Intracellular transport of calcium from plasma membrane to mitochondria in adrenal H295R cells: implication for steroidogenesis

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Abstract

Angiotensin II and extracellular potassium stimulate aldosterone production in adrenal glomerulosa cells by mobilizing the calcium messenger system. This response requires calcium influx across the plasma membrane, followed by calcium uptake into the mitochondria. It has been proposed that calcium is transported to the mitochondria via the lumen of the endoplasmic reticulum, acting as a kind of intracellular calcium pipeline. This hypothesis has been tested in the present study by measuring intramitochondrial calcium variations in H295R cells with a new fluorescent calcium probe, ratiometric pericam. Calyculin A, a protein phosphatase inhibitor, induced the formation of a large cortical layer of actin filaments, removing the peripheral endoplasmic reticulum away from the plasma membrane and thereby physically uncoupling the calcium channels from the pipeline. The mitochondrial calcium response to potassium was markedly reduced after calyculin treatment, but that of AngII was unaffected. Under the same conditions, potassium-stimulated pregnenolone and aldosterone production was significantly reduced, whereas the [...]
Intracellular Transport of Calcium from Plasma Membrane to Mitochondria in Adrenal H295R Cells: Implication for Steroidogenesis

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Angiotensin II and extracellular potassium stimulate aldosterone production in adrenal glomerulosa cells by mobilizing the calcium messenger system. This response requires calcium influx across the plasma membrane, followed by calcium uptake into the mitochondria. It has been proposed that calcium is transported to the mitochondria via the lumen of the endoplasmic reticulum, acting as a kind of intracellular calcium pipeline. This hypothesis has been tested in the present study by measuring intramitochondrial calcium variations in H295R cells with a new fluorescent calcium probe, ratiometric pericam. Calyculin A, a protein phosphatase inhibitor, induced the formation of a large cortical layer of actin filaments, removing the peripheral endoplasmic reticulum away from the plasma membrane and thereby physically uncoupling the calcium channels from the pipeline. The mitochondrial calcium response to potassium was markedly reduced after calyculin treatment, but that of AngII was unaffected. Under the same conditions, potassium-stimulated pregnenolone and aldosterone production was significantly reduced, whereas the steroidogenic response to AngII remained unchanged. The inhibitory action of calyculin A on the responses to potassium was not mediated by a modification of the calcium channel activity and was not accompanied by a reduction of the cytosolic calcium response. It therefore appears that, in H295R cells, the organization of the actin cytoskeleton at the cell periphery influences the steroidogenic action of potassium, but not the response to angiotensin II. The response to potassium is proposed to be dependent on the endoplasmic reticulum-mediated transfer of calcium entering through plasma membrane calcium channels to the mitochondria. (Endocrinology 144: 4575–4585, 2003)

Aldosterone production by adrenal glomerulosa cells in response to angiotensin II or extracellular potassium requires calcium influx, followed by calcium uptake into the mitochondria. Because both agents are known to depolarize the cells (1), in addition to enhance Ca\(^{2+}\) influx (2, 3), voltage-operated Ca\(^{2+}\) channels appear to be directly involved in the control of steroidogenesis. In glomerulosa cells, both low threshold (T-type) and high threshold (L-type) voltage-operated calcium channels have been characterized (4, 5). Recently, a clear dissociation between L- and T-type channel functions has been established, and Ca\(^{2+}\) entering through each channel appears to have distinct roles and destinations (6). L-Type channels are major mediators of the large cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{cyt}\)) variations observed in response to small, physiological increases in extracellular potassium concentrations, but selective inhibition of these channels does not markedly affect steroidogenesis (6, 7). In contrast, despite of the fact that the cytosolic Ca\(^{2+}\) increase resulting from T channel opening is barely detectable, the activity of T channels has been shown to be more closely related to aldosterone production (8, 9).

Although the angiotensin II- (AngII) and potassium-induced control of aldosterone synthesis and the involvement of voltage-operated calcium channels in this process have been extensively documented, much less is known about the mechanism by which calcium entering through T channels is forwarded to mitochondria, the cellular site where it will control rate-limiting steps of steroidogenesis. To take into account the functional specificity of calcium channels in glomerulosa cells and the poor diffusion of Ca\(^{2+}\) through the cytosol, we have proposed a model in which calcium would be transported from the T channels, embedded in the plasma membrane, to the mitochondria via the lumen of the endoplasmic reticulum (e.r.). The e.r. would therefore act like a kind of intracellular calcium pipeline, specifically connected to T channels (10). This hypothesis, however, implies 1) a nonrandom distribution of the calcium channels within the plasma membrane, and 2) the maintenance of a close physical relation between T channel-enriched regions of the plasma membrane and specific portions of the e.r., able to rapidly accumulate local ambient Ca\(^{2+}\). This second assumption has been tested in the present study using the human adrenocarcinoma aldosterone-producing cell line, H295R (11), by removing the e.r. away from the plasma membrane with calyculin A and therefore preventing the transfer of calcium from the channel into this putative pipeline.

Calyculin A is a serine/threonine phosphatase inhibitor that specifically blocks PP1 and PP2A enzymes and induces
major cytoskeletal reorganization within the cell. Indeed, it has been shown in several cell types that after treatment with this drug, normal stress fibers disappear rapidly from the center of the cytoplasm, actin filaments are redistributed at the periphery, and actin becomes tightly condensed near the plasma membrane (12, 13). In addition, condensed actin bundles can be observed inside the cells, which may reflect the interaction of actin filaments with intermediate filaments (14). Such in situ modification of the actin cytoskeleton by steric obstruction prevents coupling between the e.r. and the plasma membrane Ca2+ channels, while not affecting the inositol 1,4,5-trisphosphate-mediated chemical coupling between stimulated receptors and the release of stored Ca2+ (15). This approach has been recently used to demonstrate the requirement for a conformational interaction between intracellular Ca2+ stores and the plasma membrane during activation of the Ca2+ capacitative influx (15). Moreover, calyculin action was reversible, and disassembly of the cortical actin layer resulting from washing out the drug permitted reestablishment of coupling and reactivation of Ca2+ entry. Thus, a physical actin barrier, while permitting transmission of the diffusible messenger inositol 1,4,5-trisphosphate, prevents the close interaction between e.r. and the plasma membrane that is required to activate Ca2+ entry. Recently, a new fluorescent calcium probe, derived from circularly permuted green fluorescent proteins (GFP) and engineered to sense calcium, has been described for monitoring calcium signaling within cell organelles (16). Indeed, like any protein, ratiometric pericam can be specifically targeted, for example, to the mitochondria, where its fluorescent spectral properties (excitation wavelength) change reversibly with intramitochondrial calcium fluctuations. Thanks to its high fluorescence yield, the signals elicited by ratiometric pericam can be easily detected and analyzed at the subcellular levels.

In the present study the consequence of calyculin A treatment on the mitochondrial calcium signal triggered by extracellular potassium was assessed in H295R cells by recording intramitochondrial calcium variations with ratiometric pericam. Here we show that the formation of a large actin layer at the periphery of the cell significantly reduces the mitochondrial Ca2+ signaling and the activation of steroidogenesis in response to potassium, but only minimally affects the response to AngII. Moreover, this treatment does not modify channel activation elicited by cell depolarization or the rise of cytosolic calcium concentration induced by potassium, suggesting that only the transfer of Ca2+ from the channel to the mitochondria is affected by calyculin A.

Materials and Methods

ATP, BSA, tetraethylammonium-Cl, and HFPEs were purchased from Sigma-Aldrich Corp. (St. Louis, MO), and AngII was obtained from Bachem AG (Bubendorf, Switzerland). [H]Pregnenolone was obtained from NEN Life Science Products (Geneva, Switzerland), and antipregnenolone antiserum was purchased from Biogenes Ltd. (Poole, UK). Calyculin A was purchased from Alexis Biochemicals (Lausen, Switzerland), and fura-2/AM, MitoTracker Red, and Cs4-1,2-bis-(2-amino-phenoxy)ethane-N,N,N′,N′-tetracetic acid were obtained from Molecular Probes (Eugene, OR). WIN 19738 was purchased from Farillon (London, UK). Plasmids (pcDNA3) containing DNA coding for the mitochondrial ratiometric pericam or the mitochondria-targeted red fluorescent protein (mtRFP) were provided by Dr. Tullio Pozzan (University of Padova, Padova, Italy).

Cell culture

H295R cells were obtained from Dr. W. E. Rainey (University of Texas, Dallas, TX). Cells were grown in DMEM/Ham’s F-12 (1:1) medium with 15 mM HEPES, supplemented with 120 IU/ml penicillin, 120 µg/ml streptomycin, 6 IU/ml nystatin (Life Technologies, Inc., Gaithersburg, MD), 0.5 µg/ml fungizone (Squibb AG, Zurich, Switzerland), 40 µg/ml garamycin (ESSEX Chemie AG, Lucerne, Switzerland), 1% ITS Plus (insulin, transferrin, selenium, BD Biosciences, Bedford, MA), and 2% Ulotroser SF (BioSera SA, Villeneuve la Garenne, France). Cells were routinely grown in 75-cm2 flasks in a humidified atmosphere at 37 C (95% air/5% CO2). The medium was changed every 3 d.

Before experiments, cells were detached with 0.25% trypsin-EDTA and plated into petri dishes or on glass coverslips. A few days after trypsinization, the medium was replaced by DMEM/Ham’s F-12 without insulin/transferrin/selenium and Ulotroser during the 24 h preceding the start of the various treatments.

Endoplasmic reticulum, mitochondria, and actin filament fluorescence labeling

The e.r. was labeled by transfecting the H295R cells with a cytomegalo-virus promoter (pCMV) plasmid coding for an e.r.-targeted GFP (pCMV/myc/ER/GFP, Invitrogen, San Diego, CA). H295R cells (~200,000) were plated on 25-mm glass coverslips, and at about 60% confluence, they were incubated for 6 h at 37 C with the plasmid (340 ng/ml) mixed with Effecten and reagents (Qiagen, Basel, Switzerland), as indicated by the manufacturer. After transfection, cells were washed and left in culture medium supplemented with serum for 2-3 additional d before being employed for confocal microscopic analysis. In some preparations, cells were cotransfected with pCMV/myc/ER/GFP (260 ng/ml) and a pcDNA3 plasmid (80 ng/ml) containing an mtRFP. To visualize actin filaments in e.r.-labeled cells, transfected cells were grown on glass coverslips for 3 d before being fixed with 2% paraformaldehyde (Polysciences, Inc., Warrington, PA) in PBS (Life Technologies, Inc.) for 10 min at room temperature and washed three times in the same buffer with 8% NaCl for 10 min. Cells were then permeabilized in 0.5% Triton X-100 (Fluka Chemie AG, Buchs, Switzerland) in PBS for 10 min, washed three times in PBS with 1% BSA, and incubated for 10 min in this solution. Coverslips were subsequently inverted on a drop of 10 µg/ml rhodamine (TRITC)-phalloidin (Sigma-Aldrich Corp.) in PBS in the dark for 1 h at room temperature. Finally, coverslips were washed three times in PBS, and observation was begun after 20 min.

High resolution confocal microscopy

High resolution image acquisition was performed on a spinning disk confocal microscope. The incident beam from a 2.5-W Kr/Ar water-cooled ion laser in multline mode (Innova 70C Spectrum, Coherent, Santa Clara, CA) was directed on an Acousto-Optical Tunable Filter controlled for wavelength selection and intensity modulation by a four-channel multifrequency driver (AOTF from Visitech International, Sunderland, UK). The AOTF output was coupled by a single-mode fiber optic (Oz Optics, Ontario, Canada) to a Yokogawa spinning disk confocal scan-head (QLC100 from Visitech International, Sunderland, UK) mounted on an inverted microscope (Axiovert 200M, Carl Zeiss AG, Feldbach, Switzerland). Fluorescence images were collected by a ×100 1.4 NA Plan-Apochromat oil immersion objective (Carl Zeiss AG) and captured with a cooled, 12-bit TE/CCD interlined Cool Snap HQ Photometrics camera (Roper Scientific, Trenton, NJ). To record simultaneously mitochondria-targeted (mt) Pericam and Mitotracker Red fluorescence, both markers were excited at 488 nm, and emissions were separated with a 565 DCXR dichroic filter (Chroma Technology Corp., Brattleboro, VT) mounted on a Dual-View Micro-Imager (Optical Insights, LLC, Santa Fe, NM), which permits simultaneous acquisition of green and red emissions (D525/50m and D615/60m emission filters from Chroma Technology Corp.) on each half of the CCD image sensor. Wavelength selection with the AOTF, camera control, and all motorized functions of the microscope were controlled for image acquisition by
Mitochondrial calcium measurements

Mitochondrial calcium fluctuations were recorded using mt ratio-metric pericam probes (16). Plasmid (pcDNA3) containing DNA coding for ratiometric pericam fused in-frame on the 5′ extremity to the first 36 nucleotides of the mitochondrial cytochrome C oxidase subunit IV (Cox IV) was introduced in H295R cells by transient transfection with Effectene, as described above for r.e. labeling. After transfection (plasmid at 340 ng/ml for 9–16 h), cells were washed and left in culture medium supplemented with serum for 2–3 additional d before being employed in the experiments. Specific targeting of mt Pericam within the mitochondria was demonstrated in some cells by co-staining mt Pericam-expressing H295R cells with MitoTracker Red, CMXRos (Molecular Probes Europe BV, Leiden, The Netherlands). This cell-permeant, specific mitochondrial marker was introduced simply by incubating the cells in PBS medium for 2 min at room temperature in the presence of the dye (500 nM).

Variations in mitochondrial calcium concentrations were recorded by imaging pericam fluorescence on an Axiovert S100TV microscope, using a ×100 (or ×40), 1.3NA oil immersion objective (Carl Zeiss AG). Excitation was alternatively performed at 480 ± 10 and 410 ± 10 nm with the use of a DeltaRamp monochromator (Photon Technology International, Inc., Monmouth Junction, NJ) through a 505 DCXR dichroic mirror (Chroma Technology Corp.) and emission filtered at 535 nm (Spectra, Inc., Sage, VT). Image acquisition (1 image every 10–15 sec at each excitation wavelength), and analysis was performed with the Metamorph/Metalfiuor 4.1.2 software. Fluorescence ratio (fluorescence intensity at 480 nm minus background)/fluorescence intensity at 410 nm minus background) was calculated pixel by pixel and averaged within each manually delimited entire cell. Changes in fluorescence ratio (reflecting mitochondrial calcium variations) were then expressed as a function of time.

Electrophysiological recordings

Patch-clamp recordings were performed in the whole cell configuration using an Axopatch 1D patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA). Membrane currents, elicited by cell depolarization as indicated in the figure legends, were filtered at 1–2 kHz and sampled at 5 kHz using a TL-1–125 interface (Axon Instruments). The bath solution contained 117 mM tetraethylammonium chloride, 20 mM BaCl₂, 0.5 mM MgCl₂, and 37 mM sucrose and was buffered to pH 7.5 with 10 mM HEPES (CsOH). The patch pipette (3–6 MOhm; Clark 150T, Reading, UK) were filled with 85 mM CsCl, 10 mM tetraethylammonium chloride, 6 mM MgCl₂, 5 mM Mg-ATP, 0.04 mM Na-GTP, 0.9 mM CaCl₂, and 11 mM Cs₂-1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; pH was adjusted to 7.2 with 20 mM HEPES (CsOH). Cells were relatively homogenous in size, with a membrane capacitance of 8.19 ± 1.86 pF (mean ± se, n = 29). The leak current was automatically subtracted by a P/4 protocol (pClamp V.6, Axon Instruments, Inc.).

Cytosolic calcium measurements

For cytosolic calcium measurements, H295R cells were trypsinized and maintained in solution (6 × 10⁶ cells/ml) in a Krebs medium (containing 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 1.2 mM CaCl₂, and 5.5 mM g-glucose, buffered to pH 7.4 with 20 mM HEPES) supplemented with 1% BSA. Cells were incubated for 45 min at 37 C in the presence of 2 μM fura-2/AM. After washing the excess dye, cells were incubated for 20 min at 37 C in Krebs medium before being used for determination of the fura-2 fluorescence (after correction at 340 and 380 nm and emission at 500 nm) in a CAF-110 fluorometer (Jasco, Hachioji City, Japan). The fluorescence signal was digitized (DaQSys 2.0, Sicmu, University of Geneva), and cytosolic calcium was calibrated using the ratio values of emitted fluorescence (340/380 nm) as described by Grynkiewicz et al. (17).

Determination of steroid production

Pregnenolone and aldosterone productions were determined by direct RIA of the steroids in the cell medium. Cells were incubated in a Krebs medium containing either 3 mM KCl (basal production) and 12 mM KC1 or 3 mM KCl and 10⁻² M AngII. For this purpose, H295R cells were cultured for 3 d in 24-well plates. The day of the experiment, 10 mM calyculin A or vehicle (control cells) was added in wells for 3.5 h. At the time of stimulation, cells were washed with a Krebs buffer and incubated for 1.5 h in the same buffer, supplemented or not with 9 mM KCl (12 mM final concentration) and 10⁻² M AngII, and with calyculin A or vehicle. For pregnenolone measurement, WIN 17578 (5 μM) was present in wells throughout the stimulation period to prevent the conversion of pregnenolone to progesterone. At the end of the incubation, the ambient medium was removed for pregnenolone and aldosterone assays, as previously described (18, 19). Aldosterone RIA was performed using a commercially available kit (Diagnostic Systems Laboratories, Inc., Webster, TX). Adherent-stimulated cells were washed and detached in NaOH (0.5 M) to determine protein content by the Coomassie Blue method (Bio-Rad Laboratories, Inc., Munich, Germany).

Statistics

Results are expressed as the mean ± sem unless stated otherwise. The statistical significance of the changes induced by treatments was analyzed by unpaired t tests.

Results

Effect of calyculin A on H295R cell morphology and actin distribution

Calyculin A was used to create a physical barrier of actin between the cell plasma membrane and the mitochondria to disturb the intracellular calcium pathway putatively responsible for steroid synthesis. We first assessed, by fluorescence imaging, whether calyculin A effectively acts on actin filament distribution in H295R cells as previously described in other cell types. For this purpose, the e.r. of H295R cells was first labeled in green by transfecting the cells with a pcMV plasmid coding for an e.r.-targeted GFP, and actin microfilaments were then stained in red, after fixing the cells, with rhodamine-phalloidin (Fig. 1, A–F). Confocal Nipkow microscopy analysis revealed that under control conditions, actin filaments are evenly dispersed throughout the cytosol between the e.r. network (Fig. 1A), and most importantly, that the peripheral e.r. freely reaches the edge of the cell, where it apparently can make physical contact in some places with the plasma membrane (Fig. 1B). In contrast, after treatment with calyculin A (10 nM, for 3.5 h), a significant reorganization of the actin microfilaments toward the periphery of the cell was observed (Fig. 1C). Indeed, a redistribution of actin staining into a tight cortical layer, subjacent to the plasma membrane, was visible and prevented the e.r. from interacting with the plasma membrane by physically excluding the organelle from the vicinity of the membrane (Fig. 1D). After treatment, some cells became more rounded and sometimes plasma membrane surface blebs were apparent (Fig. 1E). These budding structures were also delimited by a ring of filamentous actin and contained a portion of the e.r. This effect, associated with the presence of a thicker actin layer (Fig. 1F) and a propensity of the cells to detach from petri dishes, was attributed to the disappearance of stress fibers.
and was observed in an increased number of cells upon longer treatments or when higher concentrations of calyculin were used. However, the effect of the drug was reversed within 1 h after washing out calyculin A (not shown).

The presence of actin-labeled cells that are negative for e.r.-GFP in the various images (Fig. 1, A, C, and E) reflects the 100% efficiency of actin labeling after cell fixation compared with the modest e.r. labeling by transfection in 35–45% of the cells.

We also tested the possible effect of calyculin A on the interaction between the e.r. and the mitochondria after cotransfecting the cells with the e.r.-GFP and an mtRFP. The distribution of mitochondria, which appears as red oblong structures by confocal microscopy (Fig. 1G), among the e.r. network suggests the presence in control H295R cells of multiple sites of interaction between these organelles, as we have previously observed at the level of the electron microscopy resolution in bovine adrenal glomerulosa cells (20). The effect of calyculin A was determined by analyzing the distribution of the e.r. (Fig. 1, H and I) and the mitochondria...
(Fig. 1, J and K) in the same cell, before and after the addition of the drug. Despite a clear rounding of the cell induced by calyculin, no obvious redistribution of the organelles was observed, suggesting that the interaction between the e.r. and the mitochondria should not be affected by the treatment.

**Inhibition by calyculin A of the potassium-induced mitochondrial calcium response**

We then assessed the effect of calyculin A treatment on mitochondrial calcium in H295R cells by recording the fluctuations in the calcium concentration within the mitochondrial matrix with the fluorescent probe, ratiometric pericam. Three days after cell transfection with DNA coding for the mitochondrial pericam, the fluorescence emitted by this probe was punctuated and essentially perinuclear in most of the cells, limited to regions of the cytoplasm known to be rich in mitochondria (Fig. 1M). As expected, the nucleus was devoid of fluorescence. Nevertheless, the specificity of pericam targeting within the right organelles was confirmed by comparing, in the same cells, its distribution with that of MitoTracker Red, a recognized mitochondrial marker (Fig. 1L). Images of ratiometric pericam and of MitoTracker fluorescence distribution obtained by confocal microscopy were almost identical, confirming that the pericam expressed in H295R cells under our experimental conditions was correctly addressed to the mitochondria. The cell-permeant Mito-Tracker Red labeled mitochondria in all cells, whereas only 35–45% cells were transfected with Pericam, which explains the presence of a red signal in some cells negative for pericam.

Typical mitochondrial responses to stimulation by potassium and AngII in control (untreated) and calyculin A-treated H295R cells are shown in Fig. 2A. The stimulation of control cells by 12 mM KCl led to a biphasic response, with an immediate transient increase in the mitochondrial calcium concentration ([Ca^{2+}]_{m}) followed by a more sustained phase. This response was markedly reduced in cells treated for 3.5 h with 10 nM calyculin A (lower trace). In contrast, the [Ca^{2+}]_{m} response to AngII (100 nM), which was also biphasic, was only minimally affected by calyculin A treatment. After AngII stimulation, 1 μM carbonyl cyanide m-chlorophenyl-hydrazone, a proton ionophore, was added in the medium to abolish the mitochondrial H^{+} gradient and therefore the driving force for Ca^{2+} uptake into the organelles. This inhibition led to a rapid decrease in the fluorescence ratio, confirming the mitochondrial localization of pericam. Finally, ionomycin (2 μM), a calcium ionophore, was used to elicit a maximal calcium increase within the mitochondria, which was used to normalize the previous responses.

The amplitude of the response to each agonist, expressed as a percentage of the response to ionomycin, was highly variable from one cell to the other, with a coefficient of
variation greater than 75%. Some control cells (20%) responded better to AngII than to KCl, whereas others (30%) were more sensitive to potassium. Nevertheless, as shown in Fig. 2B, calyculin A pretreatment induced a significant decrease in the $[\text{Ca}^{2+}]_{\text{m}}$ response to KCl in H295R cells. On the average, the response was reduced by 77% compared with that of control cells. In contrast, the response to AngII in the same cells was much less affected (17%) by calyculin A, a decrease that was not statistically significant.

To determine whether the inhibition of the mitochondrial signal induced by calyculin A was due to the formation of the cortical actin layer or to the reduction of the microfilament network, we used additional experiments in which cells (n = 11) were pretreated for 1.5 h with a cocktail of cytoskeleton inhibitors composed of 10 μM cytochalasin B, 20 μM colchicine, 10 μM demecolcine, and 10 μM nocodazole. The effect of this treatment, which normally does not affect the interaction between the e.r. and the plasma membrane (15), could be easily visualized by a change in cell morphology (not shown) and was accompanied by a reduction of the mitochondrial calcium response to both potassium and AngII, by approximately 70% and 35%, respectively. These data suggest that the integrity of the cytoskeleton at the proximity of the mitochondria could also be required to maintain the e.r. closely connected to these organelles and to allow the transfer of calcium from the pipeline into the mitochondria.

**Lack of effect of calyculin A on the activation of voltage-operated calcium channels**

To exclude the possibility that the action of calyculin A on the $[\text{Ca}^{2+}]_{\text{m}}$ response to KCl was simply due to impairment of channel activation upon cell depolarization, we analyzed channel activity in control and treated H295R cells with the patch-clamp technique, in the whole cell configuration. The presence of both low threshold, T-type, and high threshold, L-type, calcium channels in these cells has been previously demonstrated (21). To discriminate between T- and L-type currents, we used distinct electrophysiological properties of the corresponding channels, namely the slower deactivation of T channels and the slower inactivation of L channels (21).

Thus, for determining T-type channel activity, tail current was elicited by repolarizing the cell at $-65 \text{ mV}$ after a 20-msec depolarization at $+20 \text{ mV}$ from a HP of $-90 \text{ mV}$ (Fig. 3A). The slowly deactivating Ca$^{2+}$ current evoked upon cell repolarization was exclusively due to T channel activity because of the very rapid L channel deactivation at this voltage ($t_{1/2} \sim 0.2 \text{ msec}$). Tail current was fitted to a single exponential function, and the value of T current present at the time of membrane repolarization was then determined by extrapolation.

To specifically determine L-type current amplitude, we depolarized the cell from a holding potential (HP) of $-90 \text{ mV}$ to 0 mV and maintained this potential for 600 msec (Fig. 3B). The amplitude of the inward current was measured 500 msec after the depolarization, when T-type channels were almost completely inactivated.

The density of T-tail currents (i.e. the amplitude of the current divided by the cell capacitance) was $22.5 \pm 3.4 \text{ pA/pF}$ (mean $\pm \text{SEM}; n = 13$), and that of L-type currents was $9.0 \pm 1.3 \text{ pA/pF}$ ($n = 9$) in control cells. We did not observe any significant modification in T-type (24.4 $\pm 3.4 \text{ pA/pF}; n = 11$) or L-type ($6.1 \pm 0.9 \text{ pA/pF}; n = 9$) current densities after treatment of the cells for 3.5 h with 10 nM calyculin A (Fig. 3C).

Because T-type channels have been directly involved in the stimulation of steroidogenesis by potassium in adrenal glomerulosa and H295R cells, we also verified that calyculin A did not modify other electrophysiological properties of this channel. The dependence of T channel opening on the membrane potential was assessed by establishing activation and steady state inactivation curves as a function of voltage, as previously described in detail (22). As shown in Fig. 3D, the half-potential of channel activation was similar in normal ($-31.3 \pm 0.9 \text{ mV}; n = 12$) and calyculin A-treated cells ($-28.5 \pm 1.15 \text{ mV}; n = 10$). Similarly, calyculin A did not affect the half-potential of channel steady state inactivation, which was $-47.4 \pm 1.2 \text{ mV}$ in control cells ($n = 6$) and $-44.7 \pm 1.1$ in treated cells ($n = 8$). As a consequence, the size of the permissive window of voltage, in which activation and inactivation curves overlap and which allows a sustained influx of calcium through T channels, was not reduced after calyculin A treatment.

**Lack of effect of calyculin A on the cytosolic calcium responses induced by KCl or AngII**

We also carried out experiments to assess whether the cytosolic calcium ($[\text{Ca}^{2+}]_c$) rise induced by KCl and AngII in H295R cells was affected by calyculin A treatment. For this purpose, fura-2-loaded cells were stimulated, after 3.5-h pretreatment in the presence or absence of 10 nM calyculin A, consecutively with 12 mM KCl, 100 nM AngII, and 2 μM ionomycin. As shown in Fig. 4A, KCl-induced a small, but relatively sustained, $[\text{Ca}^{2+}]_c$ response in both control and calyculin A-treated cells. The amplitude of this response to KCl, which has been shown to be essentially due to activation of L-type channels in adrenal glomerulosa cells (6), was not significantly different in the cell groups (Fig. 4B). Compared with the response induced in the same cells by the calcium ionophore ionomycin, the $[\text{Ca}^{2+}]_c$ response elicited by KCl was $9 \pm 2\%$ ($n = 11$) in control cells and $7 \pm 1\%$ ($n = 12$) in calyculin A-treated cells.

The response to AngII was clearly biphasic and involved Ca$^{2+}$ release from intracellular stores as well as voltage-dependent and -independent calcium entry pathways (19). Once again, calyculin A treatment had no significant effect on the size of the peak responses, which, on the average, were $32 \pm 4\%$ ($n = 11$) and $27 \pm 3\%$ ($n = 12$) of the response to ionomycin in control and calyculin A-treated cells, respectively.

**Inhibition of KCl- and AngII-stimulated steroidogenesis by calyculin A**

Finally, we investigated the ability of calyculin A to inhibit the steroidogenic function of H295R cells. For this purpose, we carried out experiments to evaluate the production of two steroids: pregnenolone, which is the first metabolite of cholesterol produced in the steroidogenic cascade, and aldoste-
roneline (Fig. 5). Basal steroid production was not inhibited by 3.5-h pretreatment with 10 nM calyculin A. In contrast, calyculin A significantly decreased both pregnenolone (Fig. 5A) and aldosterone (Fig. 5B) responses to 12 mM KCl by 57% and 39%, respectively (the drug effect on the response was calculated after subtracting basal values). In the case of stimulation with AngII (100 nM), pregnenolone formation was only slightly inhibited (by 24%) by calyculin A, whereas aldosterone was not affected by the drug. To exclude the possibility that calyculin A, by increasing the phosphorylation level of the cell, directly affects the activity of the various steroidogenic enzymes (what would be, however, in apparent contradiction with the lack of effect of the drug on the aldosterone response to AngII), we also tested the effect of calyculin on the aldosterone production supported by an analog of cholesterol. Indeed, 25-hydroxycholesterol freely enters the mitochondria and can therefore be converted into steroids independently from agonist stimulation and calcium mobilization (23). We found in this independent series of experiments that the aldosterone produced upon stimulation with KCl was reduced to 59.5 ± 13.5% (n = 3) of control values after treatment with calyculin, but the conversion of

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**Fig. 3.** Lack of effect of calyculin A treatment on voltage-gated calcium currents in H295R cells. Cells plated on glass coverslips were treated for 3.5 h with 10 nM calyculin A or vehicle immediately before being used for the patch-clamp experiments in the whole cell configuration. When appropriate, calyculin A was maintained in the recording solution. A, Example of a slowly deactivating tail current elicited upon repolarization of a control cell to −65 mV after a 20-msec period of channel activation at +20 mV from a HP of −90 mV. The amplitude of the maximal T-type current was determined by extrapolating the tail current fitted to a single exponential. B, Inward current activated, in the same cell as in A, by a step depolarization from −90 to 0 mV and maintained for 600 msec. The amplitude of the L-type current was measured at 500 msec after depolarization. C, The amplitude of T- and L-type currents was determined in control and treated cells using the voltage protocols indicated in A and B and then normalized for the cell capacitance (mean ± SEM; n = 9–13 cells). D, The voltage-dependent activation and steady state inactivation properties of T channels were examined in the same cells with the patch-clamp technique as previously described (22). Slowly deactivating currents were recorded at −65 mV after 20-msec activation at various potentials or after steady state inactivation for 10 sec at various potentials, followed by a 20-msec stimulation at +20 mV. The amplitude of the maximal tail current occurring upon repolarization to −65 mV was determined and plotted as a function of test potential to be fitted to Boltzmann's equation. The parameters of the equation were then determined for each cell, and the currents were normalized to the maximal current (I0) before being averaged. Mean activation (○ and ■) and steady state inactivation (□) and ■) curves were established for control and calyculin A-treated cells (mean ± SEM; n = 6–12 cells).
The parallel and reversible inhibition of the capacitative influx observed in these studies strongly suggested that a physical interaction with a portion of the e.r. was mandatory for activating store-operated calcium channels, and a model of regulation of the capacitative influx, based on vesicle trafficking and docking on plasma membrane, has been proposed (15).

A similar mechanism is believed to occur in aldosterone-producing cells, where a specific interaction between plasma membrane T-type calcium channels and the e.r., acting in these cells as a sort of intracellular calcium pipeline to convey the cation to the mitochondria (10), would be required to maintain the functional specificity attributed to the various calcium channels (6, 7). Indeed, the fact that selective activation of L channels leads to a large increase in \([\text{Ca}^{2+}]_c\), but to a poor steroidogenic response, strongly suggests that rate-limiting steps controlled by calcium are not located within the cytosol. This assumption is reinforced by the observation that stimulation of steroid production in response to in-

Discussion

In this study we show that restructuring actin cytoskeletal organization at the periphery of H295R cells with calyculin A leads to an impairment of calcium signal transduction from the plasma membrane to the mitochondria that normally occurs upon cell stimulation with extracellular potassium and therefore reduces the steroidogenic response to this agonist. This inhibitory action of calyculin A was observed without any change in calcium channel activity or modification of the size of the cytosolic calcium response.

This approach had been previously used by others to demonstrate, in smooth muscle (15), platelet (24), and lymphocyte (25) cell lines, that in situ redistribution of filamentous actin into a tight cortical layer subjacent to the plasma membrane displaces cortical e.r. and prevents coupling between the e.r. and the plasma membrane \(\text{Ca}^{2+}\) channels while not affecting inositol 1,4,5-trisphosphate-mediated store release.

10 \(\mu\text{M}\) 25-hydroxycholesterol into aldosterone in calyculin-treated cells was 105.3 ± 17.7% (\(n = 3\)) of that measured in the respective control cells. Aldosterone production from 25-hydroxycholesterol-supplemented cells was quantitatively similar to that elicited by KCl in untreated cells. Thus, this result demonstrates that the ability of the steroidogenic enzymes to convert cholesterol into aldosterone remains intact in H295R cells after treatment with calyculin A.

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creased ambient calcium in permeabilized glomerulosa cells is sensitive to ruthenium red, an efficient blocker of mitochondrial calcium uptake (26), as well as by the demonstration of activation of mitochondrial cholesterol transport by increasing cytosolic calcium (23, 27). In addition, important modification of steroidogenesis follows pharmacological modulation of T-type channel activity, which results in barely detectable changes in [Ca\(^{2+}\)]\(_{\text{c}}\) (8, 9, 21, 28). It was therefore proposed that calcium entering the cell through T channels (the steroidogenic calcium) must be conveyed to the mitochondria (the steroidogenic organelles) without crossing the cytosol, in which the diffusion of the cation is known to be only limited to microdomains (Fig. 6A). Microscopic morphological analysis of adrenal glomerulosa cells revealed the presence of a continuous e.r. network in contact with both regions of the plasma membrane and many mitochondria (20), suggesting that the e.r. could be responsible for this intracellular calcium transport, and a specific docking process of the e.r. close to T channels has been proposed as an important factor allowing the cell to discriminate between calcium flowing through T and non-T channels.

We propose that upon treatment of the cells with calyculin A, removing the e.r. away from the plasma membrane by condensation of cortical actin results in a failure to fill up the pipeline and therefore to supply mitochondria with calcium. This model is fully supported by our observation that the [Ca\(^{2+}\)]\(_{\text{m}}\) response to potassium is significantly reduced by calyculin A as well as the production of steroids, particularly that of an early intermediate such as pregnenolone, which is highly sensitive to calcium variations (23, 29).

The mitochondrial response to AngII was much less affected by calyculin A. In fact, we analyzed the peak response to the hormone, reflecting essentially the release phase of the signal. This lack of effect of calyculin A confirmed the observation previously made in other cell types that the inositol 1,4,5-trisphosphate response is insensitive to this drug (15, 25) and that calcium stores are not altered, nor is the ability of the mitochondria to accumulate calcium. The plateau phase of the response to AngII was not taken into account because of the complexity of the factors contributing to its maintenance. Indeed, the plateau level is not only the integration of the activation of multiple voltage-dependent and

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**Fig. 6.** Hypothetical model of intracellular calcium transport from T channels to mitochondria. A, Control cells: docking of a portion of the e.r. network in regions of the plasma membrane specifically enriched in T channels facilitates the transport of calcium entering the cell through these channels to the mitochondria, where the cation stimulates early and limiting steps of steroidogenesis. Other channels, not linked to the pipeline, are less efficient for stimulating steroid production. B, Calyculin A treatment: after reorganization of the cortical actin filaments induced by calyculin A, this physical barrier uncouples T channels from the pipeline, decreasing the transfer of calcium to the mitochondria, and therefore steroidogenesis. Calyculin treatment is not expected to modify channel activity or the cytosolic response.
-independent influx pathways, but is also modulated by the activity of the calcium extrusion mechanisms (30). AngII has been shown to modulate most of these factors (31), in many cases through the control of protein kinases and phosphatases (22, 32, 33), that could be a priori directly affected by calyculin A treatment. Moreover, steroid production upon AngII challenge is not really expected to faithfully reflect Ca$^{2+}$ transport into the mitochondria because Ca$^{2+}$-dependent and -independent mechanisms have been clearly shown to coexist in the control of steroidogenesis by the hormone (23, 32, 34). However, AngII has been used in this study essentially to demonstrate that calyculin A by itself does not impair calcium release from the stores, calcium uptake into the mitochondria, or the steroidogenic capacity of the cells.

In contrast, the response elicited by extracellular potassium in H295R cells and leads to the activation of steroidogenesis. Although not proving it, these observations support our model of intracellular transport of the steroidogenic calcium from the plasma membrane to the mitochondria through a physical pipeline formed by the e.r. network. Additional work is now necessary to identify the molecular determinants responsible for maintaining this pipeline in place and for regulating its activity.

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