Measurement of perimitochondrial Ca^{2+} concentration in bovine adrenal glomerulosa cells with aequorin targeted to the outer mitochondrial membrane

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Microdomains of high cytosolic free Ca^{2+} concentration in the proximity of mitochondria might have an important role in the stimulation of steroidogenesis in bovine adrenal glomerulosa cells. In the present study we have investigated local changes of free Ca^{2+} concentration near the outer mitochondrial membrane ($[\text{Ca}^{2+}]_{\text{om}}$) under stimulation with angiotensin II (Ang II) and K^+ . Glomerulosa cells in primary culture were transfected with a recombinant cDNA encoding the N-terminal region of the human translocase protein 20 of the outer mitochondrial membrane, in frame with the Ca^{2+} -sensitive photoprotein aequorin. This chimaeric aequorin (TomAeq) was associated with mitochondria-enriched subcellular fractions of transfected COS-7 cells and was susceptible to proteinase K, showing that it was targeted to the outer mitochondrial membrane, facing the cytosolic space. In bovine adrenal glomerulosa cells transfected with TomAeq cDNA, Ang II induced a transient $[\text{Ca}^{2+}]_{\text{om}}$ peak reaching

$1.42 \pm 0.28 \mu\text{M}$, which decreased immediately to the basal resting value. The peak response to Ang II was strikingly lower than the peak response of mitochondrial free Ca^{2+} concentration, which increased to $5.4 \pm 1.2 \mu\text{M}$. The smaller response of $[\text{Ca}^{2+}]_{\text{om}}$ to Ang II compared with the elevated matrix response did not result from buffering effects of the organelle, from altered mechanisms of intramitochondrial Ca^{2+} transport or from differences in the affinity of the chimaeric aequorins for Ca^{2+} . This approach has allowed us to follow perimitochondrial Ca^{2+} homeostasis in bovine glomerulosa cells under stimulation with Ca^{2+} -mobilizing agonists and to reveal a strong gradient of Ca^{2+} concentration between the mitochondrial matrix and the immediate environment of the organelle.

Key words: aldosterone, angiotensin II, mitochondrion, Tom20 (translocase protein 20).

INTRODUCTION

In the zona glomerulosa cell of the adrenal cortex, the octapeptide hormone angiotensin II (Ang II) and K^+ ions stimulate aldosterone biosynthesis [1]. The acute activation of steroidogenesis is mediated by increases in intracellular $[\text{Ca}^{2+}]$ elicited by these two agonists through distinct mechanisms [2–6].

Ang II triggers a biphasic response of intracellular $[\text{Ca}^{2+}]$. On binding to its receptor, the hormone stimulates the formation of $\text{Ins}(1,4,5)\text{P}_3$, which is responsible for the release of Ca^{2+} from intracellular stores. The depletion of intracellular Ca^{2+} pools is followed by a sustained plateau of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{c}}$) resulting from the activation of two distinct membrane Ca^{2+} channels: the capacitative, Ca^{2+} release-activated Ca^{2+} (CRAC) channels and the voltage-operated Ca^{2+} (VOC) channels [7]. In contrast, K^+ opens exclusively VOC channels by depolarizing the cell membrane [8,9], involving an activation of both low-threshold (T-type) and high-threshold (L-type) Ca^{2+} channels. Although the precise intracellular target(s) of the Ca^{2+}

messenger have not yet been identified, it seems likely that the mitochondrion, which contains essential steroidogenic enzymes, is an important site of regulation for steroidogenesis [10–12].

The study of mitochondrial free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{m}}$) changes occurring in intact cells under stimulation with physiological agonists can therefore yield important clues to understanding how Ca^{2+} induces the activation of crucial steps of mineralocorticoid biosynthesis. Indeed, we have previously investigated the $[\text{Ca}^{2+}]_{\text{m}}$ responses to Ang II and K^+ in bovine adrenal glomerulosa cells transfected with a mitochondrial matrix-targeted aequorin (mtAeq) cDNA [11]. The use of this Ca^{2+} -sensitive photoprotein showed that $[\text{Ca}^{2+}]_{\text{m}}$ rises occur concomitantly with the $[\text{Ca}^{2+}]_{\text{c}}$ changes when cells are stimulated with either Ang II or K^+ . The transient $[\text{Ca}^{2+}]_{\text{c}}$ response to Ang II was amplified approx. 5-fold within the mitochondria, whereas the K^+ -induced sustained $[\text{Ca}^{2+}]_{\text{c}}$ elevations were similar in both the cytosol and the mitochondria. These observations were in agreement with results showing that vasopressin- or Ang II-induced intracellular Ca^{2+} release was more efficient than the

Abbreviations used: Ang II, angiotensin II; $[\text{Ca}^{2+}]_{\text{c}}$, cytosolic free Ca^{2+} concentration; $[\text{Ca}^{2+}]_{\text{om}}$, perimitochondrial free Ca^{2+} concentration; $[\text{Ca}^{2+}]_{\text{m}}$, mitochondrial free Ca^{2+} concentration; cytAeq, mtAeq lacking the targeting mitochondrial presequence cDNA ('non-targeted aequorin'); DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; hTom20, human translocase protein 20 of the outer mitochondrial membrane; mtAeq, mitochondrial matrix-targeted aequorin; TomAeq, aequorin targeted to the outer mitochondrial membrane; VOC channel, voltage-operated Ca^{2+} channel.

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Ca^{2+} influx evoked by K^+ in stimulating Ca^{2+} -dependent mitochondrial NAD(P)H formation in rat adrenal glomerulosa cells [13].

Several other groups have reported, for various cell types, that stimulation by $\text{Ins}(1,4,5)\text{P}_3$ -producing agonists evoked a transient Ca^{2+} response of much higher amplitude in the mitochondria than in the cytosol. This phenomenon has been tentatively attributed to a close proximity of mitochondria and Ca^{2+} storage compartments of the endoplasmic reticulum, resulting in so-called microdomains of high Ca^{2+} concentration [14–17].

In theory, it should be possible to test the above hypothesis by anchoring a Ca^{2+} probe in the outer mitochondrial membrane, facing the cytosol. Here we describe the construction of a new chimaeric aequorin specifically targeted to the outer mitochondrial membrane, by fusing aequorin with the transmembrane domain of a component of the translocase of the outer mitochondrial membrane machinery. This approach allowed us to follow perimitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{om}}$) changes in bovine glomerulosa cells under stimulation with Ca^{2+} -mobilizing agonists.

MATERIALS AND METHODS

Materials

Ionomycin was purchased from Calbiochem (Lucerne, Switzerland) and $[\text{Ile}^5]\text{Ang II}$ from Bachem (Bubendorf, Switzerland). Nifedipine, digitonin, cyclosporin A, 2-mercaptoethanol, dithiothreitol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP), proteinase K, PMSF and Triton X-100 were supplied by Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and L-glutamine were purchased from Gibco (Grand Island, NY, U.S.A.). Coelenterazine was obtained from Molecular Probes (Leiden, The Netherlands). Glycerol and Bromophenol Blue were purchased from Aldrich (Buchs, Switzerland) and Tween 20 from Merck (Dietlikon, Switzerland). BayK8644 was supplied by Bayer (Leverkusen, Germany). Restriction enzymes *Ava*II and *Hind*III were obtained from Promega (Madison, WI, U.S.A.), the ^{32}P Sequencing[™] Kit from Pharmacia Biotech (Uppsala, Sweden) and pcDNA1.1 from InVitrogen (Leek, The Netherlands). The other reagents used for molecular cloning and cell culture reactants have been described elsewhere [2,18].

Chimaeric aequorin cDNA constructs

$[\text{Ca}^{2+}]_{\text{m}}$ was measured in bovine adrenal glomerulosa cells transfected with a plasmid coding for mtAeq and $[\text{Ca}^{2+}]_{\text{c}}$ was determined in cells transfected with the same plasmid lacking the targeting mitochondrial presequence cDNA (cytAeq), as described previously [11].

To construct the chimaeric cDNA for aequorin targeted to the outer mitochondrial membrane (TomAeq), we isolated the *Hind*III cDNA fragment coding for the first 46 residues of the human translocase protein 20 of the outer mitochondrial membrane (hTom20) from the pBluescript SK⁺ vector containing the entire hTom20 cDNA sequence [19], inserted at the *Eco*RV–*Not*I site. We subsequently cloned this *Hind*III fragment into the *Hind*III site of the pcDNA1.1 plasmid coding for cytAeq [20]. Screening of positive recombinant clones was done by enzymic digestion with *Ava*II to verify the correct orientation of the insert. Positive clones were then transfected into glomerulosa cells as described below, leading to luminescence when cells were lysed in the presence of CaCl_2 , whereas clones containing the insert in the reverse orientation did not express functional

aequorin. The subcloned DNA sequence was further characterized by using the Sanger chain-termination method [21].

Cell culture and transfections

COS-7 cells (ATCC, Rockville, MD, U.S.A.) were cultured in DMEM with 4 mM L-glutamine, 18.1 mM sodium bicarbonate, 25 mM glucose, 1.0 mM sodium pyruvate, 10% (v/v) fetal calf serum, streptomycin (120 $\mu\text{g}/\text{ml}$), penicillin (120 i.u./ml), gentamycin (20 $\mu\text{g}/\text{ml}$) and nystatin (6 i.u./ml). At 2 days before transfection, 10^6 cells were resuspended in 10 ml of DMEM and plated in 10 cm plastic Petri dishes. For transfections, cells were incubated in the presence of 10 μg of the appropriate plasmid and 60 μl of Superfect (Qiagen, Basel, Switzerland) in accordance with the specifications of the manufacturer.

Primary cultures of bovine adrenal zona glomerulosa cells were obtained by enzymic dispersion as described previously [22], except that the cells (500 000 per well) were plated on round 13 mm plastic Thermanox coverslips (Nalge Nunc International, Naperville, IL, U.S.A.). On the next day, the medium was removed and replaced with serum-free DMEM; the cells were then transfected with the phosphate Ca^{2+} precipitation method, as described previously [11].

Isolation of COS-7 mitochondria and digestion by proteinase K

On the third day after transfection, COS-7 cells were homogenized on ice with a Potter–Elvehjem homogenizer (1200 rev./min, 45 strokes) in 5 mM Tris/HCl buffer, pH 7.4, containing 275 mM sucrose. The homogenate was centrifuged at 200 *g* for 15 min at 4 °C to remove large debris and nuclei. Further centrifugation of the supernatant at 10 000 *g* for 10 min at 4 °C yielded the mitochondria. The mitochondrial pellet was washed twice, with centrifugation at 800 *g* for 10 min at 4 °C, with the same buffer.

Mitochondria were resuspended in the sucrose-containing buffer to obtain a protein concentration of 1 mg/ml, as quantified with a protein microassay (Bio-Rad, Munich, Germany). The mitochondria-enriched subcellular fractions were incubated on ice for 2 h in the presence or the absence of proteinase K (30 $\mu\text{g}/\text{ml}$) with or without 0.05% (v/v) Triton X-100. At the end of the digestion period, PMSF was added to a final concentration of 2 mM.

SDS/PAGE

SDS/PAGE was performed by the method of Laemmli [23]. Subcellular fraction proteins (15 μg per 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/0.01% Bromophenol Blue] and loaded on an SDS/10% (w/v) polyacrylamide minigel (Mini-Protein II system; Bio-Rad, Munich, Germany). Electrophoresis was performed at 100 V for 1.5 h.

Blotting procedure and immunodetection of chimaeric aequorins

Proteins resolved by SDS/PAGE were transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell, Riehen, Switzerland) by the method of Towbin et al. [24]. After transfer, the membrane was incubated in a blocking buffer consisting of PBS containing 0.4% (v/v) Tween 20 and 5% (w/v) non-fat dried milk, for 1 h at room temperature. The nitrocellulose membrane was further incubated for 2 h in PBS/Tween 20 buffer with an antiserum generated by CovalAb (Oullins, France) against the entire 22 kDa recombinant aequorin protein expressed in *Escherichia coli* (Molecular Probes, Leiden, The Netherlands). The membrane was thoroughly washed four

times with the same buffer (10 min each), then incubated for 1 h with horseradish peroxidase-labelled goat anti-rabbit IgG (CovalAb). The nitrocellulose sheet was then washed four times (15 min each) with PBS/Tween 20 and the antigen-antibody complexes were revealed by enhanced chemiluminescence, with the Western blotting detection kit and Hyper-ECL[®] film from Amersham (Zurich, Switzerland).

Measurement of aequorin luminescence

On the day of the experiment, the apoaequorin-coelenterazine complex was reconstituted by incubating bovine glomerulosa cells in primary culture for at least 2 h at 37 °C, in the presence of 200 μl of 2.5 μM coelenterazine dissolved in DMEM. Cells were superfused with a Ca^{2+} -containing Krebs-Ringer buffer unless otherwise stated (concentrations in mM: CaCl_2 1.2, NaCl 136, NaHCO_3 5, KH_2PO_4 1.2, MgSO_4 1.2, KCl 1.8, D-glucose 5.5, Hepes 20; pH 7.4). Light emitted by the cells was detected and amplified by a photomultiplier tube (Thorn-EMI, Ruislip, Middx., U.K.) [25]. The counts recorded were calibrated in terms of $[\text{Ca}^{2+}]$ as already described [11].

Electron microscopy

Freshly isolated bovine adrenal glomerulosa cells were fixed in a Sørensen phosphate buffer [26 mM KH_2PO_4 /40 mM Na_2HPO_4 (pH 7.0)] containing 2.5 % (w/v) glutaraldehyde for 1 h at room temperature. They were rinsed three times (5, 10 and 15 min respectively) in the phosphate buffer and then post-fixed in 2 % (w/v) osmium tetroxide in the Sørensen phosphate buffer for 1 h. The cells were then washed again three times, embedded in a 2 % (w/v) agar solution and soaked for 30 min in 2 % (w/v) uranyl acetate to improve the fixation of cellular membranes. They were dehydrated in a graded ethanol series from 25 % to 100 % (v/v) at room temperature, kept in ethanol at 4 °C overnight and then progressively infiltrated with Spurr resin, in which they were finally embedded in gelatin capsules. Ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome and collected on copper grids. The sections were stained with 5 % (w/v) uranyl acetate for 25 min, then with Reynolds [26] lead citrate for 20 min. They were viewed and photographed with a Zeiss EM10 electron microscope.

Analysis of data

Results are expressed as means $\pm$ S.E.M. unless stated otherwise. Means were compared by analysis of variance with the Fisher or Scheffé *F* test for balanced or unbalanced groups respectively. A value of $P < 0.05$ was considered statistically significant. Where appropriate, concentration-response curves were fitted to a four-parameter logistic function. Quantification of immunoblots was performed with a Molecular Dynamics (Sunnyvale, CA, U.S.A.) computing densitometer.

RESULTS

Localization of TomAeq in the outer mitochondrial membrane

The efficiency of transient transfections in bovine adrenal glomerulosa cells with the chimaeric aequorin cDNA species was sufficient for adequate measurements of chemiluminescent signals and for the determination of intracellular Ca^{2+} concentrations. However, the low expression levels of the chimaeric aequorins did not permit reliable detection of the proteins by Western blotting of cellular extracts, immunoprecipitation of aequorin in cells labelled metabolically with [^{35}S]methionine or by immuno-

Figure 1 Localization of mtAeq and TomAeq in mitochondria of transfected COS-7 cells

(A) Mitochondrial and cytosolic fractions of TomAeq and mtAeq cDNA-transfected COS-7 cells were purified and analysed by SDS/PAGE and immunoblotting as described in the Materials and methods section. A representative Western blot from one of four similar experiments is shown. Lane 1, 5 ng of recombinant aequorin (R); lane 2, 15 μg of protein of mitochondrial extract from mock-transfected cells (M); lanes 3 and 4, 15 μg of protein of cytosol and mitochondrial extract respectively from mtAeq cDNA-transfected cells; lanes 5 and 6, 15 μg of protein of mitochondrial extract and cytosol respectively from TomAeq cDNA-transfected cells. (B) Immunospecific bands for aequorin proteins from cells transfected with TomAeq and mtAeq cDNA were quantified by densitometry in 4 independent experiments. Integrated optical density (I.O.D.) values are expressed as a percentage of that measured in the mitochondria-enriched fraction. *** Significantly different from the mitochondrial fraction ($P < 0.001$).

fluorescence. To circumvent this difficulty, transient transfections were performed in COS-7 cells, in which the proteins could be easily detected and localized by subcellular fractionation and immunoblotting as described in the Materials and methods section.

As expected, recombinant aequorin migrated with a molecular mass of 22 kDa (Figure 1A, lane 1). When COS-7 cells were transfected with the mtAeq cDNA, the antiserum raised against aequorin revealed a band of approx. 26 kDa in the mitochondrial fraction (Figure 1A, lane 4), which was absent from the mitochondria-enriched fraction of mock-transfected cells (Figure 1A, lane 2). A small amount (less than 3 %) of contaminating

Figure 2 Targeting of mtAeq to the mitochondrial matrix and of TomAeq to the outer mitochondrial membrane

(A) A Western blot of mtAeq (lanes 6–8) and of TomAeq (lanes 3–5) in mitochondria-enriched fractions of transfected COS-7 cells is shown. Recombinant aequorin (rAeq) (lanes 1 and 2) or isolated mitochondria were treated with 30 μ g/ml proteinase K (lanes 2, 4, 5, 7 and 8) in the presence (lanes 5 and 8) or in the absence of Triton X-100 (0.05%, v/v) as described in the Materials and methods section. (B) The immunospecific bands for aequorin were quantified by densitometry in three independent experiments. Integrated optical density (I.O.D.) values are expressed as a percentage of that measured in the respective control samples that were treated neither with proteinase K nor with Triton X-100. *, *** Significantly different from controls at $P < 0.05$ and $P < 0.001$ respectively; # significantly different from mitochondria treated with proteinase K in the absence of Triton X-100 ($P < 0.05$).

mtAeq was detectable in the fraction containing the microsomes and the cytosolic soluble proteins of transfected COS-7 cells (Figure 1A, lane 3, and Figure 1B). In cells transfected with TomAeq cDNA, chimaeric aequorin was detected mainly in the mitochondrial fraction, with a molecular mass of approx. 29 kDa (Figure 1A, lane 5). Small amounts of TomAeq were also observed in the cytosolic protein fraction ($20 \pm 17\%$ of the mitochondrial fraction, $P < 0.001$) (Figure 1A, lane 6, and Figure 1B).

Treatment with proteinase K of mitochondria-enriched fractions from TomAeq cDNA-transfected cells led to a partial digestion of the chimaeric aequorin ($31.2 \pm 14.7\%$ of control, $P < 0.05$) (Figure 2A, lanes 3 and 4, and Figure 2B). Permeabilization of mitochondrial membranes with Triton X-100 did not significantly affect the digestion of TomAeq by proteinase K ($49.0 \pm 19.1\%$ of control) (Figure 2A, lane 5, and Figure 2B). In contrast, mtAeq was not susceptible to digestion with proteinase K when mitochondrial membranes were left intact (Figure 2A, lanes 6 and 7, and Figure 2B). When mitochondria were treated with proteinase K in the presence of Triton X-100, mtAeq was completely degraded (Figure 2A, lane 8, and Figure 2B).

These results show clearly that mtAeq and TomAeq could be targeted to distinct mitochondrial compartments of COS-7 cells: TomAeq faced the cytosolic space, whereas mtAeq was confined

Figure 3 Response of $[Ca^{2+}]_{om}$ to Ang II

(A) Effect of Ang II on $[Ca^{2+}]_{om}$ in bovine adrenal glomerulosa cells transfected with TomAeq. Glomerulosa cells in primary culture were transfected with TomAeq cDNA as described in the Materials and methods section. After loading with coelenterazine, cells were challenged with 10 nM Ang II and $[Ca^{2+}]_{om}$ was determined as described [11]. This trace is representative of similar results obtained with 23 separate cell preparations. (B) Concentration dependence of the peak Ca^{2+} response to Ang II in cells transfected with either mtAeq (solid line, $\square$), cytAeq (broken line, $\blacktriangle$) or TomAeq (dotted line, $\circ$). Each point is the mean of data from 2–51 experiments. **, *** Significantly different from $[Ca^{2+}]_{om}$ peak responses at $P < 0.01$ and $P < 0.001$ respectively.

to the mitochondrial matrix and was therefore not susceptible to proteinase K in intact mitochondria.

Perimitochondrial Ca^{2+} response to Ang II

In glomerulosa cells transfected with TomAeq cDNA, the resting $[Ca^{2+}]_{om}$ was 337 ± 107 nM ($n = 38$), a value slightly lower than, but not significantly different from, the basal $[Ca^{2+}]$ previously reported in the cytosol and in the mitochondria of cells transfected with non-targeted aequorin (cytAeq) cDNA and mtAeq cDNA respectively [11].

When the cells were challenged with Ang II (10 nM), a transient $[Ca^{2+}]_{om}$ response was observed. After a rapid rise reaching a mean maximal value of 1.42 ± 0.28 μ M ($n = 23$), $[Ca^{2+}]_{om}$ rapidly returned to basal values (Figure 3A).

The amplitude of the cytosolic, mitochondrial and perimitochondrial Ca^{2+} peaks depended on hormone concentration, with very similar sensitivities (Figure 3B). However, the amplitude of the transient $[Ca^{2+}]_{om}$ response to Ang II was significantly higher

Figure 4 Perimitchondrial and mitochondrial Ca^{2+} responses to K^+ , BayK8644 and Ang II

(A) Effect of BayK8644 (100 nM) on cells transfected with TomAeq cDNA (dotted line) or mtAeq cDNA (solid line) and previously subjected to an increase in extracellular $[\text{K}^+]$ from 3 to 13 mM in the superfusion medium. Traces are means of eight and nine independent experiments for TomAeq and mtAeq cDNA-transfected cells respectively. (B) Effect of 1 nM Ang II on cells transfected with TomAeq cDNA (dotted line) or mtAeq cDNA (solid line). Traces are means of five and six independent experiments for TomAeq and mtAeq cDNA-transfected cells respectively.

than those of both the cytosolic and the perimitchondrial Ca^{2+} peaks, which were comparable.

Comparison of mitochondrial and perimitchondrial Ca^{2+} responses to K^+ , BayK8644 and Ang II

A small, sustained but not significant rise of $[\text{Ca}^{2+}]_{\text{om}}$ (from a mean resting value of 260 ± 108 nM to a plateau of 308 ± 123 nM; $n = 10$) was observed in bovine glomerulosa cells when extracellular K^+ was increased from 3 to 13 mM (results not shown). The addition of BayK8644 (100 nM), a dihydropyridine that increases the opening probability of L-type Ca^{2+} channels [27], potentiated the effect of K^+ on $[\text{Ca}^{2+}]_{\text{om}}$, increasing it to a mean transient value of 580 ± 350 nM, followed by a mean plateau of 455 ± 178 nM (Figure 4A) ($n = 8$, $P < 0.001$ compared with the basal value). This sustained Ca^{2+} response was partly abolished in the presence of 100 nM nifedipine (382 ± 158 nM, $n = 7$; results not shown). When used at this concentration, nifedipine can be considered to be a specific blocker of L-type Ca^{2+} channels [8]. The $[\text{Ca}^{2+}]_{\text{m}}$ responses to K^+ and BayK8644 recorded in mtAeq-transfected bovine glomerulosa cells were not signifi-

Figure 5 Cytosolic, mitochondrial and perimitchondrial $[\text{Ca}^{2+}]$ changes in Ca^{2+} -clamped glomerulosa cells

(A) Recording of $[\text{Ca}^{2+}]_{\text{om}}$ in TomAeq cDNA-transfected glomerulosa cells exposed to 2 μM ionomycin and increments of added $[\text{Ca}^{2+}]$ (arrows). A representative trace from six similar independent experiments is shown. (B) The $[\text{Ca}^{2+}]$ measured in glomerulosa cells transfected with TomAeq (light grey bars), mtAeq (dark grey bars) or cytAeq (white bars) cDNA and subjected to a Ca^{2+} clamp was expressed as a function of total extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{t}}$) ($n = 3-9$ independent experiments for each $[\text{Ca}^{2+}]_{\text{t}}$). *, **, *** Significantly different from the corresponding basal $[\text{Ca}^{2+}]$ measured in a nominally Ca^{2+} -free Krebs-Ringer buffer, at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively; +, ++ significantly different from $[\text{Ca}^{2+}]_{\text{om}}$ measured at the same $[\text{Ca}^{2+}]_{\text{t}}$ at $P < 0.05$ and $P < 0.01$ respectively.

cantly different from the perimitchondrial Ca^{2+} responses ($n = 9$).

The amplitude of the transient $[\text{Ca}^{2+}]_{\text{om}}$ response to 1 nM Ang II (537 ± 65 nM, $n = 5$) was quantitatively equivalent to and not significantly different from the peak of $[\text{Ca}^{2+}]_{\text{om}}$ elicited by BayK8644 (Figure 4B). However, the amplitude of the transient $[\text{Ca}^{2+}]_{\text{m}}$ response to 1 nM Ang II, reaching a mean of 2725 ± 967 nM ($n = 6$), was significantly higher than that of the $[\text{Ca}^{2+}]_{\text{om}}$ response evoked by the same concentration of Ang II ($P < 0.001$).

These results show clearly that increases in $[\text{Ca}^{2+}]_{\text{om}}$ of similar amplitude can be amplified or not within the mitochondrial matrix in intact cells, depending on the source of Ca^{2+} .

Affinity for Ca^{2+} of the various chimaeric aequorins

The apparent amplification, within the mitochondrial matrix, of the cytosolic and perimitchondrial Ca^{2+} responses elicited by Ang II could be an artifact resulting from differences in affinity

Figure 6 Effect of the mitochondrial uncoupler FCCP on the $[Ca^{2+}]_{om}$ response to Ang II

Glomerulosa cells transfected with TomAeq cDNA were preincubated with $2.5 \mu M$ FCCP and challenged with $10 nM$ Ang II in the continuous presence of FCCP. The trace is representative of 10 similar observations.

for Ca^{2+} of the three chimaeric aequorin proteins. To assess whether the rates of consumption of cytAeq, mtAeq and TomAeq were similar, transfected glomerulosa cells were subjected to an

intracellular Ca^{2+} clamp in the presence of a Ca^{2+} ionophore. Cells were superfused with a Ca^{2+} -free medium containing $2 \mu M$ ionomycin; the extracellular $[Ca^{2+}]$ was increased stepwise by increments of 200, 100, 200, 500 μM and 1 mM.

The addition of ionomycin to transfected glomerulosa cells, incubated in a nominally Ca^{2+} -free Krebs-Ringer buffer containing $0.2 mM$ EGTA, led to a transient increase in $[Ca^{2+}]$ in the periphery of mitochondria (Figure 5A), as well as in the cytosol and in the mitochondria (results not shown). A similar increase in $[Ca^{2+}]$ has previously been observed in the cytosol of fura-2-loaded glomerulosa cells and attributed to the release of Ca^{2+} from intracellular stores [6]. Stepwise increases of extracellular $[Ca^{2+}]$ led, after a large transient peak of $[Ca^{2+}]$, to an equilibrium in which progressive and sustained elevations of $[Ca^{2+}]$ were observed in the vicinity of mitochondria (Figure 5A), as well as in the cytosol and in the mitochondrial matrix (results not shown). Quantification of the sustained elevations showed that intracellular $[Ca^{2+}]$ increased as a function of the total extracellular $[Ca^{2+}]$ in the glomerulosa cells transfected with any of the three chimaeric aequorin proteins (Figure 5B). Overall, the measured intracellular $[Ca^{2+}]$ did not show significant differences between the three intracellular compartments (cytosol, periphery of mitochondria and mitochondrial matrix). Similar results were observed when comparing the peak Ca^{2+} responses triggered after each increase in extracellular $[Ca^{2+}]$ (results not shown), indicating that the Ca^{2+} -dependent consumption rate of the chimaeric aequorin proteins was not affected by either the N-

Figure 7 Electron microscopy of a bovine adrenal glomerulosa cell

Isolated bovine glomerulosa cells were fixed and stained as described in the Materials and methods section. Arrowheads point to regions of close but partial apposition of the outer mitochondrial membrane and endoplasmic reticulum structures.

terminal-targeting sequences of the proteins or by differences in the ionic milieu surrounding the various aequorins.

Effect of uncoupling mitochondria on $[\text{Ca}^{2+}]_{\text{om}}$ responses

The mitochondrial uncoupler FCCP has previously been shown to suppress the mitochondrial Ca^{2+} response to Ang II completely without affecting the cytosolic response [11]. This H^+ ionophore is therefore an appropriate pharmacological tool to inhibit Ca^{2+} entry into the mitochondrial matrix of intact cells and to determine whether this manoeuvre affects $[\text{Ca}^{2+}]_{\text{om}}$ responses.

When glomerulosa cells were superfused with FCCP, a transient increase in $[\text{Ca}^{2+}]_{\text{om}}$ was observed at the boundary of mitochondria, as previously noticed in the cytosol and in the mitochondrial matrix [11]. More importantly, however, the FCCP-induced depolarization of the inner mitochondrial membrane affected neither basal nor Ang II-stimulated $[\text{Ca}^{2+}]_{\text{om}}$ (Figure 6) (400 ± 176 and $1.08 \pm 0.30 \mu\text{M}$ respectively, $n = 10$; values were not significantly different from those obtained in the absence of FCCP). Similarly, the $[\text{Ca}^{2+}]_{\text{om}}$ responses induced by K^+ and BayK8644 were not affected by FCCP (results not shown).

Electron microscopy of bovine glomerulosa cells

When isolated bovine glomerulosa cells were observed under the electron microscope, numerous mitochondria, displaying the typical lamellar matrix, were seen in close apposition with endoplasmic reticulum structures (Figure 7). Interestingly, these zones of apposition were confined to only a fraction of the whole mitochondrial surface.

DISCUSSION

In a previous study we showed that the $[\text{Ca}^{2+}]_{\text{c}}$ response to Ang II is relayed and markedly amplified within the mitochondrial matrix in bovine adrenal glomerulosa cells. Various observations suggested that this mitochondrial potentiation of the Ca^{2+} response might result from microdomains of high $[\text{Ca}^{2+}]$ generated by $\text{Ins}(1,4,5)\text{P}_3$ in the vicinity of the organelles [11]. However, alternative explanations could not be entirely dismissed.

In the present study we have examined Ca^{2+} homeostasis in the immediate periphery of mitochondria, to gain information on the mechanisms of mitochondrial potentiation of the $[\text{Ca}^{2+}]_{\text{c}}$ response. We therefore transfected bovine glomerulosa cells in primary culture with a chimaeric aequorin targeted to the outer mitochondrial membrane.

The cDNA encoding the N-terminus of hTom20 was fused in frame with the cDNA coding for aequorin. Tom20 is a component of the outer mitochondrial import receptor complex that recognizes nuclear-encoded mitochondrial preproteins and facilitates their transport across the two mitochondrial membranes. The predicted amino acid sequence of hTom20 reveals the presence of (1) a hydrophobic segment at the N-terminus that might function as the outer mitochondrial membrane anchor, (2) a tetratricopeptide motif in the middle of the protein that would be involved in protein-protein interactions, and (3) a net negative charge at the C-terminus that is supposed to be implicated in the interaction with positively charged targeting sequences of mitochondrial preproteins [19].

Two pieces of evidence indicated that TomAeq was indeed inserted in the outer mitochondrial membrane with the proper

orientation: (1) in Cos-7 cells, TomAeq was localized to the mitochondria and susceptible to enzymic degradation by proteinase K, whereas mtAeq was not; (2) disruption of mitochondrial membrane potential with FCCP in glomerulosa cells did not alter the TomAeq signal while suppressing that of mtAeq.

One of the major findings of the present study is the fact that the amplitude of the $[\text{Ca}^{2+}]_{\text{om}}$ peak response to Ang II was identical with that of the $[\text{Ca}^{2+}]_{\text{c}}$ response, whereas the transient $[\text{Ca}^{2+}]_{\text{m}}$ rise was severalfold higher. It is worth mentioning that although Ca^{2+} responses in glomerulosa cells transfected with cytAeq cDNA are very similar to those recorded in cells transfected with TomAeq cDNA, the amount of TomAeq expressed is much higher than that of cytAeq, as measured by the total emitted luminescence (results not shown). This is reminiscent of a study showing that aequorin is broken down differently depending on the intracellular compartment in which it is localized [28] and provides an additional indication about the localization of TomAeq, which seems unlikely to reside in the bulk cytosol.

Various hypotheses have been considered here that could explain the quantitative similarity of the $[\text{Ca}^{2+}]_{\text{c}}$ and $[\text{Ca}^{2+}]_{\text{om}}$ responses to Ang.

1. The consumption rate of aequorin could be different between the chimaeric proteins because of a modification of their intrinsic properties and/or a change in their ionic environment. However, the perimitochondrial and mitochondrial Ca^{2+} responses to successive stimulations with K^+ and BayK8644 were very similar. This indicates that the Ca^{2+} -dependent luminescent reaction of aequorin is identical, whether aequorin is targeted to the mitochondrial matrix or to the outer mitochondrial membrane. This was confirmed by clamping intracellular Ca^{2+} in glomerulosa cells with ionomycin and various concentrations of extracellular Ca^{2+} (Figure 5). $[\text{Ca}^{2+}]_{\text{om}}$ could thus be raised to a sustained plateau equivalent to the transient perimitochondrial response elicited by 10 nM Ang II. Under the same conditions, $[\text{Ca}^{2+}]_{\text{m}}$ was not significantly different in Ca^{2+} -clamped cells (Figure 5B). Therefore our results show clearly that the difference between the peak $[\text{Ca}^{2+}]_{\text{om}}$ and $[\text{Ca}^{2+}]_{\text{m}}$ responses to Ang II does not result from different affinities of the chimaeric aequorins for Ca^{2+} .

2. Microdomains of high $[\text{Ca}^{2+}]$ around mitochondria could be masked owing to the faculty of the organelles for buffering Ca^{2+} released from the $\text{Ins}(1,4,5)\text{P}_3$ -dependent stores. Indeed, several studies in different cell types have reported that mitochondria have an important role in the clearance of cytosolic Ca^{2+} after an imposed large intracellular elevation in $[\text{Ca}^{2+}]$ [29–31]. In fact, the observed $[\text{Ca}^{2+}]_{\text{om}}$ response in adrenal glomerulosa cells could represent the summation of two opposite effects: the release of a high Ca^{2+} burst from stores of neighbouring mitochondria and the concomitant 'mopping up' of perimitochondrial Ca^{2+} by rapid mitochondrial uptake. However, this hypothesis was invalidated by the fact that the resting $[\text{Ca}^{2+}]_{\text{om}}$ value, as well as the $[\text{Ca}^{2+}]_{\text{om}}$ responses to Ang II (Figure 6) or BayK8644 (results not shown), remained unaffected by FCCP, a proton ionophore that completely suppresses the $[\text{Ca}^{2+}]_{\text{m}}$ response to Ang II [11].

3. Moreover, in separate experiments performed with CGP 37157, a blocker of the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger, or with a fluorescent probe for mitochondrial membrane potential, we observed that the difference in amplitude between the $[\text{Ca}^{2+}]_{\text{c}}$ response and the $[\text{Ca}^{2+}]_{\text{m}}$ response to Ang II in bovine adrenal glomerulosa cells does not result from characteristics of the intramitochondrial Ca^{2+} transport system (results not shown).

In pioneering studies with organic fluorescent Ca^{2+} probes such as fura-2 or indo-1 in mitochondria, various groups have shown that, above a $[\text{Ca}^{2+}]_{\text{c}}$ of approx. $0.4\text{--}0.6 \mu\text{M}$ (the so-called

mitochondrial set-point) the gradient of Ca^{2+} concentration (in/out of mitochondria) was strikingly more pronounced, owing to the sigmoidal shape of the mitochondrial Ca^{2+} uptake curve [32–35]. However, such a mechanism, which does not require the existence of Ca^{2+} microdomains, cannot alone explain all the results that we have obtained in glomerulosa cells. Indeed, in these cells, the mitochondrial set-point was estimated to be approx. $0.6 \mu\text{M}$ [36] and we observed that the mitochondrial response to Ang II was amplified, even at $[\text{Ca}^{2+}]_c$ values lower than the mitochondrial set-point (Figure 3). Moreover, the Ca^{2+} signal evoked by BayK 8644 seemed to be relayed within the mitochondrial matrix practically without amplification, in spite of the fact that the $[\text{Ca}^{2+}]_{om}$ reached were similar to those observed with Ang II (Figure 4).

Two possible explanations can be proposed that explain the above results.

1. The $[\text{Ca}^{2+}]_{om}$ measured during stimulation with Ang II might reflect an average between high $[\text{Ca}^{2+}]$ microdomains and low peak responses. One can speculate that, in TomAeq-transfected cells, mitochondria are uniformly coated with TomAeq, whereas in fact the outer membrane of the organelles might only partly face microdomains linked to intracellular Ca^{2+} stores. Consistent with this hypothesis is electron microscopy of bovine adrenal glomerulosa cells that shows that mitochondria are in close proximity, although not over their entire surface, to endoplasmic reticulum structures. In this respect it is worth mentioning that Rizzuto et al. [37] obtained almost similar results in HeLa cells with a chimaeric aequorin targeted to the mitochondrial intermembrane space. Indeed, the peak $[\text{Ca}^{2+}]$ increase elicited by histamine, an $\text{Ins}(1,4,5)\text{P}_3$ -generating agonist, was much smaller than that measured in the mitochondrial matrix, although slightly larger than the signal detected in the cytosol. Finally, one should also consider the possibility that TomAeq might be excluded from the 'synaptic cleft'-like space formed between the mitochondria and the endoplasmic reticulum.

2. Alternatively, there might exist distinct subpopulations of mitochondria that do not sense and relay identical $[\text{Ca}^{2+}]_c$ changes. Such different behaviours of mitochondrial populations within individual cells have been observed in CHO.T cells [38].

The concept of confinement of intracellular Ca^{2+} signalling is now well accepted [39]. In adrenal zona glomerulosa cells, the distinctly compartmentalized sources of the Ca^{2+} messenger most probably act on different intracellular sites, leading to a convergent stimulation of aldosterone biosynthesis. Several observations illustrate this heterogeneity of action of Ca^{2+} coming from different sources. Capacitative Ca^{2+} influx, but not Ca^{2+} entry through VOC channels, potentiates the activity of a Ca^{2+} -dependent adenylate cyclase isotype stimulated by adrenocorticotrophic hormone in bovine glomerulosa cells [40]. Moreover, an increase in extracellular $[\text{K}^+]$ activates both T-type and L-type Ca^{2+} channels in glomerulosa cells. However, only T-type channel activation seems to be relevant for the acute stimulation of aldosterone production, although the measured $[\text{Ca}^{2+}]_c$ increase is more closely related to L-type channel opening [8]. Finally, mitochondrial NADPH, which is an important cofactor for several mitochondrial enzymes of the mineralocorticoid cascade, has been shown to be more efficiently produced by the release of Ca^{2+} from intracellular stores than by K^+ -evoked Ca^{2+} influx in rat adrenal glomerulosa cells [13].

The confinement of the Ca^{2+} messenger could represent an effective way for the cell to compensate for the inability of Ca^{2+} to diffuse freely in the cytosolic space while coping with a multiplicity of intracellular targets. Indeed, intracellular Ca^{2+} is thought to have at least a dual site of action in the acute

stimulation of mineralocorticoid synthesis: besides its intra-mitochondrial effects, Ca^{2+} exerts rapid genomic actions at the transcriptional level, resulting in synthesis *de novo* of the steroidogenic acute regulatory protein, StAR, an important factor for the regulation of steroidogenesis [41–43].

In conclusion, we have shown that aequorin can be specifically targeted to the outer mitochondrial membrane for the recording of perimitochondrial changes in $[\text{Ca}^{2+}]$. Although we observed an amplification of the Ang II-induced $[\text{Ca}^{2+}]_c$ response in the mitochondrial matrix, the concomitant transient $[\text{Ca}^{2+}]_{om}$ rise was very similar to the $[\text{Ca}^{2+}]_c$ response in adrenal glomerulosa cells. Further work with targeted Ca^{2+} probes allowing a discrimination of the Ca^{2+} signal within the mitochondrial population, such as the 'cameleon' (*sic*) probes [44], will tell whether mitochondria are assigned distinct functions within individual adrenal glomerulosa cells. In more general terms, the possibility of anchoring proteins in the outer mitochondrial membrane might be useful for the study of a series of processes involving mitochondrial transport mechanisms.

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