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Abstract: Glomerulosa cells secrete aldosterone in response to the Ca2+ - mediated agonists angiotensin II, K+ and the cAMP - mediated agonist corticotrophin. A recently recognized interaction between Ca2+ and cAMP is the Ca2+ - induced cAMP formation in the mitochondrial matrix. Here we describe that soluble adenylyl cyclase (sAC) is expressed in H295R adrenocortical cells. Mitochondrial cAMP formation, monitored with a mitochondria-targeted fluorescent sensor (4mtH30), is enhanced by HCO3- and the Ca2+ mobilizing agonist angiotensin II. The effect of angiotensin II is inhibited by 2-OHE, an inhibitor of sAC and by RNA interference of sAC whereas is enhanced by an inhibitor of phosphodiesterase PDE2A. Heterologous expression of the Ca2+ binding protein S100G within the mitochondrial matrix attenuates the agonist - induced mitochondrial cAMP formation. Inhibition and knockdown of sAC significantly reduce angiotensin II - induced aldosterone production. These data provide the first evidence for a cell-specific functional role of mitochondrial cAMP.

The recent discovery of the existence of an intramitochondrial cAMP signaling machinery (soluble adenylyl cyclase (sAC) and a cAMP phosphodiesterase) has been followed by the direct demonstration that in HeLa and CHO cells as well as in cardiomyocytes a rise in cAMP can be triggered by an increase in HCO_3^- and Ca^{2+} in the matrix, acting synergically. Intramitochondrial cAMP rise is paralleled by a significant increase of the matrix level of ATP. On the contrary, no consensus exists as to the target(s) of cAMP in the mitochondrial matrix. Moreover, the possible functional effects of this cAMP increase are still largely unexplored. In the present manuscript we report on our experiments revealing that sAC is expressed in the mitochondria of adrenocortical cells. Mitochondrial Ca^{2+} signal activates the cyclase whereas knockout or inhibition of sAC attenuates the Ca^{2+} - induced production of aldosterone. These data which provide the first evidence for a cell-specific functional role of mitochondrial cAMP, may also be clinically relevant in elucidating the progress of various cardiovascular, renal and inflammatory diseases.

Calcium-dependent mitochondrial cAMP production enhances aldosterone secretion

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Abbreviations: COX-IV, cytochrome *c* oxidase IV; mt-cAMP, mitochondrial cAMP; 2-OHE, 2-hydroxy-estradiol; sAC, soluble adenylyl cyclase

ABSTRACT

Glomerulosa cells secrete aldosterone in response to the Ca^{2+} - mediated agonists angiotensin II, K^+ and the cAMP – mediated agonist corticotrophin. A recently recognized interaction between Ca^{2+} and cAMP is the Ca^{2+} - induced cAMP formation in the mitochondrial matrix. Here we describe that soluble adenylyl cyclase (sAC) is expressed in H295R adrenocortical cells. Mitochondrial cAMP formation, monitored with a mitochondria-targeted fluorescent sensor (4mtH30), is enhanced by HCO_3^- and the Ca^{2+} mobilizing agonist angiotensin II. The effect of angiotensin II is inhibited by 2-OHE, an inhibitor of sAC and by RNA interference of sAC whereas is enhanced by an inhibitor of phosphodiesterase PDE2A. Heterologous expression of the Ca^{2+} binding protein S100G within the mitochondrial matrix attenuates the agonist – induced mitochondrial cAMP formation. Inhibition and knockdown of sAC significantly reduce angiotensin II – induced aldosterone production. These data provide the first evidence for a cell-specific functional role of mitochondrial cAMP.

1. Introduction

Cyclic AMP and Ca^{2+} are the two most common second messengers in eukaryotic cells and they control a variety of cellular functions as diverse as secretion, contraction, cell movement and death. These two messengers may act synergistically, as observed e.g. in cardiac myocytes, whereas their action may be antagonistic in other tissues, as in smooth muscle cells (Bolton et al., 1999;Schaub and Kunz, 1986;Sperelakis, 1990). In aldosterone producing adrenal glomerulosa cells angiotensin II and extracellular K^+ concentration control aldosterone secretion via Ca^{2+}

signaling whereas the action of corticotrophin (ACTH) is mediated by cAMP (Spät and Hunyady, 2004). Under acute stimulatory conditions the site of action is, in both cases, the Steroidogenic Acute Regulatory Protein (StAR) (reviewed in (Hattangady et al., 2011;Spät et al., 2004)) that facilitates the cholesterol transport to the cholesterol side chain cleaving enzyme (cytochrome $P450_{scc}$), located on the inner mitochondrial membrane. In addition, Ca^{2+} exerts intramitochondrial action(s) as well. By activating mitochondrial dehydrogenases (McCormack et al., 1990), elevation of mitochondrial matrix [Ca^{2+}] enhances the formation of reduced pyridine nucleotides in rat (Pralong et al., 1992;Pralong et al., 1994;Rohács et al., 1997) and human (Spät et al., 2012) glomerulosa cells and stimulates aldosterone secretion in cells from both species (Spät et al., 2012;Wiederkehr et al., 2011).

Although ACTH and angiotensin II act synergistically on aldosterone secretion (Spät et al., 2004), surprisingly angiotensin II, through the activation of the inhibitory G-protein, G_i (Enyedi et al., 1986;Hausdorff et al., 1987;Lu et al., 1996;Maturana et al., 1999;Rocco et al., 1990), reduces basal and ACTH-induced cAMP production (Bell et al., 1981){Marie, 1983 7799 /id}(Begeot et al., 1987;Marie and Jard, 1983;Woodcock and Johnston, 1984) (but see Baukal et al., 1994;Burnay et al., 1998)). The recent discovery in HeLa cells and cardiac myocytes that agonist - induced mitochondrial Ca^{2+} signals can induce the formation of cAMP and ATP within the mitochondrial matrix (Di Benedetto et al., 2013) may offer an explanation for this apparent paradox. Indeed an increase of cAMP in a small compartment, e.g. mitochondria (that represent ~ 25 % of the cytoplasmic volume in glomerulosa cells (Nussdorfer, 1980)), may be masked by the decrease of *total* intracellular cAMP. Accordingly we decided to investigate whether mitochondrial cAMP is modulated by angiotensin II-induced mitochondrial Ca^{2+} increases in adrenocortical cells and, more decisively, if mitochondrial cAMP can influence the secretion of

steroid hormones. Here we take advantage of a fluorescent cAMP sensor selectively targeted to the mitochondrial matrix (4mtH30) and show that in an adrenocortical cell line, H295R cells (Bird et al., 1993;Rainey et al., 1994), not only angiotensin II - induced Ca^{2+} release from the endoplasmic reticulum results in a significant increase of mt-cAMP formation, but also that the mt-cAMP increase contributes to hormone production.

2. Materials and methods

2.1 Materials

OPTI-MEM, Lipofectamine 2000, RNAiMax, Fluo-4 AM, Rhod-2 AM, SNARF AM and MitoTracker Deep Red were purchased from Life Technologies (Paisley, UK). siRNA for silencing sAC (MR2, (Di Benedetto et al., 2013)) and Control siRNA (Universal Negative Control, SIC001) were obtained from Sigma-Aldrich (St. Louis, MO, USA). UltroSer G was from Bio Sepra (Cergy-Saint-Christophe, France). Coat-A-Count RIA kit was purchased from Siemens Health Care Diagnostic (Deerfield, IL).

S100G cDNA, fused to a mitochondrial targeting sequence and cloned into an adenovirus vector under the control of the tetON promoter (mitoS100G) was prepared by Dr. A. Wiederkehr (Geneva, Switzerland). H30 was from K. Jalink's lab (Ponsioen et al., 2004), 4mt-H30 and mtAlphi were constructed by the authors (G.D. & T.P.) as described (Cano Abad et al., 2013).

Primary antibodies were purchased as follows: anti-soluble adenylyl cyclase (R21.002) was from CEP Biotech (Tamarac, FL), monoclonal anti-β-actin (A5316) was from Sigma-

Aldrich, anti-COX IV (sc-69359) was from Santa Cruz (Dallas, TX). The secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate, R-05071-500) was purchased from Advansta (Menlo Park, CA). Other chemicals were obtained from Sigma-Aldrich.

2.2 Cell culture and transfection

H295R cells (CRL-2128, ATCC, Manassas, VA) were cultured in DMEM/Ham's F12 (1:1 v/v) containing 1% ITS^+, 2% UltroSer G, 100 U/ml penicillin and 100 µg/ml streptomycin. One day before aldosterone experiments the concentration of Ultroser G was reduced to 0.5 %.

Cells (2.5 - $4*10^4$ H295R) were plated onto 25-mm diameter circular glass coverslips on day 1. For FRET experiments the cells were transfected on day 2 with 3 µg 4mtH30 or 1.6 µg H30 DNA (per coverslip) using Lipofectamine 2000 in OPTI-MEM. FRET measurements were performed on day 5 (4mtH30) or day 4 (H30). For silencing sAC, on day 3 the cells were transfected with 100 pmol MR2 siRNA (Di Benedetto et al., 2013) or 100 pmol contrRNA using lipofectamin RNAiMax in OPTI-MEM. The experiments were conducted on day 5. In Western blot studies cell lysis was performed 2 days after transfection with control or siRNA. For aldosterone measurements see subsection 2.7.

For binding Ca^{2+} in the mitochondrial matrix $3*10^4$ cells were plated and infected 1 day later with Ad-mitoS100G (60 IFU/cell) together with 30 IFU/cell of Ad-tetON. Previous immunocytochemical examination revealed the expression of S100G in 63% of the cells (Wiederkehr et al., 2011).

2.3 Immunoblotting

Cell lysis, SDS-PAGE and immunoblotting were performed as described (Fülöp et al., 2011) with the following modifications. Cell lysis was performed with protease-completed RIPA buffer. PMSF was used at a concentration of 1 mM. 10 % SDS-PAGE was used.

2.4 Confocal microscopy

A Zeiss LSM710 confocal laser scanning microscope (operated with ZEN 11.0 software) and a 63*/1.3 oil immersion objective (Plan-Apochromat, Zeiss) were used. Subcellular localisation of cAMP sensors (H30 or 4mt-H30) was examined in cells preloaded with MitoTracker Deep Red (MTDR). Applying multitrack mode the YFP component of the sensor was excited at 514 nm, emitted light was measured between 640 and 740 nm whereas MTDR was excited at 633 nm and monitored at 640-740 nm. The images were deconvoluted using ImageJ 1.6.0.

For monitoring cytosolic Ca^{2+} signals and mitochondrial pH the cells were preloaded with Fluo-4 or transfected with mtAlphi, the applied excitation wavelength was 488 nm, the emitted light was monitored between 500-550 nm. For monitoring cytosolic pH the cells were preloaded with SNARF AM, the dye was excited at 488 and the intensity of 612-699 nm emitted light was divided with that of the 560-600 nm light.

The optical slice was 4 μm in the cytosolic measurements and 1.5 μm in the mitochondrial ones. As exception, H30 localisation examinations 0.8 or 1 μm optical slice was applied. Image acquisition frequency was 0.1 Hz. In all kinetic studies fluorescence intensity was normalized to the average 60-s intensity measured before stimulation (F_o).

2.5 Measurements with fluorescent wide-field microscopy

cAMP level in the mitochondrial matrix and in the cytosol were monitored by means of FRET, using 4mt-H30 and H30, respectively. These measurements as well as those of mitochondrial [Ca^{2+}] were performed on an inverted microscope (Axio Observer D1, Zeiss) equipped with a 40x1.4 Plan-Apochromat oil immersion objective (Zeiss) and a Cascade II camera (Photometrics). Excitation wavelengths were set by a random access monochromator connected to a xenon arc lamp (DeltaRAM, Photon Technology International). For ratiometric FRET measurements of H30 or 4mt-H30 excitation wavelength of 435 nm was selected along with a Dual-View emission splitting system (505dcxr, 480/30 and 535/30; Photometrics) enabling the acquisition of simultaneous donor (eCFP) and raw FRET emissions. For measuring eYFP an excitation wavelength of 500 nm was used, the light passed a 500-nm dichroic filter and emission was measured at 535/30 nm. (YFP image was used off-line for masking the chosen cells (ROIs).) FRET signals were evaluated with Metamorph Offline Version 7.7.0.0.

Since binding of cAMP to the sensor reduced FRET intensity, cAMP - induced fluorescence was computed as 100 * (R_o - R)/ R_o and expressed as $\Delta R/R_o$ (%), where R was the ratio of background-subtracted fluorescence intensity of raw FRET and eCFP, R_o was the mean ratio of the 100-sec period prior to stimulation. With regard to the often instable base-line, 4mtH30 signal was regarded as increased cAMP level if the integrated fluorescence intensity within at least a 100-sec period during the 5-min post-stimulation period was significantly greater ($p < 0.05$) than a similar period preceding the stimulation. Due to this restriction the number of responding cells may have been underestimated.

Rhod excitation wavelength 550 nm was applied. The emitted light passed as 570-nm dichroic mirror and was measured with a 610/50 filter. The expressed emission values (F) were normalized to the mean F value of the 100-sec period prior to stimulation.

2.6 Superfusion

Microscopic experiments were carried at room temperature. Following a preincubation for 30 min (in the absence or presence of any inhibitor) the coverslips were superfused with a modified Krebs-Ringer solution containing 140 mM Na^+, 4.5 mM K^+, 1.2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 5 mM Hepes and 2 mM HCO_3^- (pH 7.4). The flow rate was ~ 1ml/min. The solutions were applied with a solenoid valve-equipped, gravity-driven superfusion system, terminating at ~2 mm from the selected cells.

2.7 Aldosterone production

Cells (150 - 300 thousand/well) were plated in a 24-well culture dish (day 1). On day 3 the UltroSer G content of the culture medium was reduced to 0.1 %. On day 4, after a 30-min preincubation in serum-free medium that contained 2-OHE or solvent, the cells were incubated at 37^o for 2 hours in a similar medium, with or without angiotensin II. When the cells were transfected with control or siRNA on day 2, UltroSer G content was reduced on day 4 and final incubation, as detailed above, took place in day 5.

Aldosterone and protein content of the supernatant were determined with Coat-A-Count RIA kit and Bradford assay, respectively.

2.8 Statistics

Means + S.E.M. are shown. With the exception of cytosolic pH measurements all the experiments were performed on cells derived from at least two different cell passages. For estimating significance of differences, Student's unpaired *t*-test, sign test, chi-square test or factorial ANOVA was used, as appropriate. Data were analyzed with Statistica 9.

3. Results

3.1 Expression of soluble adenylyl cyclase in H295R cells

A specific antibody against sAC (Zippin et al., 2003) revealed an approximately 50 kDa protein band in Western-blots of total lysates from both HeLa and H295R cells. When comparing samples of identical protein mass the average density of the sAC band in H295R cells attained 60 $\pm$ 9 % of that in HeLa cells (n=5). Two days after transfecting H295R cells with a specific siRNA the density of the sAC band (related to that of actin) was reduced to 74 $\pm$ 18 % of that of control siRNA-transfected cells (n=3, Fig. 1). After permeabilizing the cells with digitonin (25 µg/ml, 5 min, 4^{o}C) the majority of immunoreactive sAC (related to the mitochondrial marker COX-IV) remained in the particulate fraction (n=3, data not shown).

3.2 Mitochondrial cAMP as measured with 4mtH30 in H295R cells

The cAMP sensor 4mtH30 consists of 4 copies of human COX VIII targeting sequence fused to the N terminus of the cAMP sensor H30. The latter contains, between ECFP and EYFP, the whole Epac1 protein, rendered catalytically inactive with two mutations in the Rap1 binding

domain and deprived of the membrane-targeting DEP domain (Ponsioen et al., 2004). Confocal microscopy revealed that in H295R cells, 3 days after transfection, 4mtH30 shows, as previously described in other cells, an excellent colocalization with the classical mitochondrial marker Mitotracker Deep Red (Fig. 2).

Cyclic AMP binding to H30 reduces the efficacy of FRET between CFP and YFP. Accordingly, upon binding, the fluorescence of CFP (emission 480 nm) increases and that of YFP (emission 540 nm) decreases and thus the ratio between the intensity of the light emitted at 540 and 480 nm decreases proportionally to cAMP elevation. In order to test whether intramitochondrial cAMP level can be modulated by activation of matrix sAC, H295R cells, transfected with 4mtH30, were superfused with an isosmotic medium containing 50 mM HCO_3^- (as sAC is known to be activated by bicarbonate ions (Buck et al., 1999;Jaiswal and Conti, 2003;Litvin et al., 2003;Steegborn et al., 2005)). An increase in the 480/540 ratio was observed in 16 out of 20 cells. Noteworthy, in parallel, the measurement of matrix pH with mtAlphi (Cano Abad et al., 2004) revealed a net alkalinisation (n=20, Fig. 3). Given that pH alkalinization results in an increase of YFP fluorescence (Di Benedetto et al., 2013), the total rise of mt-cAMP is underestimated.

3.3 Effect of angiotensin II on mitochondrial cAMP formation

In preliminary experiments we examined the effect of 1 and 10 nM angiotensin II on the generation of mt-cAMP. The effect of 1 nM was recorded in 133 cells and an increase in mt-cAMP was observed in 53 cells, whereas a small decrease was detected in 8 cases ($p < 0.01$).

In the next series of experiments we studied the effects of 10 nM angiotensin II. First angiotensin - induced changes in cytosolic (Fluo-4) and mitochondrial (Rhod-2) [Ca^{2+}] were

measured in the same cells. At the single cell level the cytosolic Ca^{2+} signals were variable, yet they were faithfully transferred to the mitochondrial matrix (Supplementary Fig. 1). The peptide brought about mt-cAMP elevation in 38 cells (Fig. 4), an inverse signal in 4 cells (increase vs. decrease: $p < 0.0001$) and no change was observed in 11 cells. The average $\Delta R/R_o$ ratio of a 100-sec period increased by 2.28 $\pm$ 0.23 % as compared with that of the 100-secretion pre-stimulation period (n=34). (Due to a decreasing ratio during the control period no response could be calculated in 4 cells). Eleven cells showed no cAMP response and 4 cells showed a decrease. The difference between the number of cells with a positive or reverse signal was highly significant ($p<0.0001$). Given that the percentage of responding cells in the 10 nM angiotensin II group was significantly higher than in the 1 nM group ($p = 0.0001$), in subsequent experiments we focused on the effects of 10 nM angiotensin II.

Considering the variance of cAMP rises in different cells and different preparations both in terms of amplitude and kinetics, the most reliable parameter to compare different conditions appeared to be the percentage of responsive cells. This parameter was, unless otherwise indicated, used in evaluating the following experiments. In the experiments presented in Supplementary Fig. 2 and Fig. 5, cells were stimulated with 10 nM angiotensin II in the presence or absence of the sAC inhibitor 2-OHE (20 μM) (Steegborn et al., 2005). Supplementary Fig. 1 demonstrates that the drug did not modify either the cytosolic or mitochondrial Ca^{2+} response to angiotensin II; however, at the same dose, 2-OHE reduced the percentage of cells showing mt-cAMP increase to angiotensin II by 34 % ($p = 0.0135$, Fig. 5A). The specificity of the inhibitor was confirmed by silencing sAC with MR2, a specific siRNA for the sAC gene (Di Benedetto et al., 2013); in this latter case the percentage of responding cells decreased by 67 % ($p = 0.006$, Fig. 5B). Next we examined the effect of EHNA (erythro-9-(2-hydroxyl-3-nonyl)adenine), an

inhibitor of the mitochondrial matrix cAMP phosphodiesterase PDE2A (Acin-Perez et al. 2011), on angiotensin II – induced changes in 4mtH30 FRET. Although there was no significant change in the percentage of responsive cells, the peak amplitude of the response was significantly higher in the presence of 10 μM EHNA ($p = 0.007$, Fig. 5D).

In order to confirm that angiotensin II - induced mt-cAMP response was induced by increased mitochondrial [Ca^{2+}], mitochondrial Ca^{2+} peaks were attenuated with heterologously expressed mitoS100G, a mitochondria-targeted Ca^{2+} binding protein. S100G was fused to a mitochondrial targeting sequence and cloned into an adenovirus vector under the control of doxycycline (DOX) - inducible tetOn promoter (Wiederkehr et al., 2011). In DOX-negative control cells 9 out of 14 cells showed angiotensin II - induced mt-cAMP increase whereas only 3 out of 12 cells showed similar response after DOX-dependent infection. The difference between the two groups was significant ($p = 0.045$, Fig. 5C).

3.4 Role of mitochondrial cAMP in the control of aldosterone production

We next investigated whether mt-cAMP plays any role in steroid hormone secretion, the specific function of adrenocortical cells. H295R cells were exposed for 2 hours to angiotensin II, applied at maximal (1 nM) or supramaximal concentration (10 nM) and their aldosterone production was measured. Angiotensin II evoked a mean 2.7 fold increase in aldosterone secretion whereas the increase in 2-OHE treated cells was reduced to 1.5 fold on average (Fig. 6A). Statistical analysis after pooling the 1 and 10 nM data showed that the effect of 2-OHE was highly significant ($p = 10^{-6}$) and, more importantly, the negative interaction of the agonist and the enzyme inhibitor was also highly significant ($p = 10^{-6}$).

The observation obtained with pharmacological inhibition of sAC has been confirmed by transfection with siRNA. The 2-hour hormone production was examined 3 days after transfection with MR2. As shown in Fig. 6B aldosterone secretion in siRNA-transfected cells was significantly smaller than in control RNA-transfected cells ($p = 0.002$) and, more importantly, the extent of stimulation was reduced from an average of 1.61 to 1.47, reflecting a significant negative interaction of gene silencing with the agonist ($p = 0.041$).

sAc is localized both in the cytosol (Braun and Dods, 1975;Jaiswal and Conti, 2001) and in the mitochondrial matrix (Acin-Perez et al., 2009). The question then arises as to whether the effects of sAC inhibition on aldosterone production depend on mt-sAC or cytosolic sAC or both. In order to distinguish among these possibilities cells were transfected with H30, the cytosolic version of the cAMP sensor. The probe, as expected, was selectively localized in the cytosol and excluded from mitochondria (Supplementary 3 A and B). 10 nM angiotensin II induced a clear cytosolic cAMP rise in 16 out of 22 cells (Fig. S2C) in controls, the average $\Delta R/R_o$ of a 100-sec stimulation period in the responding control cells showed a 3.93 $\pm$ 0.45 % increase. The sAC inhibitor 2-OHE failed to influence this cytosolic cAMP response: 12 out of the 16 examined 2-OHE-treated cells responded to angiotensin with cAMP signal, the average $\Delta R/R_o$ showed a 3.85 $\pm$ 0.35 % increase (Supplementary Fig. 3D).

This observation demonstrates that angiotensin II is capable of increasing cytosolic cAMP level by activating a 2-OHE insensitive AC. In addition, superfusion with 50 mM bicarbonate (that induces an increase in mt-cAMP) is associated with a drop in cAMP level in the cytosol (n = 12 out of 16 cells, Fig. S2E). This drop is probably only apparent as bicarbonate induces an alkalinization of cytosolic pH, similar in amplitude to that caused in the mitochondrial matrix (not shown). We thus conclude that in H295R cells: i) the activity of sAC in the cytosol is

negligible and ii) inhibition of angiotensin II-induced aldosterone production by 2-OHE depends on its inhibition of mitochondrial sAC.

4. Discussion

The recent discovery of the existence of an autonomous intramitochondrial cAMP signaling machinery (composed by a sAC isoform and a cAMP degrading mechanism sensitive to both IBMX and EHNA, a PDE2 specific inhibitor (Acin-Perez et al., 2009)) has been followed by the direct demonstration that in HeLa and CHO cells as well as in cardiomyocytes a rise in cAMP can be triggered by an increase in HCO_3^- and Ca^{2+} in the matrix, acting synergically (Di Benedetto et al., 2013). Intramitochondrial cAMP rise is paralleled by a significant increase of the matrix level of ATP (Acin-Perez et al., 2009;Di Benedetto et al., 2013). On the contrary, no consensus exists as to the target(s) of cAMP in the mitochondrial matrix (see for example (Acin-Perez et al., 2009;Di Benedetto et al., 2013;Lefkimmiatis et al., 2013)). Moreover, and most importantly, the possible functional effects of this cAMP increase are still largely unexplored.

In the present paper we have investigated whether the matrix cAMP signaling toolkit is expressed also in other differentiated cell types and whether functions other than ATP production can be modulated by the cAMP concentration in the matrix. To this end we used an adrenocortical cell line, H295R cells (Bird et al., 1993;Rainey et al., 1994), a model system widely employed to investigate the function of glomerulosa cell, especially the agonist dependent production of aldosterone.

In lysates of these cells a protein band of approximately 50 kDa, corresponding to the truncated, fully active form of sAC (Buck et al., 1999), was detected by immunoblotting with anti-sAC antibody. The density of this band (related to actin) was reduced by a specific sAC

siRNA. In cells transfected with the mitochondrial targeted cAMP sensor 4mtH30 superfusion with bicarbonate, a known activator of sAC (Buck et al., 1999;Jaiswal et al., 2003;Litvin et al., 2003;Steegborn et al., 2005) resulted in increased mt-cAMP level in 80 % of the cells.

It has been demonstrated that sAC in vitro is activated not only by bicarbonate but also by Ca^{2+} (reviewed in (Di Benedetto et al., 2014) (Valsecchi et al., 2014). Depending on its concentration, Ca^{2+} increases the V_{max} (Jaiswal et al., 2003) and substrate affinity (Litvin et al., 2003) of the enzyme. We have also shown that sAC in live cells can be activated by an increase in mitochondrial matrix Ca^{2+} concentration (Di Benedetto et al., 2013). We and other groups have previously shown that the peptide angiotensin II induces an important (often oscillatory) cytosolic Ca^{2+} increase that is rapidly transferred to the mitochondrial matrix of glomerulosa cells (Brandenburger et al., 1996;Lalevee et al., 2003;Spät et al., 2004;Spät and Pitter, 2004); under the same conditions angiotensin II causes a reduction of mitochondrial pyridine nucleotides (Pralong et al., 1992;Pralong et al., 1994;Rohács et al., 1997) and a potentiation of cytosolic Ca^{2+} - evoked aldosterone production (Wiederkehr et al., 2011). As predicted, angiotensin II induces an increase in mt-cAMP. The cAMP response was smaller than previously reported (Di Benedetto et al., 2013), a phenomenon that may be accounted for by two factors: i) expression level of sAC (related to total protein content) in H295R cells was only about half of that measured in e.g. HeLa cells and, ii) agonist – induced mitochondrial Ca^{2+} signals (measured with similar technique) are smaller in H295R cells than in other cell lines (e.g. HeLa (Fülöp et al., 2011)). Moreover, when mitochondrial Ca^{2+} signaling was attenuated by expression of mitoS100G, a Ca^{2+} binding protein targeted into the mitochondrial matrix (Wiederkehr et al., 2011), the percentage of cells showing a mt-cAMP response was significantly reduced. Finally, confirming the role played by sAC, the increase in the matrix cAMP generation is markedly attenuated by the sAC inhibitor 2-OHE and by specific siRNA and accentuated by EHNA, a specific inhibitor of phosphodiesterase PDE2A.

The mitochondria targeted Ca^{2+} binding protein S100G which attenuates exclusively the mitochondrial Ca^{2+} signal (Wiederkehr et al., 2011), interfered with the mitochondrial cAMP response to angiotensin II. The mitochondrial cAMP sensor 4mtH30 and the cytosolic sensor H30 responded with opposite signals to bicarbonate. And importantly, the sAC inhibitor 2-OHE added with angiotensin II, attenuated the mt-cAMP signal, but had no effect on the cytosolic cAMP signal. These data indicate that under the present experimental conditions, 4mtH30 and H30 monitored specifically, and respectively, the mitochondrial and the cytosolic compartment.

The final key question concerns the functional significance of the mt-cAMP formation. In order to address this point we examined the primary biological function of adrenocortical cells, namely hormone production. Pharmacological evidence support the notion that sAC - produced mt-cAMP plays a modulatory role on Ca^{2+} dependent aldosterone production. In fact, 2-OHE significantly reduced hormone production elicited by 1 or 10 nM angiotensin II. Most importantly, knockdown of the sAC also attenuated control and angiotensin II - induced aldosterone production.

The interaction of the ACTH - cAMP and angiotensin II - Ca^{2+} signaling systems in the control of aldosterone production has been thoroughly investigated in the past and conflicting results have been reported. The Tait group showed a K^+ - evoked cAMP formation in rat glomerulosa cells (Hyatt et al., 1986). Several years later the expression of the Ca^{2+} - activable isoform 1 of adenylyl cyclase (AC1) was shown to be expressed in human glomerulosa and fasciculata cells (Cote et al., 2001) whereas another isoform (AC3), possibly activated by Ca^{2+}, was found in human (Cote et al., 2001), rat (Nishimoto et al., 2013) and bovine (Burnay et al., 1998) glomerulosa cells. On the contrary, a reduced cAMP formation was reported in angiotensin – stimulated rat glomerulosa (Bell et al., 1981;Woodcock et al., 1984) or bovine adrenocortical cells (Begeot et al., 1988;Marie et al., 1983). In our hands, in H295R cells, angiotensin II caused

a clear and reproducible increase in cytosolic cAMP. It is not easy to reconcile these apparently contradictory findings. It may be proposed that coexpression of various amounts of Ca^{2+}-activated and Ca^{2+}-inhibited isoforms (AC5 and AC6, (human: (Cote et al., 2001), rat: (Shen et al., 1997)) is a species specific feature and may account for the different results obtained in different models. Our data provide evidence that the synergic action of Ca^{2+} and cAMP plays a role also within the mitochondrial matrix, regulating aldosterone biosynthesis. In mitochondria, however, the synthesis of cAMP depends on sAC that in turn is activated by Ca^{2+} and bicarbonate.

Summarizing the present observations, sAC is expressed in the mitochondria of adrenocortical cells. In addition to a possible activation of a transmembrane adenylyl cyclase by the cytosolic Ca^{2+} signal, the mitochondrial cyclase is activated by mitochondrial Ca^{2+} signal as well. Activation of sAC enhances the Ca^{2+} - induced production of aldosterone, a hormone controlling not only salt-water balance but also playing a significant role in the control of blood pressure as well as in the progress of various cardiovascular, renal and inflammatory diseases (Andersen, 2013;Briet and Schiffrin, 2010;Gomez-Sanchez, 2014;Tomaschitz et al., 2010). These data provide the first evidence that cAMP rise in mitochondria has a functional role. This observation has significance both for cell biology and endocrinology.

Authors' contribution

A.S. designed the experiment and wrote the manuscript. A.S. and D.K. performed the microscopic examinations, off-line evaluations and statistical calculations. A.R. performed cellular and analytical works. G. D. B. and T. P. designed and prepared 4mtH30, and contributed with critical remarks and advice to the whole work.

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Appendix. Supplementary material

Supplemental data associated with this article can be found, in the online version at …..

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Highlights:

- HCO_3^- activates mitochondrial adenylyl cyclase (sAC) in human adrenocortical cells
- angiotensin II causes a mitochondrial [cAMP] rise in a $[Ca^{2+}]_{mito}$-dependent way
- cAMP signal is reduced by siRNA for sAC, and enhanced by PDE2-inhibitor
- inhibition of sAC attenuates angiotensin II-induced aldosterone secretion

Figure 1

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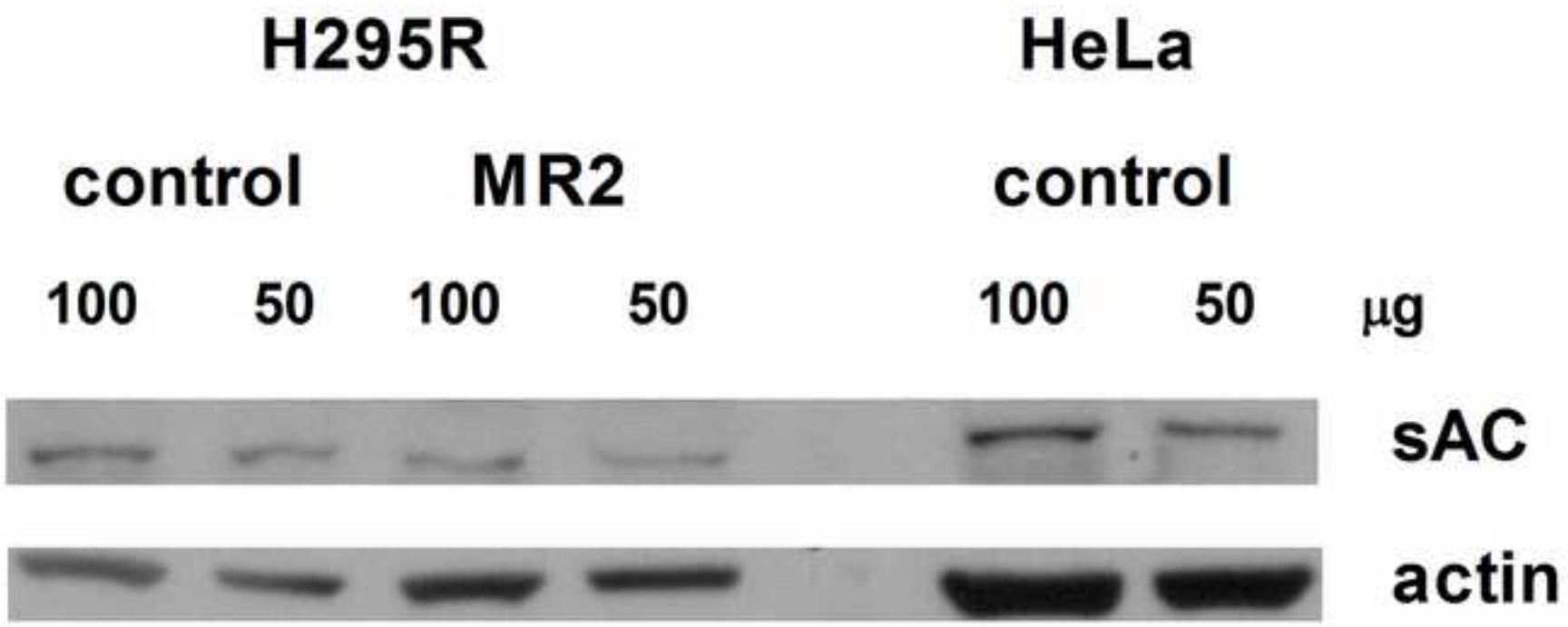

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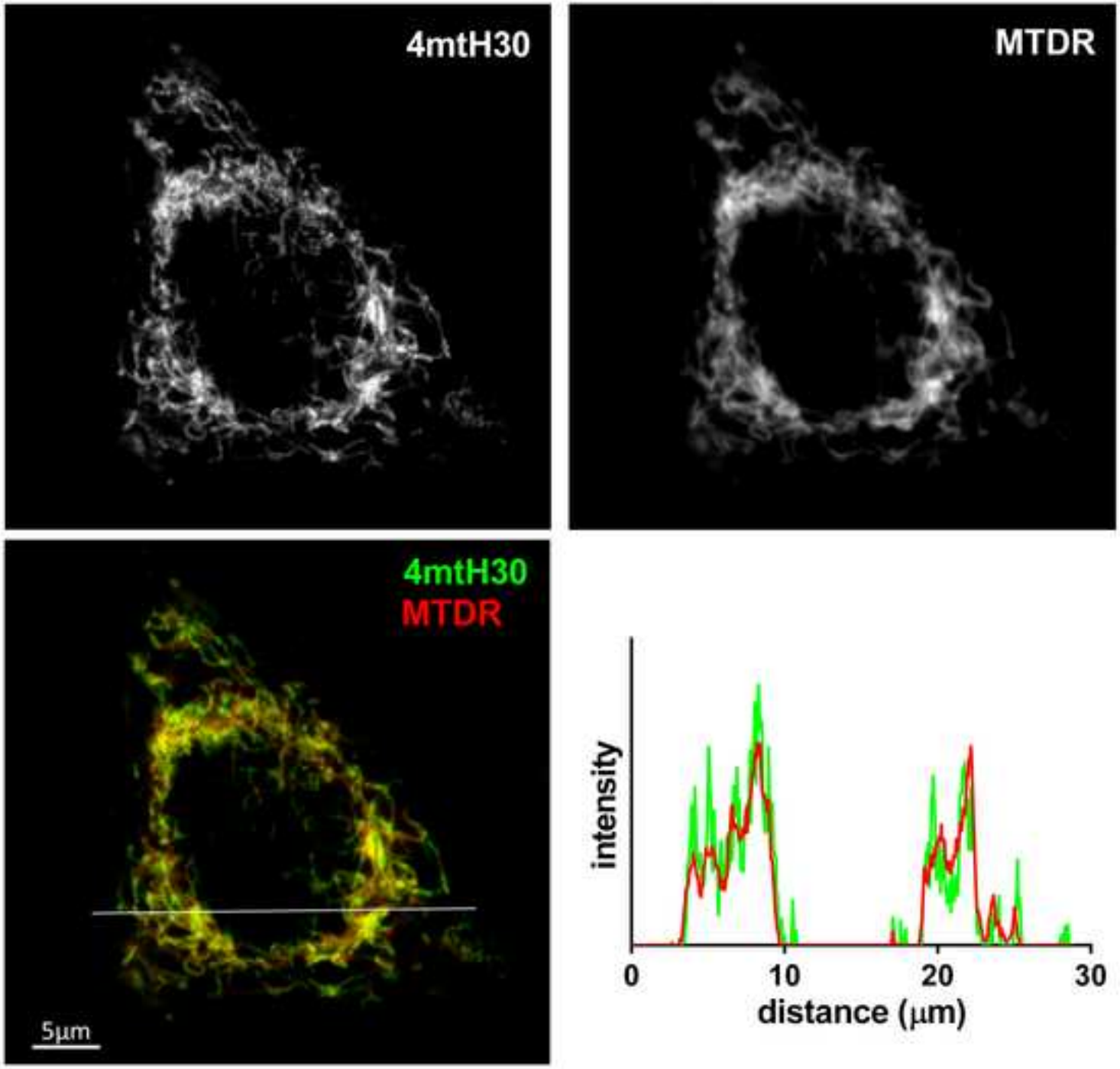

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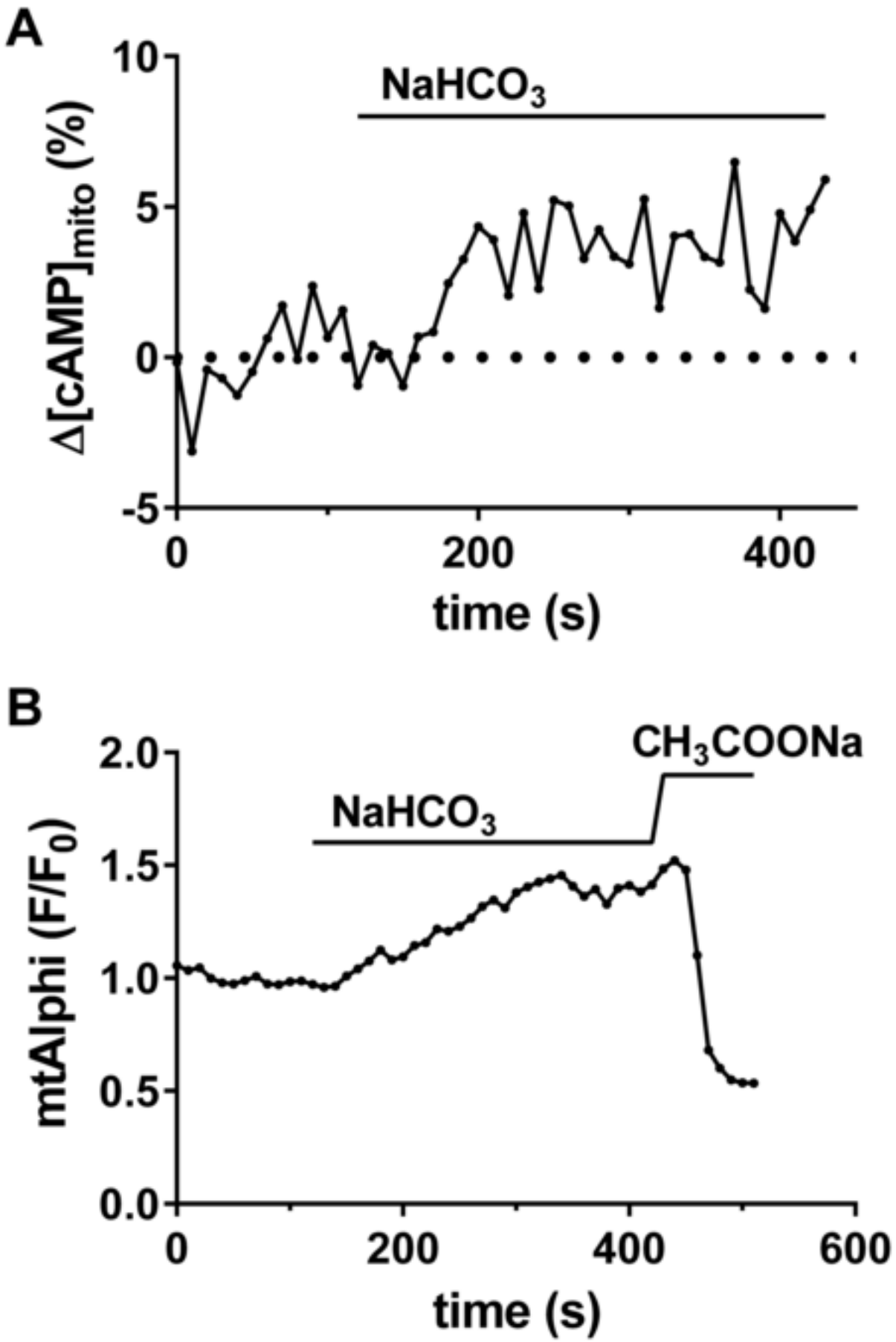

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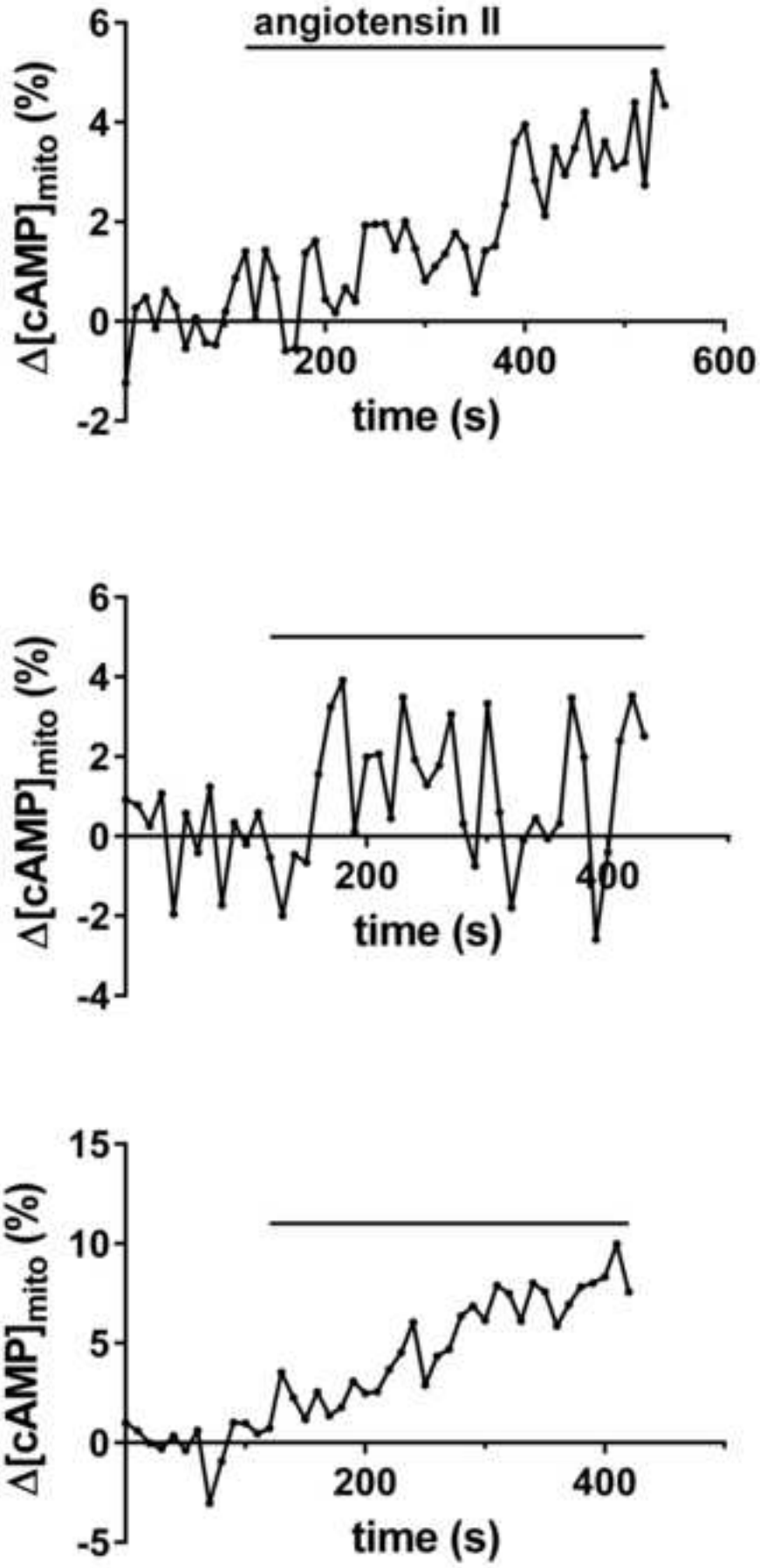

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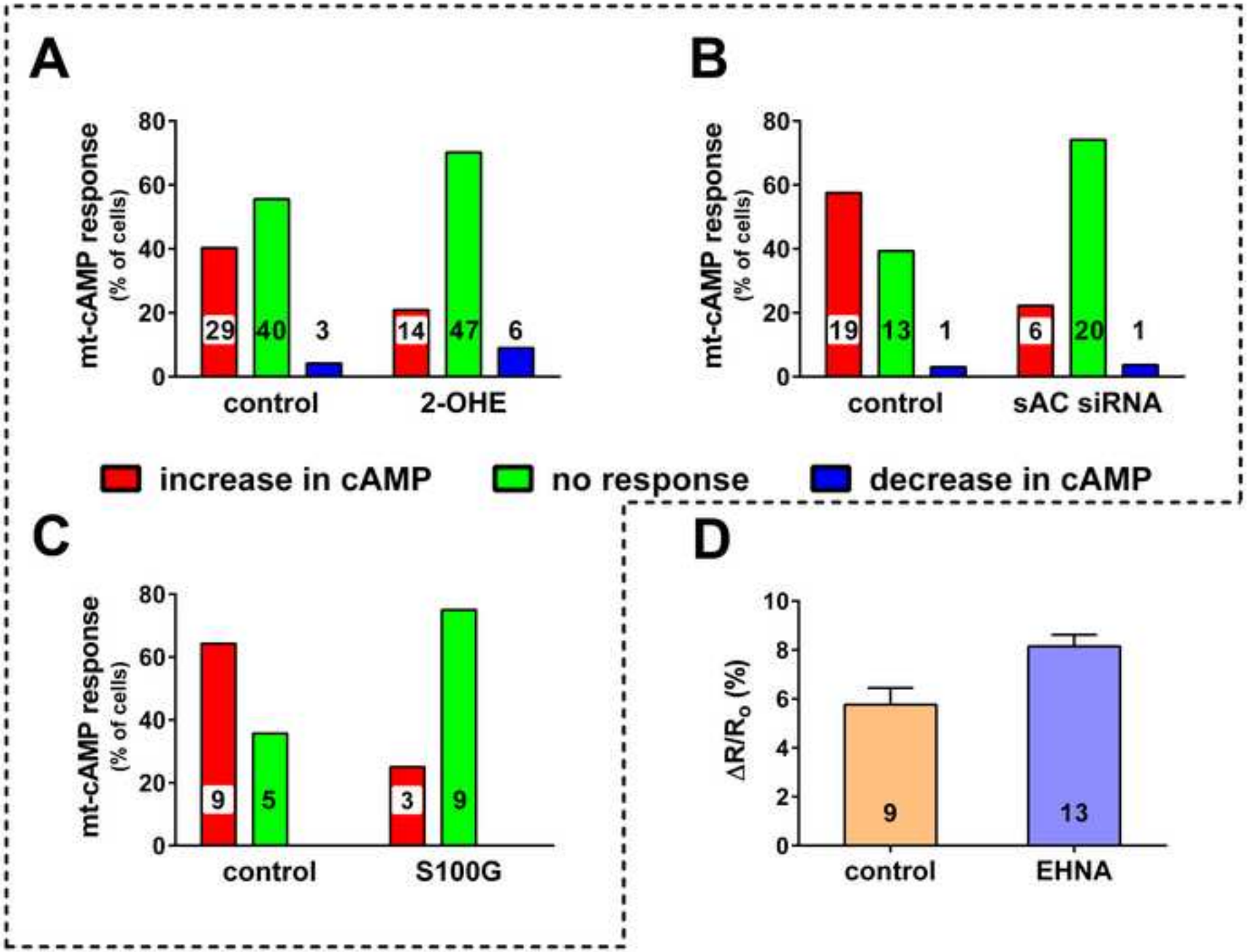

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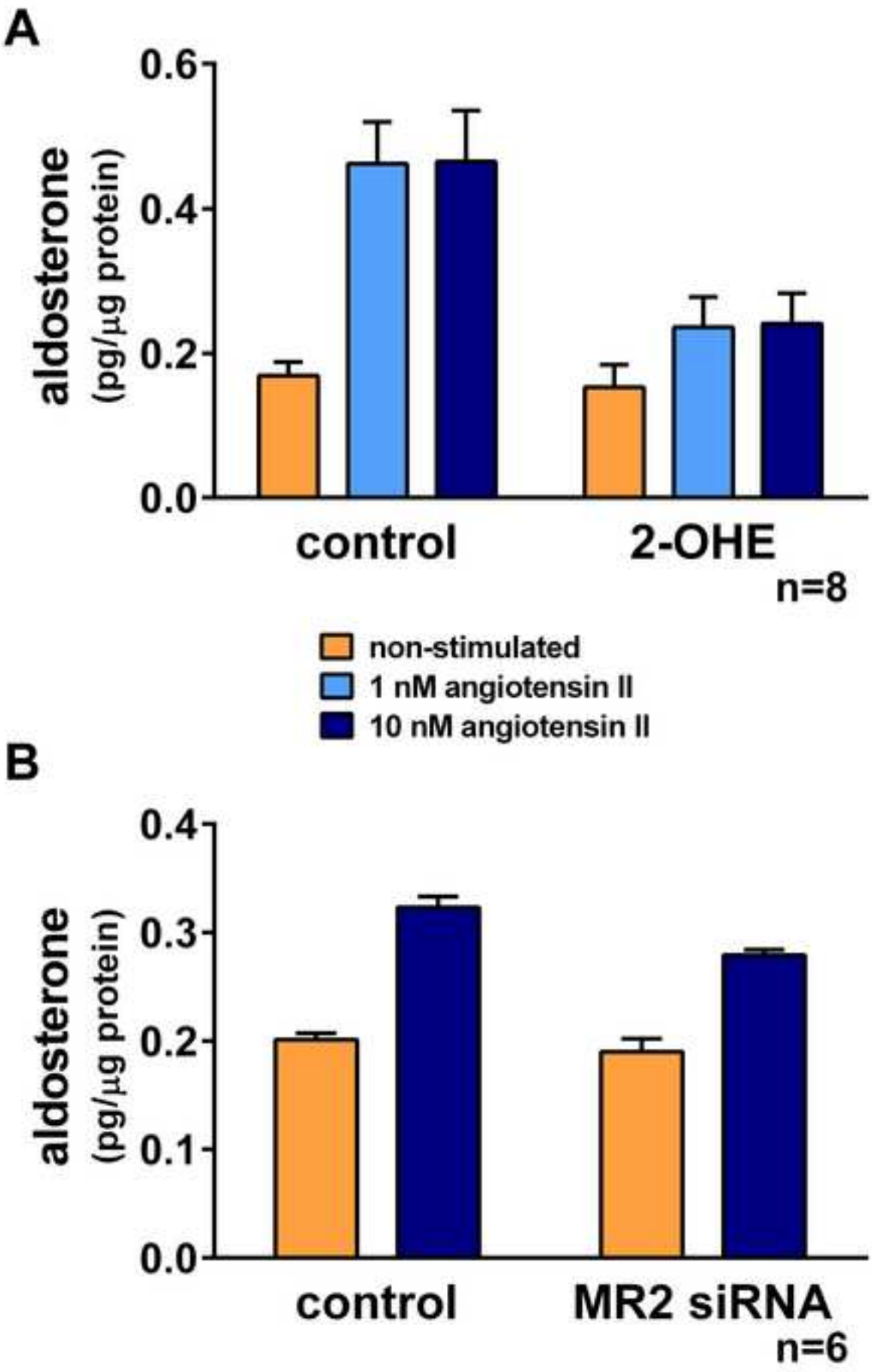

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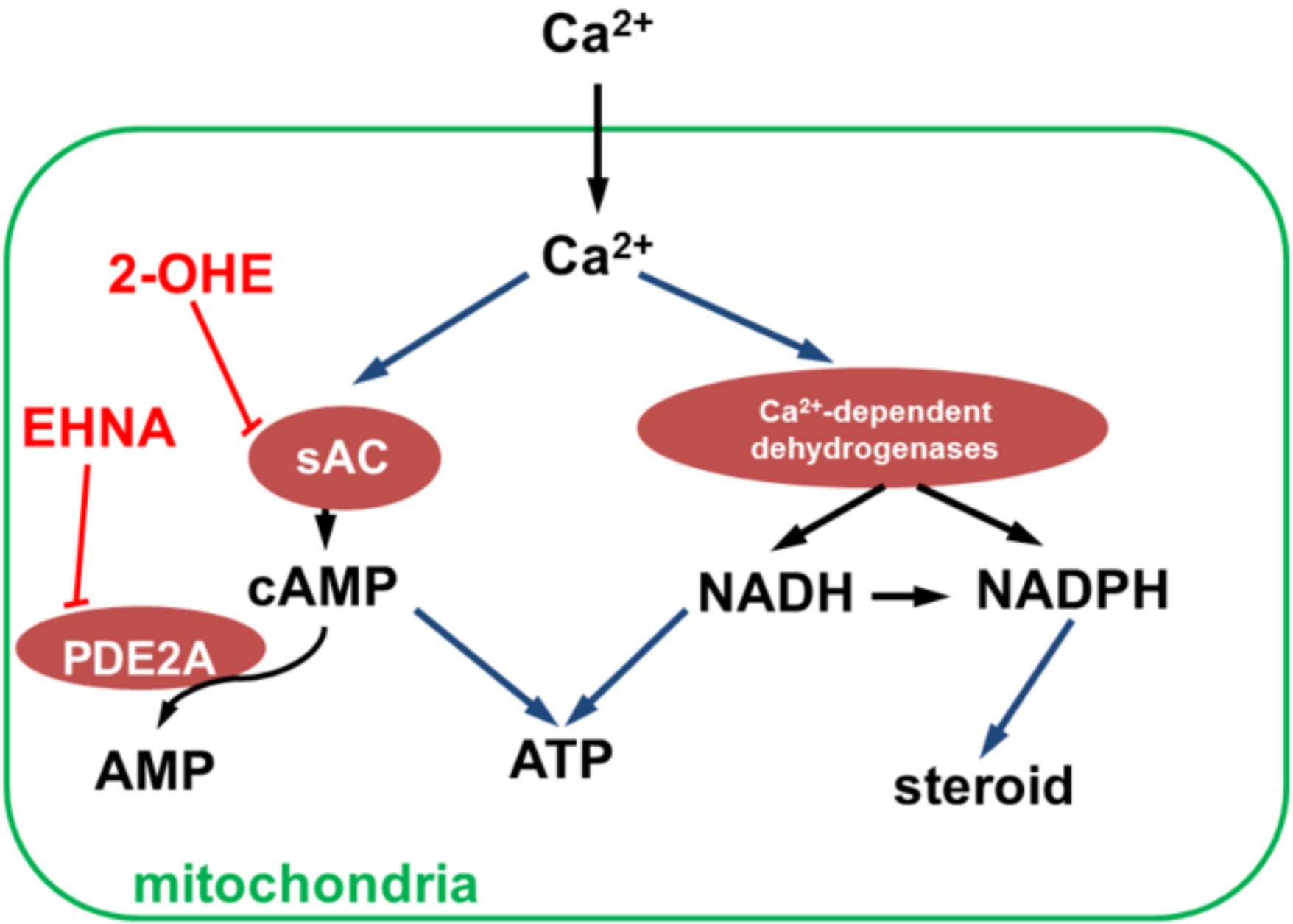

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Conflict of Interest

The authors declare that they have no conflict of interest