

**Mineralocorticoid modulation of cardiac ryanodine receptor activity is associated with downregulation of FK506-binding proteins.**

Ana María Gómez, Angélica Rueda, Yannis Sainte-Marie, Laetitia Pereira, Spyros Zissimopoulos, Xinsheng Zhu, Roxane Schaub, Emeline Perrier, Romain Perrier, Céline Latouche, et al.

► **To cite this version:**

Ana María Gómez, Angélica Rueda, Yannis Sainte-Marie, Laetitia Pereira, Spyros Zissimopoulos, et al.. Mineralocorticoid modulation of cardiac ryanodine receptor activity is associated with downregulation of FK506-binding proteins.. *Circulation*, American Heart Association, 2009, 119 (16), pp.2179-87. <10.1161/CIRCULATIONAHA.108.805804>. <inserm-00422024>

**HAL Id: inserm-00422024**

**<http://www.hal.inserm.fr/inserm-00422024>**

Submitted on 5 Oct 2009

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Mineralocorticoid modulation of cardiac ryanodine receptor activity is associated with FKBP  
down regulation.**

Gómez: Aldosterone modulation of Ca<sup>2+</sup> sparks

Ana María Gómez, PhD<sup>1</sup>, Angélica Rueda, PhD<sup>1</sup>, Yannis Sainte-Marie, PhD<sup>2</sup>, Laetitia Pereira PhD<sup>1</sup>, Spyros Zissimopoulos PhD<sup>3</sup>, Xinsheng Zhu, PhD<sup>4</sup>, Roxane Schaub, MD<sup>5</sup>, Emeline Perrier, PhD<sup>1</sup>, Romain Perrier PhD<sup>1</sup>, Céline Latouche, MS<sup>2</sup>, Sylvain Richard, PhD<sup>1</sup>, Marie-Christine Picot, MD PhD<sup>5</sup>, Frederic Jaisser, MD PhD<sup>2</sup>, F Anthony Lai, PhD<sup>3</sup>, Héctor H Valdivia, MD PhD<sup>4</sup> and Jean-Pierre Benitah, PhD<sup>1</sup>

<sup>1</sup>INSERM, U637, Université Montpellier, France.

<sup>2</sup>INSERM, U772, Collège de France, Paris, France.

<sup>3</sup>Wales Heart Research Institute, Cardiff University School of Medicine, Cardiff, UK.

<sup>4</sup>Department of Physiology, University of Wisconsin Medical School, Madison, USA.

<sup>5</sup>Département de l'Information Médicale, Hôpital Lapeyronie, CHU Montpellier, France

**Corresponding author**

Jean-Pierre Benitah

Laboratoire de physiopathologie cardiovasculaire

INSERM U637

CHU Arnaud de Villeneuve

34295 MONTPELLIER, France

Tel 33 4 67 41 52 38

Fax 33 4 67 41 52 42

Email [jean-pierre.benitah@inserm.fr](mailto:jean-pierre.benitah@inserm.fr)

**The total word count** 5981

**Subject Codes** [136] [152]

## **Abstract**

**Background.** The mineralocorticoid pathway is involved in cardiac arrhythmias associated with heart failure (HF) through yet incompletely understood mechanisms. Defective regulation of the cardiac ryanodine receptor (RyR) is an important cause in the initiation of arrhythmias. Here, we examined whether the aldosterone pathway might modulate RyR function.

**Methods and results.** Using whole-cell patch-clamp, we observed an increase in the occurrence of delayed after-depolarizations during action potential recordings in isolated adult rat ventricular myocytes exposed 48 hours to 100 nmol/L aldosterone, in freshly isolated myocytes from transgenic mice with human mineralocorticoid receptor expression in the heart and in wild-type littermates treated with aldosterone. Sarcoplasmic reticulum  $\text{Ca}^{2+}$  load and RyR expression were not altered, however, RyR activity, visualized *in situ* by confocal microscopy, was increased in all these cells, as evidenced by an increased occurrence and redistribution to long-lasting and broader populations of spontaneous  $\text{Ca}^{2+}$  sparks. These changes were associated with down-regulation of FK506-binding proteins (FKBP12 and 12.6), regulatory proteins of the RyR macromolecular complex.

**Conclusions.** We suggest that, in addition to modulation of  $\text{Ca}^{2+}$  influx, over stimulation of the cardiac mineralocorticoid pathway in the heart might be a major upstream factor for aberrant  $\text{Ca}^{2+}$  release during diastole, contributing to cardiac arrhythmia in HF.

**Key Words:** aldosterone,  $\text{Ca}^{2+}$  sparks, FKBP, arrhythmia

## Introduction

During the past decade, research has focused on the actions of aldosterone in target organs beyond the kidney, expanding the role of the aldosterone pathway in cardiovascular pathogenesis<sup>1</sup>. Indeed, mineralocorticoid receptors (MR), which mainly underlie aldosterone action, have been detected in a range of non-renal tissues including the brain, blood vessels and the heart<sup>2</sup>, suggesting a broader pattern of biological activity for aldosterone than previously anticipated. The pivotal role of aldosterone, causing sodium retention with expansion of the extracellular volume, resulting in deterioration of hemodynamic responsiveness and a fall in cardiac output, has long been recognized in heart failure (HF)<sup>3,4</sup>. In addition, accumulating experimental and clinical evidence suggests that aldosterone has direct adverse cardiac effects independent of its effects on blood pressure, especially an increased risk of arrhythmic death<sup>3,4</sup>. Interestingly, major clinical trials involving MR antagonists have shown significant benefits on risk of cardiovascular events, in particular sudden death in HF<sup>5,6</sup>. A pertinent question is, therefore, how activation of cardiac MR participates in life-threatening arrhythmias?

Most fatal arrhythmias in experimental HF initiate by nonreentrant mechanisms arising from abnormal ventricular automaticity or triggered activity<sup>7,8</sup>. The latter consists of either early afterdepolarizations (EADs) occurring in the plateau phase of the action potential (AP) or delayed afterdepolarizations (DADs) occurring at repolarized membrane potentials<sup>9</sup>. EADs typically occur in the setting of prolonged repolarization due to alterations in ionic currents and to reactivation of  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ ). DADs are caused by membrane depolarization initiated by spontaneous  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR). We have accumulated evidence, both *ex* and *in vivo*, that the modulation of  $\text{Ca}^{2+}$  influx is a central factor in the cardiac action of aldosterone pathway<sup>10-14</sup> and might be involved in EAD-related fatal ventricular tachyarrhythmia<sup>14</sup>. Moreover, enhanced diastolic leak of  $\text{Ca}^{2+}$  via the RyRs generates DADs and is

a fundamental mechanism underlying several genetic or acquired arrhythmias<sup>15,16</sup>. Therefore, we tested here whether the activation of aldosterone pathway modulates diastolic RyR activity in heart.

## Methods

All experiments were carried out according to European Union Council Directives (86/609/EEC) for the care of laboratory animals. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

A detailed Methods section can be found in the online Supplemental Material.

### *Cell isolation and incubation*

Isolated ventricular myocytes from adult male Wistar rats (250-350 g) were incubated for 48 hours, with or without 100-nmol/L D-Aldosterone (Sigma). In some experiments, 10- $\mu$ mol/L RU28318 was added to aldosterone<sup>10,11</sup>.

### *hMR transgenic mice and in vivo aldosterone exposure*

Cardiac-specific expression of human MR (hMR) was obtained by crossing a tetO-hMR mouse strain with an  $\alpha$ -MHC-tTA transactivator mouse strain<sup>14</sup>. Littermate wild-type, gender-matched mice (WT) were used as controls. In some of them, after pentobarbital sodium anesthesia (30 mg/kg), osmotic minipumps (Model 2 ML4, Alza Corporation) were subcutaneously implanted for constant delivery of 50 $\mu$ g/day D-Aldosterone (dissolved in 0.9% saline) over 3 weeks. Chronic aldosterone infusion significantly increased the plasma aldosterone concentration above control levels (from 278 $\pm$ 77 [n=7] to 1374 $\pm$ 150 [n=7] pg/ml,  $P<0.05$ ). Mice were devoid of cardiac hypertrophy either on whole organ (heart weight-to-body weight ratio in mg/g: 5.9 $\pm$ 0.4 [n=15], 5.9 $\pm$ 0.3 [n=11] and 5.3 $\pm$ 0.4[n=6] in WT, aldosterone-treated and hMR mice, respectively,  $P>0.05$ ) or at cellular level (membrane capacitance in pF: 201.8 $\pm$ 6.1 [n=30], 199.6 $\pm$ 12.7 [n=21] and

199.0±11.0 [n=26] for isolated ventricular myocytes from WT, aldosterone-treated and hMR mice, respectively,  $P>0.05$ ).

#### *Action potential recording*

Whole cell patch clamp method was used in current clamp configuration to record APs using solutions and protocol as described<sup>11</sup>.

#### *Spontaneous local SR Ca<sup>2+</sup> release: Ca<sup>2+</sup> sparks*

Fluo-3AM loaded cells were imaged in Tyrode solution (in mmol/L: NaCl 130, NaH<sub>2</sub>PO<sub>4</sub> 0.4, NaHCO<sub>3</sub> 5.8, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 0.5, KCl 5.4, glucose 22, HEPES 25, insulin 0.01, pH 7.4) using a confocal microscope in line scan mode<sup>11</sup>.

#### *Cell lysate and SR-enriched membrane fraction (SR fraction)*

Cell lysates were prepared with homogenization buffer (in mmol/L: sucrose 300, NaF 20, HEPES 20, Aprotinin 5.2 10<sup>-4</sup>, Benzamidine 0.5, Leupeptin, 0.012, PMSF 0.1, pH 7.2) using a Potter-Elvehjem and spun at 2,000 g for 10 min. SR fractions were isolated by ultracentrifugation at 40,000 g for 30 min at 4°C<sup>17</sup>. <sup>17</sup>P protein concentration by Bradford method and binding of [<sup>3</sup>H]ryanodine to SR fractions were assessed<sup>17</sup>.

#### *Single-channel recordings*

Analysis of RyR single-channel activity was done by fusing SR vesicles into planar lipid bilayers using Cs<sup>+</sup> as charge carrier<sup>17</sup>. Only bilayers containing a single channel were used. Channel activity was always tested for sensitivity to EGTA, added in the cis chamber at the end of each experiment.

#### *RT-PCR analysis*

Total RNA was extracted using Trizol (InVitrogen). First strand cDNA was synthesized after DNaseI treatment (DNAfree, Ambion) using 1 µg of total RNA, random hexamers (Amersham) and Superscript II reverse transcriptase (InVitrogen). Transcripts levels were analyzed in triplicate by real time PCR with an iCycler iQ apparatus (Bio-Rad) using a qPCR Core Kit for SYBR Green I

(Eurogentec) containing 500 nmol/L of specific primers (Table S1) and 3  $\mu$ L of diluted template cDNA. Relative expression of FKBP12/12.6 and RyR were normalized by the geometric average of relative quantities for reference genes. Serial dilutions of pooled cDNA were used in each experiment to assess PCR efficiency.

#### *Immunoblots*

Immunoblots were prepared from cell lysates using anti-FKBP12/12.6 (1:1000, Santa-Cruz Biotechnology Inc), anti-RyR (1:3000, Affinity Bioreagents), and anti-actin (1:20000, Sigma) antibodies. FKBP12/12.6-RyR interaction was assessed on SR fractions using anti-FKBP12/12.6 (1:200) and anti-RyR (1:1000) antibodies from Affinity BioReagents. Immunoblots of RyR phosphorylation were performed using anti-RyR-PS2809 (1:5000) antibody from Badrilla (Leeds, West Yorkshire, UK) and anti-RyR-PS2815 (1:5000) antibody generously provided by Dr A.R. Marks (Columbia University, New York, NY).

#### *Statistics*

Preliminary descriptive analyses include frequencies for categorical variables and means $\pm$ SD for continuous ones. A conditional hierarchical linear model was used (SAS/UNIX statistical software, SAS Institute, Cary, N.C. proc mixed) to compare continuous variables between groups to take into account for multiple observations per animals. The group was a fixed effect, animals were a random effect nested in the group and, in case of repeated measures on cells, we add a random effect for cell in animal.

Data are presented as mean $\pm$ SEM and compared using Student's t test (for two groups) or ANOVA (for more groups followed up with a post-hoc pairwise Tukey's HSD test). Significance was defined at  $P < 0.05$ .

## **Results**

### *Aldosterone pathway increases occurrence of DADs.*

During AP recordings, at 0.1-Hz cycle length in ventricular myocytes isolated from transgenic mice expressing hMR in the heart (hMR mice, Fig.1A), we observed oscillations in membrane potential following completion of the driven AP (Fig.1A middle). Eventually, the DAD was large enough to reach the threshold to trigger spontaneous AP (Fig.1A right). The occurrence of DADs was greatly enhanced by the activation of aldosterone pathway (Fig.1B). *Ex vivo*, after 48-hours exposure of isolated rat ventricular myocytes to 100-nmol/L aldosterone, the occurrence of DADs increased compared with control myocytes kept 48 hours in absence of aldosterone. Likewise *in vivo*, increases of DAD occurrence appeared after 3-week minipump infusion of aldosterone in WT mice or in hMR mice, as compared to untreated WT littermates.

*Modulation of Ca<sup>2+</sup> sparks by aldosterone pathway.*

DADs are commonly initiated by non-electrically driven, spontaneous Ca<sup>2+</sup> release from the SR via the RyRs<sup>8</sup>. We thus examined the properties of RyRs *in situ*, by visualizing spontaneous Ca<sup>2+</sup> sparks that reflect brief and local Ca<sup>2+</sup> release events occurring when RyRs open<sup>18,19</sup>. Figure 2A shows line scan images of cells isolated from WT, aldosterone-treated and hMR mice. Either elevated circulating aldosterone or cardiac hMR expression resulted in ~1.6-fold increase of Ca<sup>2+</sup> spark frequency (Table S2, Fig.2B). No differences in mean values for Ca<sup>2+</sup> spark amplitude (Fig.2C) or rise time (Fig.2D) were seen between the 3 groups. As noticed in Figure 2A, besides “classical” Ca<sup>2+</sup> sparks characterized by a brief and localized increase in the fluorescence signal (a half time of decay ~30 ms and a diameter of ~2 μm)<sup>18</sup>, we also observed the appearance of widened (>4 μm) and long-lasting Ca<sup>2+</sup> release events (>80 ms). A mixed-effect model revealed increases of both averaged Ca<sup>2+</sup> spark full widths (FWHM) and durations (FDHM) at half maximal in aldosterone-treated and hMR mice (Table S2), which might reflect differences in population proportions. The distributions of FWHM and FDHM were empirically fitted to bimodal Gaussian functions (Fig.2E and F). The distributions of FWHM showed a prominent mode near 2.8 μm and a second minor mode near 4.3 μm, whatever the conditions. However, the proportion of events



showing larger width was greater in aldosterone-treated and hMR mice compared with WT (Table 1). Moreover, events lasting more than 50 ms were more frequent in aldosterone-treated and hMR mice compared with WT. Distributions of log(FDHM) were fitted by 2 Gaussian distributions with 2 distinct peaks ~32 and 87 ms, but the frequency of long-lasting signals were larger in aldosterone-treated and hMR mice than that observed in WT mice (Table 1). No correlation between Ca<sup>2+</sup> spark spatio-temporal characteristics was observed. This is consistent with occurrence of three different populations of Ca<sup>2+</sup> sparks, whose redistributions to long-lasting and larger spread area populations were increased by activation of the cardiac aldosterone pathway.

While the effects of aldosterone *in vivo* are presumably due to its direct cardiomyocyte effect, aldosterone might cause release of second messengers from non-cardiac cells that can affect Ca<sup>2+</sup> sparks. To exclude this possibility we examined the properties of spontaneous Ca<sup>2+</sup> sparks of isolated rat ventricular myocytes kept 48 hours with or without 100-nmol/L aldosterone. Three different populations of Ca<sup>2+</sup> sparks were also observed (Fig.3): the normal Ca<sup>2+</sup> sparks are narrow and brief (Fig.3A); the second population of Ca<sup>2+</sup> sparks is substantially wider in space, but only slightly longer in duration (Fig.3B); the third population of Ca<sup>2+</sup> sparks has the same width as the normal but is very much longer in duration (Fig.3C). Analysis of the distributions of FWHM (Fig.3D) and FDHM (Fig.3E) showed that *ex vivo* aldosterone exposure increases the occurrence of both wider and longer populations (Tables 1 and S2). In addition, we observed a 1.9-fold increase in Ca<sup>2+</sup> spark frequency (Table S2, Fig.3F), whereas mean Ca<sup>2+</sup> spark amplitude (Fig.3G) and rise time (Fig.3H) were not affected. Co-incubation with a specific MR antagonist, RU28318<sup>11,12,13</sup>, prevented aldosterone-induced changes in Ca<sup>2+</sup> spark properties (Fig.3).

*Aldosterone effects on Ca<sup>2+</sup> sparks might arise from modulation of RyR intrinsic properties.*

The open probability of RyRs is influenced by the amount of Ca<sup>2+</sup> stored in the SR<sup>20</sup>. A potential increase of SR Ca<sup>2+</sup> load by aldosterone could therefore underlie the observed effects on Ca<sup>2+</sup> sparks. SR Ca<sup>2+</sup> load was estimated by rapid caffeine application (10 mmol/L) in the same

intact cells used for  $\text{Ca}^{2+}$  sparks<sup>11,21</sup>. No difference ( $P>0.05$ ) was observed in hMR mice (peak  $F/F_0$ :  $4.0\pm 0.1$ ,  $n=56$ ) and in aldosterone-treated mice ( $3.8\pm 0.2$ ,  $n=43$ ) compared to WT ( $4.1\pm 0.1$ ,  $n=29$ ). As well, no difference in the caffeine-evoked  $\text{Ca}^{2+}$  transients was noticed after *ex vivo* aldosterone exposure ( $3.7\pm 0.2$  and  $4.0\pm 0.2$  in control [ $n=13$ ] and aldosterone treated myocytes [ $n=13$ ], respectively,  $P>0.05$ ). These results are consistent with the absence of alteration in  $\text{Ca}^{2+}$  spark amplitude and suggest that aldosterone does not modulate RyR activity (and hence,  $\text{Ca}^{2+}$  sparks), by altering SR content.

$\text{Ca}^{2+}$  sparks result from the opening of clusters of RyRs, thus, increased  $\text{Ca}^{2+}$  spark frequency might result from increased RyR expression. Immunoblot analysis of cell lysates showed similar RyR amounts (after normalization against control, ratios of RyR to actin levels were:  $1\pm 0.3$  [ $n=8$ ],  $0.95\pm 0.3$  [ $n=4$ ] and  $0.82\pm 0.3$  [ $n=4$ ] in WT, WT+Aldo and hMR mice, respectively,  $P>0.05$ ; and  $1\pm 0.2$  [ $n=8$ ],  $0.94\pm 0.2$  [ $n=4$ ] in rat ventricular myocytes kept 48 hours without and with aldosterone, respectively,  $P>0.05$ ). RyR expression of isolated rat cardiomyocytes kept 48 hours with or without aldosterone, examined by [ $^3\text{H}$ ]ryanodine binding to SR fractions, indicated unchanged high-affinity binding site for ryanodine ( $K_d$  values in nmol/L:  $1.5\pm 0.2$  and  $1.8\pm 0.4$  after 48 hours incubation with [ $n=6$ ] and without aldosterone [ $n=6$ ], respectively;  $P>0.05$ ) and similar expression levels of RyR after aldosterone exposure (maximal receptor density:  $0.24\pm 0.04$  and  $0.29\pm 0.03$  pmol/mg of protein, respectively,  $P>0.05$ ).

These results showed that activation of the aldosterone pathway in cardiac myocytes alters  $\text{Ca}^{2+}$  spark properties without modifying SR  $\text{Ca}^{2+}$  content or RyR expression. Therefore, aldosterone modulation of  $\text{Ca}^{2+}$  sparks might arise from changes in the intrinsic activity of the RyR complex. As an index, we fused SR fractions from control and aldosterone-treated rat cardiomyocytes into planar lipid bilayers to record single channel activity of RyR. Besides classical bimodal gating<sup>20</sup>, characterized by sequences of flickering openings with low (Fig.4A) and high (Fig.4B) open probability, a clearly distinct long and relatively stable openings with similar unitary

current amplitude were observed (Fig.4C). Measurements from holding potentials  $-35$  to  $+35$  mV showed that current-voltage relationships had similar conductance of 639, 632 and 640 pS (for Fig.4A, B and C, respectively). These results suggested that all recorded channel activity corresponded to RyRs, however, although the burst and long-lasting modes were observed in channels with and without aldosterone treatment, these modes occurred more frequently after aldosterone exposure (Fig.4D).

*FKBP12/12.6 downregulation by mineralocorticoid pathway.*

Taken together, our results indicate that aldosterone through MR increases the likelihood that RyRs open abnormally during diastole, which might reflect changes in intrinsic properties of the RyR. Activity of RyR is tightly regulated by several accessory proteins that form a macromolecular signaling complex with RyR<sup>15,16</sup>. Among these ancillary proteins, FK506-binding proteins (FKBP12 and 12.6) have been involved in the incidence of subsets of cardiac  $\text{Ca}^{2+}$  sparks with longer duration and wider spatial spread, along with increased frequency<sup>21-23</sup>. Therefore, we assessed the expression of FKBP12/12.6 protein levels. Compared to the respective controls, immunoblot analysis of cell lysates showed decreases in the amount of FKBP12/12.6 relative to actin levels after *in* or *ex vivo* aldosterone exposure and in hMR mice (Fig.5A). The RyR-FKBP12/12.6 association was indirectly assessed by measuring the ratio of FKBP12/12.6 to RyR detected in the SR fractions<sup>24-26</sup>. Immunoblot analysis indicates that the relative amounts of FKBP12/12.6 to RyR are decreased in the heart of hMR mice or WT mice treated with aldosterone compared to control WT (Fig.5B). Because RyR phosphorylation could influence FKBP12/12.6-RyR association<sup>15,16</sup>, we examined RyR phosphorylation status following activation of the mineralocorticoid pathway. RyR contains multiple phosphorylation sites including RyR-Ser2815 (phosphorylated by CaMKII)<sup>27</sup> and RyR-Ser2809 (phosphorylated by both CaMKII and PKA)<sup>28</sup>. Using 2 different phospho-specific antibodies (RyR-PS2815 and RyR-PS2809) on SR fractions, we observed no difference in the ratio of phosphorylated RyR to total RyR among groups (Fig.5C). To

distinguish FKBP12 versus FKBP12.6 expression, real-time quantitative PCR was used to assess mRNA levels with specific primers. At mRNA level, a decreased expression of both FKBP12 and 12.6 was observed in the 3 models studied compared to respective controls (Fig.5D), whereas mRNA levels of RyR were not altered (data not shown).

## **Discussion**

In summary, long-term aldosterone exposure *ex* or *in vivo*, or cardiac hMR expression in transgenic mice, increases the occurrence of DADs, in line with abnormal diastolic openings of RyR, which are associated with down-regulation of FKBP12 and 12.6. While the link might be circumstantial, several lines of evidence suggest that over-stimulation of the cardiac mineralocorticoid pathway may be a major upstream factor for aberrant Ca<sup>2+</sup> release during diastole, contributing to cardiac arrhythmia in HF.

Numerous experimental and clinical studies indicate that the aldosterone pathway participates in cardiac alterations associated with hypertension, HF, diabetes and other pathologies<sup>1,3-6,12,14</sup>. Notably, inappropriate activation of cardiac MR is a likely participant in the development of poor outcomes for patients with HF, especially those associated with cardiac arrhythmia<sup>5,6</sup>. Interestingly, hMR mice presented high mortality and increased occurrence of arrhythmia<sup>14</sup>. One of the possible cellular mechanisms for those arrhythmias is triggered activity caused by EADs and DADs, both of which are commonly associated with intracellular Ca<sup>2+</sup> mishandling. We previously reported that along with AP lengthening, EADs occur in hMR mice<sup>14</sup>. The incidence of EADs was (in %) 1, 11 and 14 in ventricular myocytes isolated from WT, aldosterone-treated and hMR littermate mice, respectively. In addition, we observed that 10% of rat ventricular myocytes incubated 48 hours with aldosterone presented EADs, whereas none was observed in control. Here, we found that the incidence of DAD is approximately 2-fold higher, thus constituting a substantial mechanism for aldosterone pathway-induced arrhythmias.

Whereas many  $\text{Ca}^{2+}$ -handling proteins are involved in DAD-related arrhythmias, abnormal opening of the RyR during diastole is an essential component<sup>7,8</sup>. We show here that aldosterone pathway increased the occurrence, and changed the spatio-temporal properties, of spontaneous  $\text{Ca}^{2+}$  sparks. Similar alterations were found either *ex vivo* by incubating adult cardiac myocytes with aldosterone, or *in vivo* in mouse hearts after chronic delivery of aldosterone or hMR expression. These consistent findings suggest that a direct activation of cardiac MR by aldosterone modifies  $\text{Ca}^{2+}$  spark phenotype, independently of other compensatory changes *in vivo*. Indeed, *ex vivo* aldosterone effects are prevented by a specific MR antagonist. In addition, hMR mice have increased aldosterone receptor activity, presumably due to physiologic aldosterone levels, as assessed by prevention of phenotype effects with MR antagonist<sup>14</sup>. Moreover, MR antagonists also prevented other aldosterone-induced cardiac effects<sup>10,11,13</sup>.

Although we cannot exclude that the effects might be secondary to other modulations in myocyte  $\text{Ca}^{2+}$  handling, several lines of evidence suggest that activation of MR by aldosterone directly affects RyR activity. The increase in  $\text{Ca}^{2+}$  spark frequency is not due to an increase in SR  $\text{Ca}^{2+}$  load but rather might reflect a modulation of the intrinsic properties of the RyR complex. Besides the increase in  $\text{Ca}^{2+}$  spark frequency, we observed a redistribution of  $\text{Ca}^{2+}$  sparks to long-lasting and broader populations. These “abnormal”  $\text{Ca}^{2+}$  sparks were also seen in control conditions, but at much lower frequency. Others have also found  $\text{Ca}^{2+}$  sparks that are longer or wider than the “classic”  $\text{Ca}^{2+}$  sparks in control conditions<sup>18,21,29-31</sup>. The incidence of wide ( $>2\ \mu\text{m}$ ) and long (60-80 ms)  $\text{Ca}^{2+}$  sparks is increased in left ventricular hypertrophy in the dog without alteration in  $\text{Ca}^{2+}$  spark amplitude<sup>32</sup>. Here, aldosterone exposure or cardiac hMR expression increased the proportions of long and wide  $\text{Ca}^{2+}$  sparks from 4-6% to 9-19% and from 4-6% to 16-30%, respectively, constituting a substantial effect. This indicates that aldosterone does not induce a new kind of  $\text{Ca}^{2+}$  sparks, but modifies the activity of functional release units.

Along with this alteration, we observed a significant down-regulation of FKBP12 and 12.6 expressions without variation in the density of RyRs. Even if other alterations may contribute to the observed modifications of Ca<sup>2+</sup> sparks characteristics, no other RyR-associated proteins have been shown to induce similar modulations as FKBP does. The FKBP12/12.6 binding to RyRs modulates the Ca<sup>2+</sup>-flux properties of the channel complex; in particular, it regulates RyR open probability and stability at rest<sup>20,33,34</sup> (but see also<sup>35</sup>). FKBP12.6 removal by pharmacological approaches<sup>22,36,37</sup> or transgenic animal models<sup>23</sup>, and conversely adenoviral short-term FKBP12.6 overexpression<sup>21,38</sup> modulated Ca<sup>2+</sup> sparks frequency and occurrence of longer and wider Ca<sup>2+</sup> sparks, in a similar manner to what we observed here. We showed that activation of cardiac aldosterone pathway produced a decreased expression of FKBP12/12.6. In addition, we have assessed the RyR-FKBP12/12.6 association in hMR transgenic and WT mice treated with aldosterone. Our results suggest that in mouse hearts, activation of the aldosterone pathway results in substantially reduced RyR-FKBP12/12.6 interaction that could be the cause of the observed RyR-mediated Ca<sup>2+</sup> leak. These effects are not associated with alteration of RyR phosphorylation status but might reflect a genomic regulation of FKBP12/12.6 by cardiac aldosterone pathway, as evidenced by the decrease in mRNA levels of FKBP12 and 12.6. Now, partial loss of FKBP12.6 from RyR in HF has been shown to cause diastolic Ca<sup>2+</sup> leak that may result in higher propensity of DADs and consequent triggered arrhythmias<sup>15,16,23,39-45</sup>. In addition, myocardial FKBP12.6 overexpression prevents triggered arrhythmias in normal hearts, probably by reducing diastolic SR Ca<sup>2+</sup> leakage<sup>46</sup>. Beyond the controversial hypothesis for excess RyR activity due to hyperphosphorylation reducing RyR affinity for FKBP12.6<sup>47</sup>, most of the studies show a reduction of FKBP protein level in HF<sup>23,39-45</sup>. Thus, we suggest that activation of the aldosterone pathway might be an essential step in the cascade of molecular events leading to FKBP deficiency that causes RyR Ca<sup>2+</sup> leakage, and trigger malignant cardiac arrhythmias in HF.

Taken together, our findings may partly explain why the use of MR antagonists on top of optimal medical therapy is associated with improved survival, and fewer sudden cardiac deaths in patients.

## Acknowledgements

## Funding Sources

This work was supported by Agence Nationale de la Recherche (project #A05071FS/APV05030FSA), Institut National de la Santé et de la Recherche Médicale (INSERM), the British Heart Foundation (PG/05/077) and the European Union (FPG, Life Science Genomics and Biotechnology for Health, #CT 2005 N°018802, CONTICA).

## Disclosures

None

## References

1. Williams JS, Williams GH. 50th anniversary of aldosterone. *J Clin Endocrinol Metab.* 2003;88:2364-72.
2. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science.* 1987;237:268-75.
3. Weber KT. Aldosterone in congestive heart failure. *N Engl J Med.* 2001;345:1689-97.
4. Rossi G, Boscaro M, Ronconi V, Funder JW. Aldosterone as a cardiovascular risk factor. *Trends Endocrinol Metab.* 2005;16:104-7.
5. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med.* 1999;341:709-17.
6. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med.* 2003;348:1309-21.
7. Bers DM. Cardiac excitation-contraction coupling. *Nature.* 2002;415:198-205.
8. Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. *Trends Cardiovasc Med.* 2004;14:61-6.
9. Clusin WT. Calcium and cardiac arrhythmias: DADs, EADs, and alternans. *Crit Rev Clin Lab Sci.* 2003;40:337-75.

10. Benitah JP, Vassort G. Aldosterone upregulates Ca<sup>2+</sup> current in adult rat cardiomyocytes. *Circ Res*. 1999;85:1139-45.
11. Benitah JP, Perrier E, Gomez AM, Vassort G. Effects of aldosterone on transient outward K<sup>+</sup> current density in rat ventricular myocytes. *J Physiol*. 2001;537:151-60.
12. Perrier E, Kerfant BG, Lalevee N, Bideaux P, Rossier MF, Richard S, Gomez AM, Benitah JP. Mineralocorticoid receptor antagonism prevents the electrical remodeling that precedes cellular hypertrophy after myocardial infarction. *Circulation*. 2004;110:776-83.
13. Perrier R, Richard S, Sainte-Marie Y, Rossier BC, Jaisser F, Hummler E, Benitah JP. A direct relationship between plasma aldosterone and cardiac L-type Ca<sup>2+</sup> current. *J Physiol*. 2005;569:153-62.
14. Ouvrard-Pascaud A, Sainte-Marie Y, Benitah JP, Perrier R, Soukaseum C, Cat AN, Royer A, Le Quang K, Charpentier F, Demolombe S, Mechta-Grigoriou F, Beggah AT, Maison-Blanche P, Oblin ME, Delcayre C, Fishman GI, Farman N, Escoubet B, Jaisser F. Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. *Circulation*. 2005;111:3025-33.
15. Wehrens XH, Lehnart SE, Marks AR. Intracellular calcium release and cardiac disease. *Annu Rev Physiol*. 2005;67:69-98.
16. Yano M, Yamamoto T, Ikeda Y, Matsuzaki M. Mechanisms of Disease: ryanodine receptor defects in heart failure and fatal arrhythmia. *Nat Clin Pract Cardiovasc Med*. 2006;3:43-52.
17. Nabhani T, Zhu X, Simeoni I, Sorrentino V, Valdivia HH, Garcia J. Imperatoxin a enhances Ca<sup>2+</sup> release in developing skeletal muscle containing ryanodine receptor type 3. *Biophys J*. 2002;82:1319-28.
18. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science*. 1993;262:740-4.
19. Bers DM. Macromolecular complexes regulating cardiac ryanodine receptor function. *J Mol Cell Cardiol*. 2004;37:417-29.
20. Fill M, Copello JA. Ryanodine receptor calcium release channels. *Physiol Rev*. 2002;82:893-922.
21. Gomez AM, Schuster I, Fauconnier J, Prestle J, Hasenfuss G, Richard S. FKBP12.6 overexpression decreases Ca<sup>2+</sup> spark amplitude but enhances [Ca<sup>2+</sup>]<sub>i</sub> transient in rat cardiac myocytes. *Am J Physiol Heart Circ Physiol*. 2004;287:H1987-93.
22. Xiao RP, Valdivia HH, Bogdanov K, Valdivia C, Lakatta EG, Cheng H. The immunophilin FK506-binding protein modulates Ca<sup>2+</sup> release channel closure in rat heart. *J Physiol*. 1997;500 ( Pt 2):343-54.
23. Xin HB, Senbonmatsu T, Cheng DS, Wang YX, Copello JA, Ji GJ, Collier ML, Deng KY, Jeyakumar LH, Magnuson MA, Inagami T, Kotlikoff MI, Fleischer S. Oestrogen protects FKBP12.6 null mice from cardiac hypertrophy. *Nature*. 2002;416:334-8.
24. Gaburjakova M, Gaburjakova J, Reiken S, Huang F, Marx SO, Rosemblyt N, Marks AR. FKBP12 binding modulates ryanodine receptor channel gating. *J Biol Chem*. 2001;276:16931-5.
25. George CH, Higgs GV, Lai FA. Ryanodine receptor mutations associated with stress-induced ventricular tachycardia mediate increased calcium release in stimulated cardiomyocytes. *Circ Res*. 2003;93:531-40.
26. Liu N, Colombi B, Memmi M, Zissimopoulos S, Rizzi N, Negri S, Imbriani M, Napolitano C, Lai FA, Priori SG. Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: insights from a RyR2 R4496C knock-in mouse model. *Circ Res*. 2006;99:292-8.
27. Wehrens XH, Lehnart SE, Reiken SR, Marks AR. Ca/calmodulin-independent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. *Circ Res*. 2004;94:e61- e70.



28. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblyt N, Marks AR. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell*. 2000;101:365-76.
29. Parker I, Wier WG. Variability in frequency and characteristics of  $\text{Ca}^{2+}$  sparks at different release sites in rat ventricular myocytes. *J Physiol*. 1997;505 ( Pt 2):337-44.
30. Blatter LA, Huser J, Rios E. Sarcoplasmic reticulum  $\text{Ca}^{2+}$  release flux underlying  $\text{Ca}^{2+}$  sparks in cardiac muscle. *Proc Natl Acad Sci U S A*. 1997;94:4176-81.
31. Shirokova N, Gonzalez A, Kirsch WG, Rios E, Pizarro G, Stern MD, Cheng H. Calcium sparks: release packets of uncertain origin and fundamental role. *J Gen Physiol*. 1999;113:377-84.
32. Song LS, Pi Y, Kim SJ, Yatani A, Guatimosim S, Kudej RK, Zhang Q, Cheng H, Hittinger L, Ghaleh B, Vatner DE, Lederer WJ, Vatner SF. Paradoxical cellular  $\text{Ca}^{2+}$  signaling in severe but compensated canine left ventricular hypertrophy. *Circ Res*. 2005;97:457-64.
33. Lehnart SE, Huang F, Marx SO, Marks AR. Immunophilins and coupled gating of ryanodine receptors. *Curr Top Med Chem*. 2003;3:1383-91.
34. Chelu MG, Danila CI, Gilman CP, Hamilton SL. Regulation of ryanodine receptors by FK506 binding proteins. *Trends Cardiovasc Med*. 2004;14:227-34.
35. Xiao J, Tian X, Jones PP, Bolstad J, Kong H, Wang R, Zhang L, Duff HJ, Gillis AM, Fleischer S, Kotlikoff M, Copello JA, Chen SR. Removal of FKBP12.6 does not alter the conductance and activation of the cardiac ryanodine receptor or the susceptibility to stress-induced ventricular arrhythmias. *J Biol Chem*. 2007;282:34828-38.
36. McCall E, Li L, Satoh H, Shannon TR, Blatter LA, Bers DM. Effects of FK-506 on contraction and  $\text{Ca}^{2+}$  transients in rat cardiac myocytes. *Circ Res*. 1996;79:1110-21.
37. Yoshihara S, Satoh H, Saotome M, Katoh H, Terada H, Watanabe H, Hayashi H. Modification of sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  release by FK506 induces defective excitation-contraction coupling only when SR  $\text{Ca}^{2+}$  recycling is disturbed. *Can J Physiol Pharmacol*. 2005;83:357-66.
38. Loughrey CM, Seidler T, Miller SL, Prestle J, MacEachern KE, Reynolds DF, Hasenfuss G, Smith GL. Over-expression of FK506-binding protein FKBP12.6 alters excitation-contraction coupling in adult rabbit cardiomyocytes. *J Physiol*. 2004;556:919-34.
39. Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng MJ, Mathews LM, Schneider MD, Hamilton SL, Matzuk MM. Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature*. 1998;391:489-92.
40. Yano M, Ono K, Ohkusa T, Suetsugu M, Kohno M, Hisaoka T, Kobayashi S, Hisamatsu Y, Yamamoto T, Noguchi N, Takasawa S, Okamoto H, Matsuzaki M. Altered stoichiometry of FKBP12.6 versus ryanodine receptor as a cause of abnormal  $\text{Ca}^{2+}$  leak through ryanodine receptor in heart failure. *Circulation*. 2000;102:2131-6.
41. Reiken S, Gaburjakova M, Gaburjakova J, He Kl KL, Prieto A, Becker E, Yi Gh GH, Wang J, Burkhoff D, Marks AR. beta-adrenergic receptor blockers restore cardiac calcium release channel (ryanodine receptor) structure and function in heart failure. *Circulation*. 2001;104:2843-8.
42. Wehrens XH, Lehnart SE, Reiken SR, Deng SX, Vest JA, Cervantes D, Coromilas J, Landry DW, Marks AR. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science*. 2004;304:292-6.
43. Yano M, Okuda S, Oda T, Tokuhisa T, Tateishi H, Mochizuki M, Noma T, Doi M, Kobayashi S, Yamamoto T, Ikeda Y, Ohkusa T, Ikemoto N, Matsuzaki M. Correction of defective interdomain interaction within ryanodine receptor by antioxidant is a new therapeutic strategy against heart failure. *Circulation*. 2005;112:3633-43.

44. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca<sup>2+</sup>/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca<sup>2+</sup> leak in heart failure. *Circ Res.* 2005;97:1314-22.
45. Huang F, Shan J, Reiken S, Wehrens XH, Marks AR. Analysis of calstabin2 (FKBP12.6)-ryanodine receptor interactions: rescue of heart failure by calstabin2 in mice. *Proc Natl Acad Sci U S A.* 2006;103:3456-61.
46. Gellen B, Fernandez-Velasco M, Briec F, Vinet L, LeQuang K, Rouet-Benzineb P, Benitah JP, Pezet M, Palais G, Pellegrin N, Zhang A, Perrier R, Escoubet B, Marniquet X, Richard S, Jaisser F, Gomez AM, Charpentier F, Mercadier JJ. Conditional FKBP12.6 overexpression in mouse cardiac myocytes prevents triggered ventricular tachycardia through specific alterations in excitation-contraction coupling. *Circulation.* 2008;117:1778-86.
47. Bers DM, Eisner DA, Valdivia HH. Sarcoplasmic reticulum Ca<sup>2+</sup> and heart failure: roles of diastolic leak and Ca<sup>2+</sup> transport. *Circ Res.* 2003;93:487-90.

### Figure Legends.

**Figure 1. Activation of aldosterone pathway increases the occurrence of delayed after-depolarizations (DADs).** A. Representative action potentials recorded from the same hMR cardiomyocyte, elicited at 0.1 Hz, showing the appearance of spontaneous depolarization (middle, DAD indicated by the arrows) and triggering spontaneous AP (right). B. Bar graph plots number of cells presenting at least 1 DAD in rat ventricular myocytes incubated 48 hours with or without 100-nmol/L aldosterone and in ventricular myocytes isolated from WT, aldosterone-treated WT and hMR littermate mice. n is the total cell number. \*  $P < 0.05$ .

**Figure 2. Change of the spatio-temporal properties of Ca<sup>2+</sup> sparks induced by mineralocorticoid in vivo.** A. Sample line-scan fluorescence images record from cells isolated from wild-type (WT, top), aldosterone-treated (WT+Aldo, middle) and hMR mice (bottom). B through D, Comparison of the frequency of occurrence, amplitude ( $F/F_0$ ), and rise time of Ca<sup>2+</sup> sparks at rest in myocytes from WT (red open bars), WT+Aldo, (light blue bars) and hMR mice (blue bars). In B, n is the cell number, whereas in C and D it is the number of Ca<sup>2+</sup> sparks. \*  $P < 0.05$ . Probability Density function (PDF) of Ca<sup>2+</sup> spark widths (E) and durations (F) at half maximal amplitude in WT, WT+Aldo, and hMR mice. Curves represent mixed Gaussians fitted to the histograms. In F,

analyses were conducted on log(FDHM) and corresponding results are presented after back transformation.

**Figure 3. *Ex vivo* aldosterone exposure promotes the occurrence of wide-spread and long-lasting  $Ca^{2+}$  sparks.** *A to C.* Representative spark images and spatio-temporal profiles from a 48-hour aldosterone-treated cell showing that spontaneous  $Ca^{2+}$  sparks can be: short (A), large (B) or long (C). The distance along the cell is represented vertically, and the time is represented horizontally. *D and E.* Pdf of FWHM and FDHM after 48 hours incubation under control (cont, red open bars), 100-nmol/L aldosterone without (Aldo; blue bars) and with 10- $\mu$ mol/L RU28318 (Aldo+RU; blue open bars) conditions. Histograms were fitted by the sum of 2 Gaussian distributions. *F to H.* Bar graphs of mean  $\pm$  SEM values for  $Ca^{2+}$  spark frequency (*F*), amplitude ( $F/F_0$ , *G*), and rise time (*H*). In *F*, *n* is the cell number whereas in *G* and *H* it is the number of  $Ca^{2+}$  sparks. \*\*  $P < 0.005$ .

**Figure 4. Single RyR channels reconstituted in lipid bilayers display three different kinetic behaviors: (A) short, (B) burst and (C) long openings.** Each panel shows representative examples of single channel behaviors of RyR reconstituted from the same SR-enriched membrane fraction of rat ventricular myocytes incubated 48 hours in the presence of aldosterone: (top) 2-min and 500-ms section of the recording on an expanded time scale of single-channel traces recorded at +25 mV with channel openings (o) in the upward direction; (bottom) Current-voltage relationship of the corresponding incorporated RyR. *D.* Bar graphs plots the percentage of the occurrence of modal behaviors of reconstituted RyR channels from SR fractions of cells kept 48 hours with (Aldo, gray bars, *n*=15) and without aldosterone (cont, open bars, *n*=11). \*\*  $P < 0.005$ .

**Figure 5. Down regulation of FKBP12 and 12.6 expressions after activation of cardiac aldosterone pathway.** *A to C.* Representative immunoblots and quantification of (A) FKBP12/12.6 protein levels in cardiac cell lysates (normalized to the corresponding actin level and normalized to respective controls), (B) FKBP12/12.6 and RyR content in SR-enriched membrane fractions (the

cumulative data presented are derived from four different heart preparations, each immunoblotted four times, and are illustrated after normalization against the WT, non-treated sample) and (C) Pooled data represent ratios of RyR-P2815 and RyR-P2809 to RyR from SR-enriched membrane fractions normalized to respective controls from WT (open bars), aldosterone-treated WT (WT+Aldo, hatched bar) and hMR (closed bars) mice; and in rat ventricular myocytes incubated 48 hours with (+Aldo, closed bar) or without (cont, open bar) 100-nmol/L aldosterone. *D*. Real time RT-PCR analysis of the cardiac tissue content of FKBP12 and 12.6.  $\beta$ 2-microglobulin, GAPDH, HPRT and UBC were used as reference genes and the relative mRNA levels were normalized to the geometric average of relative quantities for reference genes. \*  $P < 0.05$ .

**Table 1.** Mean ( $\mu$ ), standard deviation ( $\sigma$ ), proportion ( $p$  in %) and coefficient of determination ( $r^2$ ) of Gaussian fits to  $\text{Ca}^{2+}$  spark parameters.

	FWHM ( $\mu\text{m}$ )							FDHM (ms)						
	$\mu_1$	$\sigma_1$	$p_1$	$\mu_2$	$\sigma_2$	$p_2$	$r^2$	$\mu_1$	$\sigma_1$	$p_1$	$\mu_2$	$\sigma_2$	$p_2$	$r^2$
<i>Aldo in vivo</i>														
WT	2.79	0.91	93.5	4.32	0.60	6.5	0.98	30.9	5.6	96.0	85.2	23.9	4.0	0.98
WT+Aldo	2.80	0.84	83.8	4.30	1.20	16.2	0.99	32.4	5.9	91.0	87.6	30.2	9.0	0.99
hMR	2.78	0.71	70.2	4.33	1.15	28.8	0.99	33.4	5.9	81.6	89.4	27.9	18.4	0.99
<i>Aldo ex vivo</i>														
control	2.61	0.89	94.5	4.85	0.71	5.5	0.99	33.7	6.3	94.0	103.0	41.1	6.0	0.99
Aldo	2.62	0.91	72.0	4.60	1.25	28.0	0.99	33.5	6.3	81.7	106.0	50.3	18.3	0.99
Aldo+RU	2.54	0.78	95.8	4.53	0.70	4.2	0.99	33.7	5.7	95.7	105.3	35.2	4.3	0.99

FWHM and FDHM: Full width and duration at half maximal amplitude.