buffer containing 1.8 mM $CaCl_2$ in the presence (△) or absence (■) of 1 μM nifedipine. 100 nM ACTH (*C* and *E*) or 100 nM AVP (*D* and *F*) were added to the medium when indicated by the arrow. $[Ca^{2+}]_i$ was measured each minute (*C* and *E*) or each 15 s (*D* and *F*) as described above. Results are the mean of 10 to 40 $[Ca^{2+}]_i$ measurements originating from one single experiment representative of three.

Effect of ACTH on Ca^{2+} currents. As previously described by our group, human glomerulosa possess both K^+ and Ca^{2+} currents (22). T- and L-type Ca^{2+} currents were studied after blocking outward currents and using a double pulse protocol as described previously (22). From a holding potential of −80 mV, a first pulse at −30 mV triggered the T-type current while a second pulse at +50 mV elicited the L-type component. As illustrated in Fig. 7 *A*, application of 10 nM ACTH in the bath had no significant effect on the amplitude of T-type current, but increased the L-type current by 64% ($n = 4$). This effect was seen 2 min after ACTH application (Fig. 7 *B*).

ACTH binding to human glomerulosa cell membrane preparation. To determine if Ca^{2+} primarily affects the binding of ACTH to its receptor, we measured ^{125}I-labeled ACTH binding in different experimental conditions. Binding was saturable and dose dependent. Scatchard analysis of the data revealed

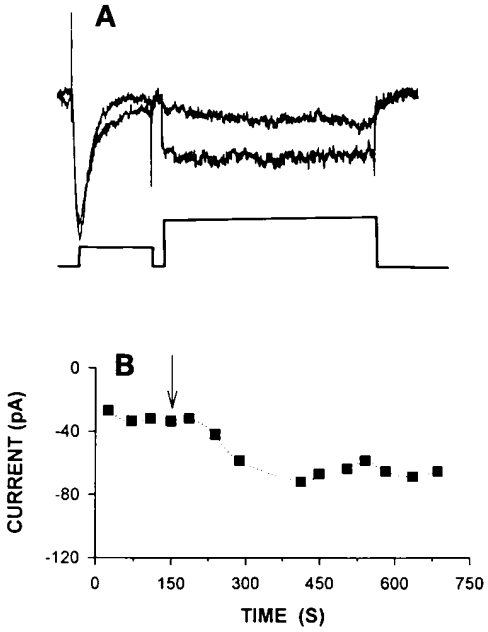

Figure 7. Effect of ACTH on calcium currents in human adrenocortical cells. Glomerulosa cells were studied after 24 h in culture according to methodology described in Methods. (*A*) T- and L-type Ca^{2+} currents were separated by using a double pulse protocol, with the first pulse at −30 mV for 150 ms and the second pulse at +50 mV for 300 ms. Scale: horizontal, 100 ms, vertical, 60 pA. (*B*) Time course of the effect of ACTH on the L-type Ca^{2+} current amplitude.

the presence of one class of binding sites exhibiting a K_d of 1.04±0.2 nM (n = 3) and maximal binding capacity (B_{max}) of 46.8±14.7 fmol/mg protein (n = 3). The absence of Ca^{2+} in the incubation medium slightly increased the affinity, but did not significantly affect the number of binding sites (K_d, 0.38±0.09 nM; B_{max}, 33.1±9.9 fmol/mg protein (n = 3). Moreover, specific binding of ACTH measured at a concentration allowing for 25% receptor occupancy was not significantly affected by the addition of either 1 μM nifedipine or 200 nM $NiCl_2$, two Ca^{2+} channel blockers that specifically block L- and T-type Ca^{2+} channels.

Discussion

The present study provides new insights into the stimulation-secretion coupling process and the mechanism by which ACTH stimulates Ca^{2+} influx in human adrenal glomerulosa cells. These studies have been conducted on enriched glomerulosa cell preparations cultured for 3 d. During this period, glomerulosa cells proliferate and maintain their ultrastuctural characteristics and functional properties and demonstrate similar or higher sensitivity to their known stimuli (reference 5 and this study). The most interesting result is that the Ca^{2+} ion is not required for ACTH binding to its receptor, but is essential for the stimulation of cAMP production. Morever, ACTH induces a Ca^{2+} influx abolished by the PKA inhibitor.

Dose-response curve analyses show that glomerulosa cells from adult human adrenal are very sensitive to ACTH. The high stimulation ratio obtained herein for aldosterone secretion (four- and sixfold) compared to that previously published (26) is probably due to the enriched cell preparation of glomerulosa cells. In the human cells, aldosterone secretion and cAMP production exhibit overlapping dose-response curves, with a stimulation threshold obtained at a concentration of 10 pM and an ED_{50} of 0.1 nM. Moreover, the absence of extracellular Ca^{2+} abolishes both cAMP production and aldosterone secretion despite the binding of ACTH to its receptor. These observations point out the crucial role of Ca^{2+} in the stimulating action of ACTH in human glomerulosa cells.

Aside from stimulation of cAMP production, ACTH also increases intracellular calcium. In contrast to that observed with AVP (or other Ca^{2+}-mobilizing hormones) (5, 32), this increase does not exhibit transient spikes. The amplitude of the increase in $[Ca^{2+}]_i$ is similar to that observed during the plateau phase of AVP and remains sustained. In fact, the intracellular increase induced by ACTH is similar to that previously observed for ACTH in rat and bovine glomerulosa cells (32, 33), as well as for parathyroid hormone in renal epithelial cells (34), or for folliculo-stimulating hormone in granulosa cells (35), all of which use cAMP as second messenger. The increase in intracellular Ca^{2+} is abolished if cells are incubated in Ca^{2+}-free medium, preincubated with H-89 or with nifedipine (Figs. 6, *A*, *C*, and *E*). These results indicate that the increase in $[Ca^{2+}]_i$ induced by ACTH is only due to Ca^{2+} influx, via an L-type Ca^{2+} channel. We therefore postulate that the activation of this dihydropyridine-sensitive Ca^{2+} channel arises from its phosphorylation, probably via the protein kinase A pathway, since we have previously observed in rat glomerulosa cells that cAMP could increase both $[Ca^{2+}]_i$ and L-type Ca^{2+} channel amplitude (24). In addition, we have recorded a depolarization of the membrane from a mean value of −35 mV to −25 mV after ACTH application (data not shown); this would favor the opening of L-type Ca^{2+} channels because of their voltage dependency.

Ca^{2+} and cAMP pathways act in synergy in human glomerulosa cells. First, we demonstrate that the increase in cAMP triggers Ca^{2+} influx. Second, many experimental evidences demonstrate that Ca^{2+} potentiates cAMP production: (*a*) in the absence of external Ca^{2+}, ACTH only transiently stimulates cAMP accumulation (Fig. 4 *A*); (*b*) blockers of Ca^{2+} channels abolish cAMP production induced by low concentrations of ACTH and decreased by as much as 50% the effect induced by maximal doses of ACTH (Fig. 5 *A*); (*c*) addition of H-89 blocks Ca^{2+} influx and decreases cAMP stimulation. One could postulate that these effects may be due to the loss of ACTH binding. However, experiments performed on membrane preparations from human glomerulosa cells indicate that ACTH binding is not Ca^{2+}-dependent. In addition, in our human glomerulosa cell preparations, nifedipine (1 μM) and $NiCl_2$ (200 nM), known to inhibit Ca^{2+} influx through L- and T-type Ca^{2+} channels, respectively (22), do not modify specific ^{125}I-labeled ACTH binding (data not shown). Data from Fig. 5 *A* indicate that threshold concentrations of ACTH are more sensitive to Ca^{2+} than at higher concentrations and suggest that adenylyl cyclase in human glomerulosa cells may be Ca^{2+} sensitive as previously shown in rat cells by Mahaffee and Ontjes (18). Indeed, recent studies clearly demonstrate that some adenylyl cyclases (types I, III, and VIII) are Ca^{2+} and Ca^{2+}-calmodulin sensitive (36, 37). Moreover, data from Fig. 5 *B* indicate that PKA activation may upregulate cAMP accumulation, probably via sustained Ca^{2+} influx.

From these complex cross talk interactions between cAMP and Ca^{2+} pathways, the following sequences of events can be proposed for the production of aldosterone in human glomer-

ulosa cells. Upon binding to its receptor, ACTH triggers exchange of GDP-GTP at the level of G_s coupling protein, thereby rapidly activating adenylyl cyclase and increasing cytosolic cAMP concentration. Cyclic AMP, by activating PKA, enables the phosphorylation of the L-type Ca^{2+} channels, which in turn increase their opening probability. This rise in opening probability increases Ca^{2+} influx, cytosolic Ca^{2+} concentration, and could participate in membrane depolarization. As ACTH-stimulated cAMP accumulation is sensitive to L-type Ca^{2+} channel blocker (Fig. 5 *A*), we can infer that Ca^{2+} influx further stimulates adenylyl cyclase and consequently steroid secretion. A positive feedback loop between adenylyl cyclase-PKA and Ca^{2+} channels ensures a slow but sustained $[Ca^{2+}]_i$ increase, as shown previously (34). These concomitant increases in intracellular calcium and cAMP act in synergy to fully stimulate steroid secretion since, as described previously, enzymes involved in the transformation of cholesterol into aldosterone or cortisol are sensitive to both second messengers (10, 11). In conclusion, our results demonstrate that, in human adrenal glomerulosa cells, ACTH-stimulated aldosterone secretion involves a tightly coupled interaction between cAMP and Ca^{2+} pathways.

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