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Specific Activation of the Alternative Cardiac Promoter of *Cacna1c* by the Mineralocorticoid Receptor

Thèse de doctorat de l'Université Paris-Saclay et de Federal
University of Sergipe, préparée à l'Université Paris-Sud XI en
Châtenay-Malabry

École doctorale n°569: innovation thérapeutique:
du fondamental à l'appliqué (ITFA)
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Titre : Activation spécifique du promoteur cardiaque alternatif du *Cacna1c* par le récepteur aux minéralocorticoïdes

Mots clés : Canaux Ca^{2+} de type L, aldostérone, récepteur aux minéralocorticoïde, Cœur, muscle lisse vasculaire.

Résumé :

Les antagonistes des récepteurs aux minéralocorticoïdes (MR) appartiennent à l'arsenal thérapeutique pour le traitement de diverses maladies cardiovasculaires, mais les mécanismes conférant leurs effets bénéfiques sont encore mal compris. Une partie de ces effets peuvent être liée à la régulation de l'expression du canal Ca^{2+} de type L $\text{Ca}_v1.2$, largement impliqué dans l'insuffisance cardiaque et l'hypertension. Nous montrons que MR fonctionne comme un facteur de transcription transformant le signal de l'aldostérone dans l'utilisation du 'cardiaque' promoteur alternatif P1, dirigeant l'expression du long N-terminal transcrit ($\text{Ca}_v1.2\text{-LNT}$)

L'aldostérone augmente de façon concentration- et de temps dépendente l'expression de $\text{Ca}_v1.2\text{-LNT}$ dans les cardiomyocytes en raison de l'activation du promoteur P1, par interactions des MR avec des séquences spécifiques de l'ADN sur le promoteur P1. Ce mécanisme de cis-régulation induit l'activation de promoteur P1 dans les cellules vasculaires conduisant à une nouvelle signature moléculaire de $\text{Ca}_v1.2\text{-LNT}$ associé à une sensibilité réduite aux bloqueurs des canaux Ca^{2+} . Ces résultats révèlent $\text{Ca}_v1.2\text{-LNT}$ comme une cible minéralocorticoïde spécifique qui pourrait influencer sur l'efficacité thérapeutique dans les maladies cardiovasculaires.

Title : Specific activation of the alternative cardiac promoter of *Cacna1c* by the mineralocorticoid receptor

Keywords : L-type Ca^{2+} channel; aldosterone; mineralocorticoid receptor; heart; vascular smooth muscle cells.

Abstract :

The mineralocorticoid receptor (MR) antagonists belong to the current therapeutic armamentarium for the management of cardiovascular diseases, but the mechanisms conferring their beneficial effects are still poorly understood. Part of these MR effects might be related to the L-type $\text{Ca}_v1.2$ Ca^{2+} channel expression regulation, critically involved in heart failure and hypertension. Here, we show that MR acts as a transcription factor triggering aldosterone signal into specific alternative 'cardiac' P1-promoter usage, given rise to long ($\text{Ca}_v1.2\text{-LNT}$) N-terminal transcripts.

Aldosterone increases $\text{Ca}_v1.2\text{-LNT}$ expression in cardiomyocytes in a time- and dose-dependent manner due to MR-dependent P1-promoter activity, through specific DNA sequence-MR interactions. This *cis*-regulatory mechanism induced a MR-dependent P1-promoter *switch* in vascular cells leading to a new $\text{Ca}_v1.2\text{-LNT}$ molecular signature with reduced Ca^{2+} channel blocker sensitivity. These findings uncover $\text{Ca}_v1.2\text{-LNT}$ as a specific mineralocorticoid target that might influence the therapeutic outcome of cardiovascular diseases.

Título : Ativação específica do promotor cardíaco alternativo do *Cacna1c* pelo receptor de mineralocorticoide

Palavras-chave : Canal para Ca^{2+} do tipo L; aldosterono; receptor de mineralocorticoide; coração; músculo liso vascular.

Resumo:

Atualmente, os antagonistas do receptor de mineralocorticoide (MR) pertencem ao arsenal terapêutico para o tratamento de diversas doenças cardiovasculares. No entanto, os mecanismos pelos quais essa classe de medicamentos exercem seus efeitos benéficos ainda não estão totalmente compreendidos. Parte desses efeitos estão relacionados à regulação da expressão do canal para Ca^{2+} do tipo L $\text{Ca}_v1.2$, amplamente envolvido na insuficiência cardíaca e hipertensão. No presente estudo, demonstramos que o MR atua como um fator de transcrição regulando a utilização alternativo do promotor P1 cardíaco, aumentando a transcrição da forma longa da região N-terminal ($\text{Ca}_v1.2\text{-LNT}$).

A aldosterona aumenta a expressão de $\text{Ca}_v1.2\text{-LNT}$ em cardiomiócitos de maneira dependente do tempo e da concentração via aumento na atividade do promotor P1, através de interações específicas do MR com sequências no DNA. Este mecanismo *cis*-regulador também induziu aumento na atividade do promotor P1 em células vasculares, levando à uma nova assinatura molecular do $\text{Ca}_v1.2\text{-LNT}$, estando associado com reduzida sensibilidade aos bloqueadores de canais para Ca^{2+} . Portanto, esses achados revelam o $\text{Ca}_v1.2\text{-LNT}$ como um novo e específico alvo do MR, podendo também influenciar a eficiência terapêutica no tratamento das doenças cardiovasculares.



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Finally, I dedicate this thesis to my mother, who gave me the bless of life and always teach me how to be a good human being. Te Amo!

Résumé Étendu

Activation spécifique du promoteur cardiaque alternatif du *Cacna1c* par le récepteur aux minéralocorticoïdes

Actuellement, les antagonistes des récepteurs aux minéralocorticoïdes (MR) appartiennent à l'arsenal thérapeutique pour le traitement de diverses maladies cardiovasculaires. Cependant, les mécanismes par lesquels cette classe de médicaments exercent leurs effets bénéfiques ne sont pas encore entièrement compris. Les recherches menées par notre groupe ont démontré à travers les expériences *in vitro*, *ex vivo* et *in vivo*, qu'une partie des effets délétères de l'aldostérone par le biais d'activation du MR implique l'augmentation de l'expression et de l'activité du $Ca_v1.2$, agissant comme un déclencheur pour le développement de pathologies cardiaques. Donc, l'objectif central de cette étude a été d'évaluer les mécanismes génomiques impliqués dans la modulation de l'expression du $Ca_v1.2$ par le MR.

Dans cette étude, nous avons démontré dans les cultures primaires de myocytes ventriculaires de rat nouveau-né (NRVM) que le traitement à l'aldostérone durant 24 h augmente de façon concentration dépendante (10^{-10} à 10^{-9} M) l'expression du $Ca_v1.2$. Il est important souligner que les sous-unités auxiliaires du $Ca_v1.2$ (β_2 et $\alpha_{2\delta 2}$) présentent également une augmentation de l'expression de leurs messagers respectifs, suggérant ainsi une activité transcriptionnelle précisément coordonnée. Faisant partie de la famille de récepteurs nucléaires, la translocation cytosol-noyau du MR a été évaluée dans les NRVM à différentes concentrations d'aldostérone (10^{-9} à 10^{-7} M) et à différents temps de traitement (30-90 min). Nous avons observé l'augmentation de la translocation nucléaire du MR à de faibles concentrations d'aldostérone (10^{-9} M) et après une courte période de temps (30 min) de traitement. En outre, nous avons validé le trafic rapide intracellulaire du MR au noyau (visible à partir de 10 min) dans les NRVMs transfectées par un plasmide contenant le hMR-GFP, sur . Il est important souligner que de hautes concentrations de dexaméthasone (10^{-8} M) – une puissante hormone glucocorticoïde synthétique – ne modifie pas la localisation cellulaire du MR.

L'expression du $Ca_v1.2$ est régulée par deux promoteurs distincts et, même si elle n'est pas totalement comprise, sa régulation se produit de manière spécifique sur certains types tissulaires. Par exemple, le promoteur "cardiaque" (P1) régule l'expression de la forme longue de la région N-terminale du $Ca_v1.2$ ($Ca_v1.2$ -LNT), tandis que le promoteur "vasculaire/cérébral" (P2) régule la forme courte de ce canal ($Ca_v1.2$ -SNT). Nous avons observé que l'aldostérone augmente la transcription du l'exon 1a ($Ca_v1.2$ -LNT) dans les NRVMs, sans altérer l'exon 1b ($Ca_v1.2$ -SNT). Ces données sont liées à l'activation du promoteur P1 de façon concentration et de temps (6-24h) dépendantes.

La spécificité de ces effets, qui implique l'activité transcriptionnelle du MR, ont été évaluées dans la culture cellulaire par le traitement simultané avec l'inhibiteur sélectif par le MR (RU28318,

concentration 100x supérieure par rapport à aldostérone) ou à travers l'inactivation (siRNA) du MR. Ainsi, nous démontrons par ces deux démarches expérimentales, que les effets de l'aldostérone sur l'augmentation de l'expression du $Ca_v1.2$ exon 1a et l'activité du promoteur P1 sont complètement abolis. Afin de confirmer ces résultats dans les cellules adultes, nous avons généré une souris génétiquement modifiée par l'expression du gène reporter luciférase sous le contrôle du promoteur P1 du *Cacna1c* (PCa.luc). De façon similaire à ce que nous observons sur les NRVMs, les cardiomyocytes ventriculaires adultes des souris PCa.luc, traités durant 24 h à l'aldostérone montrent une augmentation significative de façon concentration dépendante de l'activité de la luciférase. Ces résultats sont confirmés *in vivo* après traitement des souris PCa.luc durant 3 semaines avec de l'aldostérone. Nos données indiquent clairement que l'activation du MR provoque la transactivation du promoteur P1 et, par conséquent, la transcription $Ca_v1.2$ -LNT.

À l'aide d'analyses *in silico* et par mutagenèse, nous avons identifié que le MR interagit avec des éléments spécifiques activateurs et répresseurs de liaison à l'ADN du promoteur P1. Nous avons identifié 5 éléments de réponse aux glucocorticoïdes (GRE), dont 1 est «négatif», proche de la région de départ de la transcription du exon 1a du gène *Cacna1c*, chevauchant donc la séquence du promoteur P1 que nous utilisons. Pour évaluer la fonction de ces importantes régions, hautement spécialisées dans la régulation de l'activité du promoteur P1, chaque GRE préalablement identifiée ont été remplacées par une autre séquence (*scrambled*). Dans les NRVMs transfectés par le plasmide contenant le variant d'épissage hMR $\Delta_{5,6}$ qui ne possède pas le site de liaison au ligand, étant donc constitutivement actif, l'activité de la luciférase est augmenté de 2.5 fois. Si la mutation de la région plus distale (GRE-1) ne modifie pas l'activité, la mutation des GRE-2, GRE-3 ou nGRE, diminuée la réponse au hMR $\Delta_{5,6}$; restant cependant plus élevée par rapport aux cellules non transfectées par le hMR $\Delta_{5,6}$. En revanche, la mutation de la région plus proximale (GRE-4) augmente de 400% l'activité du promoteur P1. Ces données ont été corroborées par des expériences similaires réalisées avec l'aldostérone et le MR endogène dans le NRVM.

Étant donné que les glucocorticoïdes et les minéralocorticoïdes partagent plusieurs mécanismes moléculaires, nous avons évalué la contribution des GREs dans le contrôle du promoteur P1 en réponse à la dexaméthasone. Nous montrons que la mutation du GRE-1 ou -2 ne modifie pas la sensibilité du promoteur P1 à la dexaméthasone, alors que les mutations du GRE-3, -4 ou nGRE entraînent une augmentation de la sensibilité. Conjointement, ces essais montrent que l'aldostérone, via l'action spécifique du MR, agit en activant le promoteur P1 par la transactivation du GRE-2, -3 et nGRE et la répression du GRE-4. En outre, ces résultats mettent en évidence la différence moléculaire d'utilisation des GREs par les MRs et GRs.

Nous nous sommes ensuite demandé si cette régulation pouvait être étendue à d'autre tissus. Le MR est exprimé dans les cellules endothéliales et le muscle lisse artériel, participant au contrôle de la pression artérielle. Si des études se sont concentrées sur la régulation hémodynamique dépendante de l'endothélium (vasodilatation), les recherches récentes ont démontré la contribution du RM, situé dans le muscle lisse, dans la régulation de la pression artérielle. Dans notre étude, nous

montrons que le traitement des artères coronaires (LADCA), mésentériques et de l'aorte par l'aldostérone (10^{-8} M durant 24 h) augmente l'expression protéique du $Ca_v1.2$. Ces changements sont accompagnés de l'augmentation de la contractilité vasculaire induite par la dépolarisation ou par l'agoniste sélectif du $Ca_v1.2$ (Bay K 8644), ces réponses étant indépendantes de l'endothélium. Comme prévu, ces réponses ont été accompagnées d'une diminution de la relaxation vasculaire induite par l'acétylcholine en LADCA. De plus, nous montrons que les cellules musculaires lisses de LADCA traitées à l'aldostérone présentent une augmentation du courant Ca^{2+} s, sans changements dans leurs propriétés biophysiques.

De même que sur les cardiomyocytes, nous montrons que l'aldostérone augmente l'activité du promoteur P1, conduisant à une augmentation de la transcription de l'exon 1a et de l'exon 8a dans le tissu vasculaire. Par contre, ce traitement ne modifie pas l'expression du microARN-155 impliqué dans la régulation MR-dépendant (mais indépendamment de l'aldostérone) de l'expression du $Ca_v1.2$ -SNT dans le tissu vasculaire des animaux âgés. L'une des caractéristiques plus marquantes qui différencie le $Ca_v1.2$ -SNT et le $Ca_v1.2$ -LNT est la grande sensibilité à la nifédipine dans le tissu vasculaire ($Ca_v1.2$ -SNT). Ainsi, en confirmant nos résultats, nous montrons une perte significative de la sensibilité à la nifédipine en LADCA traitées à l'aldostérone.

Conjointement les résultats obtenus dans cette étude indiquent que l'activation du MR dans les cellules musculaires lisses – principales responsables par le maintien du tonus myogénique et le précis réglage de la pression artérielle – subissent un mécanisme sophistiqué de *switch* moléculaire d'utilisation alternative du promoteur du gène *Cacna1c* (du promoteur P2 à P1), générant donc l'apparition d'une nouvelle sous-population de $Ca_v1.2$ -LNT dans le tissu vasculaire qui se répercute sur la dysfonction vasculaire. Par ailleurs, les animaux traités à l'aldostérone/sel (0,9% NaCl pendant 3 semaines) présentent une augmentation de la pression artérielle et de l'activité du promoteur P1 cardiaque du gène *Cacna1c* dans le tissu vasculaire.

Les résultats de cette étude identifient le $Ca_v1.2$ -LNT comme une nouvelle cible spécifique du MR. Au-delà du nouveau concept mécanistique, nos résultats fournissent des évidences que les actions spécifiques d'éléments *cis*-régulateurs en réponse à l'aldostérone peuvent conduire à l'utilisation alternative du promoteur *Cacna1c* "cardiaque" dans le tissu vasculaire et, par conséquent, étendre la diversité $Ca_v1.2$ dans ce tissu. Cette étude apporte de nouvelles perspectives sur la régulation de ce mécanisme génomique dans le développement de maladies cardiovasculaires, dans lequel l'aldostérone à travers le MR joue un rôle important.

Mots-clés: Canaux Ca^{2+} de type L, aldostérone, récepteur aux minéralocorticoïde, Cœur, muscle lisse vasculaire.

Resumo Expandido

Ativação específica do promotor cardíaco alternativo do *Cacna1c* pelo receptor de mineralocorticoide

Atualmente, os antagonistas do receptor de mineralocorticoide (MR) pertencem ao arsenal terapêutico para o tratamento de diversas doenças cardiovasculares. No entanto, os mecanismos pelos quais essa classe de medicamentos exercem seus efeitos benéficos ainda não estão totalmente compreendidos. Evidências experimentais do nosso grupo têm demonstrado através de experimentos *in vitro*, *ex vivo* e *in vivo* que, parte dos efeitos deletérios da aldosterona, via ativação do MR, envolvem o aumento da expressão e atividade do $Ca_v1.2$, agindo como um importante gatilho para o desenvolvimento de patologias cardíacas. Portanto, o objetivo central deste estudo foi avaliar os mecanismos genômicos envolvidos na modulação da expressão do $Ca_v1.2$ pelo MR.

No presente estudo, demonstramos em cultura primária de miócitos ventriculares de ratos neonatais (NRVM), que o tratamento com aldosterona durante 24 h aumentou, de maneira concentração dependente (10^{-10} à 10^{-9} M) a expressão do $Ca_v1.2$. Importante destacar que as subunidades auxiliares do $Ca_v1.2$ (β_2 e $\alpha_2\delta_2$) também apresentaram aumento na expressão de seus respectivos mensageiros, sugerindo assim, uma atividade transcricional finamente coordenada. Composto parte da família de receptores nucleares, a translocação citosol-núcleo do MR foi avaliada em NRVM sob diferentes concentrações de aldosterona (10^{-9} à 10^{-7} M) e tempos de tratamento (30 – 90 min). Observamos aumento na translocação nuclear do MR em baixas concentrações de aldosterona (10^{-9} M) e curto período de tempo (30 min) de tratamento. Além disto, em NRVM transfectados com um plasmídeo contendo hMR-GFP, foi possível validar o rápido tráfico intracelular do MR para o núcleo (visível a partir de 10 min) em células estimuladas com aldosterona (10^{-9} M). Importante ressaltar que altas concentrações de dexametasona (10^{-8} M), um potente hormônio glicocorticoide sintético, não alterou a translocação nuclear do MR.

A expressão do $Ca_v1.2$ é regulada por dois promotores distintos e, apesar de não ser totalmente compreendida, sua regulação ocorre de maneira específica em determinados tipos celulares. Por exemplo, o promotor “cardíaco” (P1) regula a expressão da forma longa do $Ca_v1.2$ ($Ca_v1.2$ -LNT), enquanto que o promotor “vascular/cerebral” (P2) regula a expressão da forma curta deste canal ($Ca_v1.2$ -SNT), ambos localizados na região N-terminal. Interessantemente, a aldosterona aumentou a transcrição do exon 1a ($Ca_v1.2$ -LNT) em NRVM, sem alterar a transcrição do exon 1b ($Ca_v1.2$ -SNT). Estes dados se correlacionam com a atividade do promotor P1 de maneira concentração e tempo (6 – 24 h) dependentes.

A especificidade destes efeitos que, possivelmente, envolvem a atividade transcricional do MR, foram avaliados em cultura celular através do tratamento simultâneo com o inibidor seletivo

para o MR (RU28318, concentração 100x superior em relação à aldosterona) ou através do silenciamento (siRNA) do MR. Deste modo, demonstramos que em ambas abordagens experimentais, os efeitos da aldosterona sobre o aumento da expressão do Ca_v1.2, exon 1a e atividade do promotor P1 foram completamente abolidos. Afim de explorar o aumento da expressão do Ca_v1.2-LNT induzido pela aldosterona em células de animais adultos, geramos um camundongo transgênico expressando gene repórter luciferase sob o controle do promotor P1 do *Cacna1c* (PCa.luc). De forma similar ao encontrado em NRVM, cardiomiócitos ventriculares adultos de animais PCa.luc tratados por 24 h com aldosterona, demonstraram um aumento significativo, de modo concentração dependente, na atividade da luciferase. Coletivamente, nossos dados claramente indicam que a ativação do MR causa transativação do promotor P1 e, conseqüentemente, transcrição do Ca_v1.2-LNT.

Através da análise *in silico* e mutagênese, identificamos que o MR interage com específicos elementos ativadores e repressores de ligação ao DNA do promotor P1. Foram identificados 5 elementos de resposta aos glicocorticóides (GRE), sendo um “negativo”, próximos à região de início da transcrição do exon 1a do gene *Cacna1c*, portanto, sobrepondo-se a seqüência do promotor P1. Para avaliar a função dessas importantes regiões altamente especializadas na regulação da atividade do promotor P1, cada GRE previamente identificada foi individualmente substituído por uma outra seqüência (*scrambled*). O distúrbio causado pelas seqüências mutadas foram confirmadas através do software Genomatix. Em NRVM transfectadas com o plasmídeo contendo o *splice* variante do hMR $\Delta_{5,6}$, que não possui o domínio de ligação ao ligante, portanto, estando constitutivamente ativo, a atividade da luciferase aumentou 2,5 vezes em relação ao controle (não transfectado com *splice*). Apesar da mutação da região mais distal (GRE-1) não alterar a atividade, mutações no GRE-2, GRE-3 ou nGRE diminuiu a resposta induzida pelo hMR $\Delta_{5,6}$, embora ainda tenham permanecido elevadas em relação às células não transfectadas com hMR $\Delta_{5,6}$. Em contraste, a mutação da região mais proximal (GRE-4), aumentou em 400% a atividade do promotor P1. Estes dados foram corroborados por experimentos realizados com aldosterona e o MR endógeno em NRVM.

Devido os glicocorticóides e mineralocorticóides compartilharem diversos mecanismos moleculares, avaliamos a contribuição dos GREs no controle do promotor P1 em resposta à dexametasona. Demonstramos que a mutação do GRE-1 ou -2 não modificou a sensibilidade do promotor P1 à dexametasona, enquanto que a mutação do GRE-3, -4 ou nGRE conduziu à um aumento na sensibilidade. Em conjunto, estes ensaios mostram que a aldosterona, via ação específica do MR, age ativando o promotor P1 através da transativação do GRE-2, -3 e nGRE e repressão do GRE-4. Além disto, os resultados demonstram uma evidente diferença molecular de utilização dos GREs pelos MRs e GRs.

Expresso em células endoteliais e músculo liso arterial, o MR tem ganhado grande atenção em estudos experimentais e clínicos. Contudo, apesar de grande parte dos estudos concentrarem-se na regulação hemodinâmica dependente do endotélio, recentes estudos têm demonstrado a contribuição do MR localizado no músculo liso vascular na regulação da pressão arterial. No presente

estudo, demonstramos que o tratamento de artérias coronárias (LADCA), mesentéricas e aorta com aldosterona (10^{-9} M por 24 h) induziu aumento da expressão proteica do $Ca_v1.2$. Essa alteração foi acompanhada pelo aumento na contratilidade vascular induzida por despolarização ou pelo agonista seletivo do $Ca_v1.2$ (Bay K 8644), sendo essas respostas, independentes do endotélio. Como esperado, essas respostas foram acompanhadas por uma diminuição do relaxamento vascular induzido pela acetilcolina em LADCA. Além disso, demonstramos que LADCA tratadas com aldosterona apresentaram aumento na corrente iônica de Ca^{2+} através dos canais para Ca^{2+} do tipo L em células musculares lisas, sem alterações em suas propriedades biofísicas.

Supreendentemente, assim como NRVM, demonstramos que a aldosterona também aumentou a atividade do promotor P1, levando a um aumento da transcrição do exons 1a e 8a em tecido vascular. Embora tenha sido recentemente demonstrado o envolvimento do mir-155 na regulação da expressão do $Ca_v1.2$ -SNT mediada pelo MR em tecido vascular de animais idosos, não verificamos alteração de sua expressão em aortas de animais jovens tratadas com aldosterona (10^{-9} M por 24 h). Uma das marcantes características que diferenciam o $Ca_v1.2$ -SNT do $Ca_v1.2$ -LNT é a notável sensibilidade à nifedipina em tecido vascular ($Ca_v1.2$ -SNT). Assim, corroborando nossos resultados, demonstramos uma significativa perda da sensibilidade à nifedipina em LADCA tratadas com aldosterona. Em conjunto, os resultados obtidos nesse estudo indicam que a ativação do MR em células musculares lisas, principais responsáveis pela manutenção do tono miogênico e, o fino ajuste da pressão arterial, sofrem um sofisticado mecanismo de *switch* molecular de utilização alternativa do promotor do gene *Cacna1c* (do promotor P2 para o P1) gerando o aumento de uma nova subpopulação de $Ca_v1.2$ -LNT em tecido vascular, que repercutem no desenvolvimento da disfunção vascular aqui demonstrada. Além disto, animais tratados com aldosterona/sal (0,9% NaCl, por 3 semanas) apresentaram aumento na atividade do promotor P1 do gene *Cacna1c* no coração, associado com função ventricular melhorada e ausência de anormalidades estruturais. Finalmente, também demonstramos através da abordagem *in vivo*, aumento da pressão arterial e utilização alternativa do promotor do gene *Cacna1c* em tecido vascular.

Em conjunto, os resultados deste estudo identificaram o $Ca_v1.2$ -LNT como um novo alvo específico do MR, onde deciframos o mecanismo molecular implicado na regulação do promotor P1 do gene *Cacna1c*. Além do novo conceito mecanístico, nossos resultados fornecem evidências de que as específicas ações sob os elementos *cis*-regulatórios em resposta à aldosterona podem conduzir na utilização alternativa do promotor *Cacna1c* no tecido vascular e, conseqüentemente, ampliando a diversidade $Ca_v1.2$ neste tecido. Portanto, este estudo abre novas e interessantes perspectivas sobre a regulação deste mecanismo genômico no desenvolvimento de doenças cardiovasculares nas quais, a aldosterona através do MR, desempenha um importante papel.

Palavras-chave: Canal para Ca^{2+} do tipo L, aldosterona, receptor de mineralocorticóide, coração, músculo liso vascular.

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Chapter 1 Introduction

The mineralocorticoid receptor (MR), a ligand-dependent transcription factor belonging to the nuclear receptor superfamily, is the primary mediator of aldosterone. Classically, this steroid hormone, mainly but not exclusively produced and secreted by the adrenal cortex (MacKenzie et al., 2012), binds and activates the MR located in the cytosol of epithelial target cells. The steroid receptor (SR) complex translocates to the nucleus where it modulates gene expression and translation of specific aldosterone-induced proteins that regulate electrolytes and fluid balance and subsequent blood pressure (BP) homeostasis.

New paradigms indicate the pivotal importance of aldosterone and MR as regulators of cellular and organ function, far beyond their effects on the kidney. The MR is also expressed in non-epithelial tissues turning them sensitive to aldosterone stimulation, such as heart, blood vessels, adipose tissues, hippocampus, and cells of the immune system (Viengchareun et al., 2007). Independent of its effects on BP, aldosterone excess leads to adverse cardiovascular, renal, central nervous, and psychological effects, raising clinical and research interest (Jaisser and Farman, 2016). Collectively, experimental studies and clinical trials, suggest a detrimental association between aldosterone and life-threatening arrhythmias in heart failure (HF) patients that may be prevented by MR blockade (Pitt et al., 1999, 2003; Zannad et al., 2011). In addition, aldosterone plays a wide range of direct vascular effects, including those regulating vascular reactivity and growth or cell death. Notably, aldosterone regulates BP and exerts influence on end-organ damage directly *via* MR of vascular smooth muscle cells (VSMC) and endothelial cells (DuPont et al., 2014; Leopold et al., 2007; McCurley and Jaffe, 2012; Romagni et al., 2003).

The impressive therapeutic benefit with MR antagonists, notably in the reduction of sudden cardiac death in HF patients and the control of BP in patients with resistant hypertension (DuPont et al., 2014; Leopold et al., 2007; McCurley and Jaffe, 2012; Romagni et al., 2003; Williams et al., 2015), is however clinically limited by the concomitant blockade of the epithelial MR with the consequent development of significant hyperkalemia. There is thus clearly a need for the development of tissue-specific MR antagonists. However, signaling pathways and underlying mechanisms participating to these cardiovascular effects remain elusive. Research efforts on MR signaling have revealed a host of new pathophysiological mechanisms, both by acting on the heart and by affecting factors extrinsic to the heart, which could contribute to lethal arrhythmias (Dawson et al., 2004), in which aberrant Ca^{2+} fluxes are a recurrent theme (Benitah et al., 2010). The main mediator of cardiac Ca^{2+} influx is the L-type Ca^{2+} channel (LTCC), whose main pore-forming subunit is $\text{Ca}_v1.2$ (encoded by *Cacnalc*). LTCC plays a pivotal role in function and dysfunction of the ventricular cardiac myocytes, not only because it controls contraction, but also it contributes to rhythmogenicity, cellular

metabolism and gene transcription (Benitah et al., 2010). The cardiac MR activation by aldosterone upregulates LTCC and might be involved in triggering after depolarization-related fatal ventricular tachyarrhythmia (Bénitah and Vassort, 1999; Ouvrard-Pascaud et al., 2005; Perrier et al., 2004, 2005). Moreover, several studies have suggested effects of aldosterone on VSMC LTCCs (DuPont et al., 2014), one of the main determinants of vascular tone (Joseph et al., 2013). Indeed, VSMC-specific MR knockout in mice decreased $Ca_v1.2$ expression and activity contributing to attenuate age-related hypertension (DuPont et al., 2016; McCurley et al., 2012).

However, the molecular mechanisms and signaling pathways whereby aldosterone and MR may directly modulate $Ca_v1.2$ expression and function have yet to be identified. $Ca_v1.2$ channels are expressed as two distinct tissue-specific transcripts of *Cacna1c*, which encode, respectively, a long 'cardiac' ($Ca_v1.2$ -LNT) and a short 'vascular/brain' ($Ca_v1.2$ -SNT) N-terminal region, regulated by two alternative promoters (P1 and P2) (Hofmann et al., 2014). Here, we show that MR acts as a transcription factor to transduce aldosterone signal into specific *Cacna1c* 'cardiac' P1-promoter activation in cardiac cells but also in VSMC, in which the major $Ca_v1.2$ transcript initiation is normally controlled by the canonical P2-promoter, conferring thereby a new molecular signature to $Ca_v1.2$ in this tissue.

1.1 MR transcriptional factor

The biological effects of aldosterone are mainly mediated by the activation of the cytosolic MR (encoded by the gene *NR3C2*). Belonging to the nuclear receptor subfamily, MR is a member of the steroid-thyroid-retinoid superfamily of ligand-dependent transcription factors (Rogerson et al., 2004), being expressed in many cell types at different levels (Arriza et al., 1987; Gomez-Sanchez and Gomez-Sanchez, 2014; Gomez-Sanchez et al., 2006).

MR is composed of 984 amino acids distributed in three major domains, an N-terminal domain, a highly conserved DNA-binding domain (DBD), and a ligand-binding domain (LBD) (Arriza et al., 1987), displaying ~57% amino acid identity with GR in the LBD, and ~94% in the DBD. Moreover, progesterone receptors (PR) and androgen receptors (AR) also show significant amino acid homology (LBD: ~50%; DBD: ~90%) with MR and GR (Funder, 1997; Viengchareun et al., 2007). The DNA-binding domain contains sites for the consensus sequences, known as hormone-responsive elements (HRE), on the promoters/enhancers regions of targets genes. In common, during unliganded state MR/GR/PR/AR are associated with a complex of chaperone proteins, including the heat shock proteins (HSP), which maintain the receptors in an inactive form with high affinity for the hormone. In the cytosol, MR forms a direct complex with chaperones, mainly HSP90, but indirectly interacts with HSP70, p23, p48, FKBP-51 immunophilins and CYP40 cyclophilin (Bruner et al., 1997; Pratt

and Toft, 1997; Viengchareun et al., 2007). These pivotal chaperones interactions play a role in maintaining MR in an appropriate conformation for ligand binding. Upon ligand binding, MR dissociates from chaperone proteins, and FKBP51 is replaced by FKBP52, enabling the complex to interact with the motor protein dynein. The ligand–MR–dynein complex then translocates along microtubules from the cytosol to the nucleus supported by numerous molecular partners in a coordinated and sequential manner to ensure appropriate transcriptional regulation (Bruner et al., 1997; Nagase and Fujita, 2013; Pratt and Toft, 1997; Rogerson et al., 2004; Viengchareun et al., 2007). Once receptor/hormone complex is active, it binds to HRE in the 5'-untranslated region of aldosterone-responsive target genes to activate or repress the gene transcription (Figure 1). The control of gene transcription may also occur via other transcription factors, in which allow transcriptional control without direct interactions between receptor–steroid complex and DNA-binding sites (Karin, 1998).

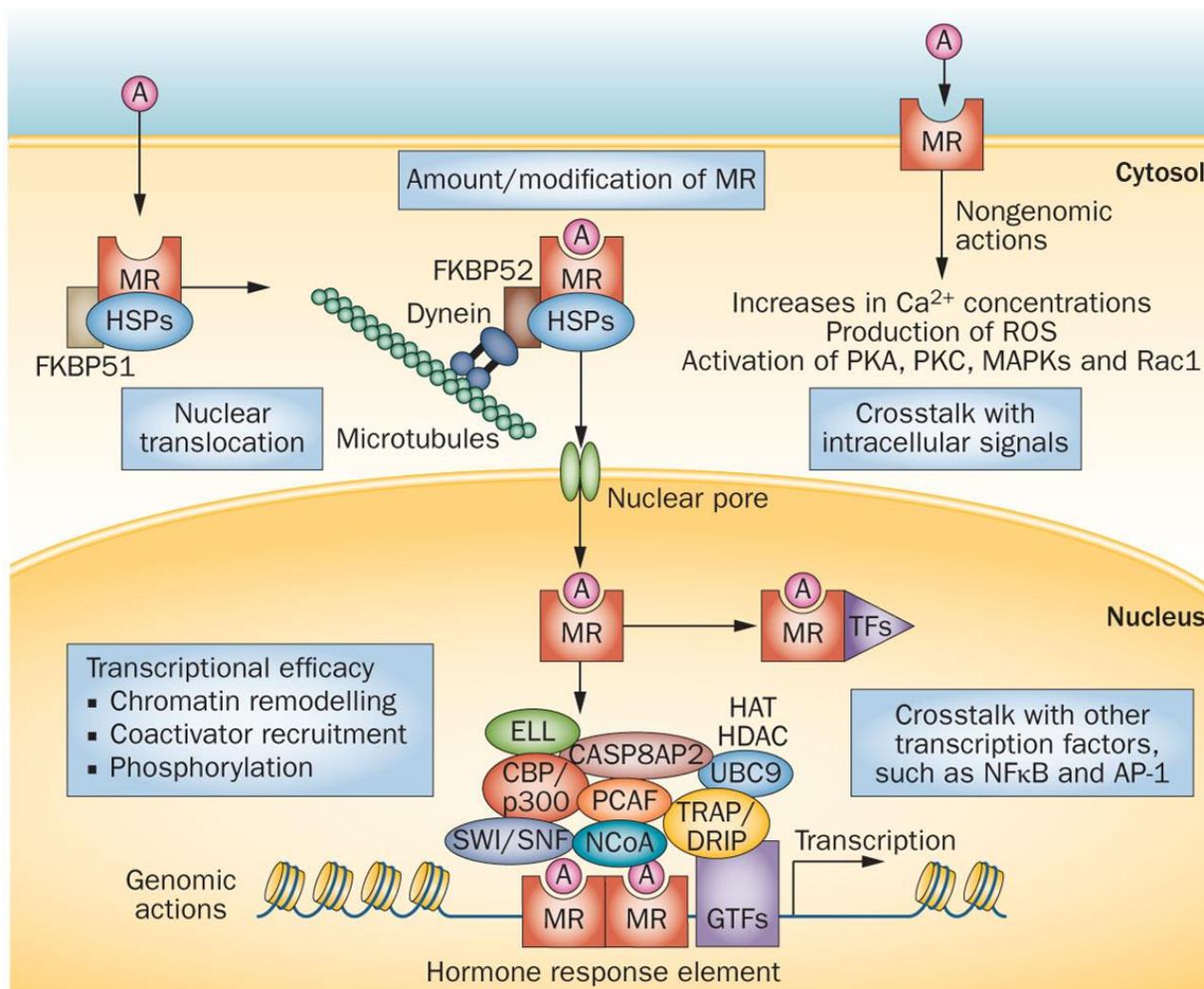


Figure 1. Mechanisms of MR activation. Upon ligand binding, aldosterone acts as a ligand-activated transcription factor translocating along microtubules from the cytosol to the nucleus, where binds to hormone response elements in the promoter regions of target genes. For the efficient initiation of gene transcription, MR

regulates transcription by controlling chromatin remodeling and the recruitment of co-activators (Nagase and Fujita, 2013).

As the result of high level of structural and functional homology, MR and GR bind to common nuclear HREs, with a consensus of 15 nucleotides sequence (AGAACA_nnnTGTTCT), which may vary despite remaining fairly conserved. These regions are referred as Glucocorticoid Responsive Elements (GRE), being composed of palindromic sequences centrally separated by three nucleotides (Arriza et al., 1988; Clyne et al., 2009; Lombès et al., 1993). This mechanism involves an orchestrated recruitment of co-regulators, known as transcriptional *cis*-activation to denote direct DNA binding to canonical HREs (Mangelsdorf et al., 1995).

MR and GR act regulating gene expression by binding to specific DNA sequences in the form of monomer or heterodimers. The binding to these regulatory elements activate or repress the transactivation. Even though that GRE operates individually, synergism between GREs has been reported (Jantzen et al., 1987; Liu et al., 1996; O'Hara et al., 2014; So et al., 2007). Moreover, MR arrangement on the DNA differs from that one displayed by GR. A single amino acid difference, at tyrosine 478 in the DBD, is responsible for the distinct structural binding conformation of MR and GR to DNA sequences. GR stimulation is also able to activate (Hollenberg et al., 1987; Webster et al., 1988) or repress (Schüle et al., 1990; Surjit et al., 2011; Yang-Yen et al., 1990) the transcription of several genes. To activate gene transcription, GR DBD cooperatively dimerizes on inverted repeat activating GREs (Hudson et al., 2014; Luisi et al., 1991).

Although the structural basis of GR-mediated transcriptional repression is still not clear, there is evidence that negative GRE (nGRE) plays a role in GR-mediated transcriptional repression (Surjit et al., 2011) by monomeric GR DBD binds to an inverted repeat with negative cooperativity (Hudson et al., 2013). Accordingly, it has been shown that GR activation downregulates the transcription of numerous anti-inflammatory genes (Kadmiel and Cidlowski, 2013). In contrast, MR activation causes proinflammatory transcriptional state (Chantong et al., 2012). Despite the opposing effects on inflammation status, GR agonists prolong the lifespan of MR null mice, indicating that GR and MR share some transcriptional sites (Berger et al., 1998). Furthermore, GR binds, with distinct conformations, to both activating GREs and nGREs within the *TSLP* promoter. Interestingly, MR was not able to repress transcription by *TSLP* nGRE, thereby confirming that GR and MR show divergence function at nGREs, which is quite remarkable considering the high sequence identity of GR and MR within the DBDs as well as the small size of the domain (75 aa) (Hudson et al., 2014). Overall, these discrepancies may explain the differential ability of MR and GR to modulate gene expression despite sharing overlapping DNA sites and ligand binding properties (Hudson et al., 2014).

In addition to the DNA sequence, the localization of GRE towards of the transcriptional start site is also critically important for the transcription initiation. GREs may be located upstream or

downstream of the transcription-initiating site, being found closely (2.5kb) or more remotely (10kb) from the promoter (Jantzen et al., 1987; Luecke and Yamamoto, 2005). In accordance, in A549 human lung cells it has been shown (So et al., 2007) that GREs are equally distributed upstream and downstream of the transcription start sites, with 63% of them >10 kb from those sites. More dramatic examples have been reported, such as an estrogen response element 144 kb upstream of the promoter of the *NRIP* gene (Carroll et al., 2005), and an intragenic region 65 kb downstream from the *Fkbp5* promoter that appears to serve as an androgen response element (Magee et al., 2006).

Regulation of transcription is not only dependent on direct interaction with HRE, but also through other transcription factors, which may act as bridge factors between the activated receptor and transcription initiation complex (Halachmi et al., 1994). For instance, GR trans-represses NFκB, AP-1 and other inflammatory transcription factor complexes without direct binding to DNA but via protein-protein tethering. These “trans” mechanisms are recognized as the primary way by which GC affects a large repertoire of inflammatory gene targets including cytokines, chemokines and adhesion molecules (Dougherty et al., 2016; Glass and Saijo, 2010; Reichardt et al., 2001; Scheinman et al., 1995). In contrast to the anti-inflammatory effects of GR stimulation, activation of MR promotes cardiovascular inflammation (Brown, 2008; Fiebeler et al., 2007; Funder, 2004). Moreover, NFκB and AP-1 are activated in hypertensive rats treated with aldosterone and high-salt diet (Fukuda et al., 2011; Tostes et al., 2002).

The MR-mediated aldosterone actions can be divided into nongenomic (few minutes) and genomic (>6 h) responses in epithelial and non-epithelial cells (Connell and Davies, 2005). About two decades ago, it was demonstrated that aldosterone, at nanomolar concentrations, produces a rapid, nongenomic effects in a variety of tissues (Christ et al., 1999; Wehling, 1995). Subsequent studies have further investigated the mechanism of rapid nongenomic effects of aldosterone on VSMC (Alzamora et al., 2000) and cardiomyocytes (Mihailidou et al., 2004). It has been reported that the rapid aldosterone action may involve the activation of protein kinase C (PKC) (Wehling et al., 1995). In human VSMC, aldosterone induces a concentration-dependent rise of intracellular $[Ca^{2+}]$ reaching plateau phase within 2–3 min (Wehling et al., 1995). Aldosterone (100 nM) has also been reported to activate αPKC in VSMC, which stimulates its translocation from the cytosol to the membrane within 5 min (Christ et al., 1995). In contrast, aldosterone decreased PKC activity in myocyte-enriched cultures from neonatal Sprague-Dawley rat hearts (Sato et al., 1997). This rapid effect was observed after 1 min of exposure as little as 1 nM of aldosterone. However, in adult ventricular myocytes $Na^+/K^+/2Cl^-$ cotransporter and the Na^+-K^+ pump are acutely regulated by aldosterone, suggesting that aldosterone regulates cotransporters or pumps by direct activation of εPKC (Mihailidou et al., 2004). Furthermore, an increase in PKC activity may also result in activation of transcription factors, and thus it is suggestive to propose that the nongenomic actions of aldosterone may influence gene

expression via aldosterone-activated PKC (Chun and Pratt, 2004).

1.1.1 Specificity of MR in target tissues

The discussion of MR specificity arises from the equal binding affinity of the MR for aldosterone and glucocorticoids (Connell and Davies, 2005). Indeed, plasmatic levels of glucocorticoids (GC) are 100- to 1000-fold higher than aldosterone. Thus, in theory, GCs should occupy more MR than aldosterone (Funder, 2009). However, there is a key mechanism to regulate MR specificity to counter-react cortisol occupation of the MR (Ferrari and Krozowski, 2000; Lombes et al., 1994). Firstly described in epithelial tissues, MR specificity is regulated by the action of the type 2 isoform of 11-hydroxysteroid dehydrogenase enzyme (11 β -HSD2) that inactivates cortisol into 11-cortisone, which cannot bind to the MR, thereby protecting MR from inappropriate activation by GC (White et al., 1997). Although this mechanism explains aldosterone specificity at epithelial tissues, MR has also been shown to be expressed in non-epithelial cells such as, in the heart, brain and blood vessels (Freel and Connell, 2004; Marney and Brown, 2007).

In the heart, MR is expressed in cardiomyocytes (Lijnen and Petrov, 2003; Lombès et al., 1995) and cardiac fibroblasts (Rombouts et al., 2001; Stockand and Meszaros, 2003). Furthermore, the expression of 11 β -HSD2 has been demonstrated in the heart, although some researchers failed to show similar results (Kayes-Wandover and White, 2000; Lombès et al., 1995; Sheppard and Autelitano, 2002; Slight et al., 1996). However, it is generally accepted that 11 β -HSD2 expression levels are much lower levels than those found in epithelial tissue (Funder, 2004; Slight et al., 1993). Therefore, it has been proposed that cardiac MR is mainly occupied by cortisol rather than aldosterone (Chai and Danser, 2006). Additionally, this hypothesis is further supported by the notion that the stoichiometric efficiency of 11 β -HSD2 to metabolize cortisol cannot be achievable to allow a comparable MR competition between GC and mineralocorticoid (Funder, 2007).

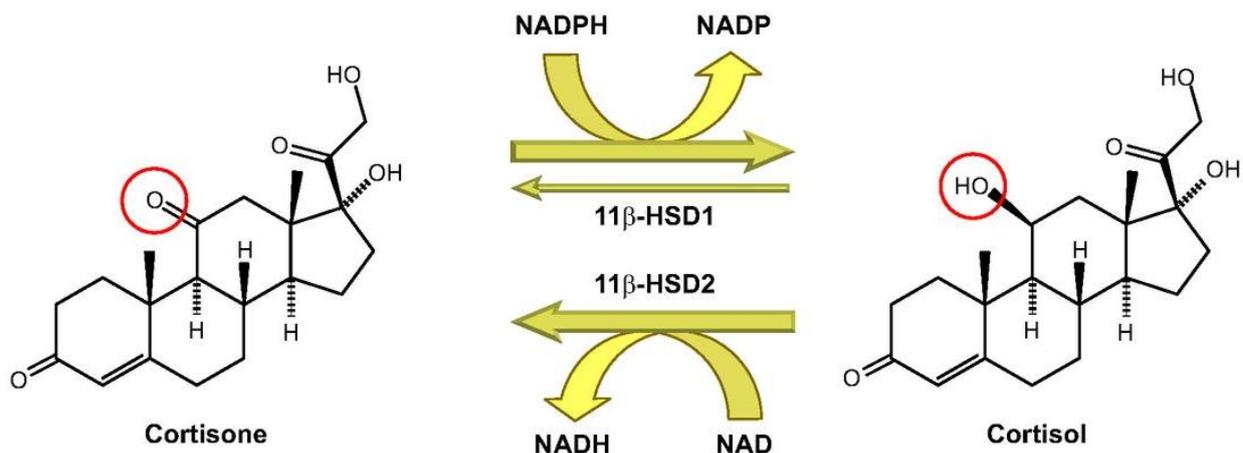


Figure 2. Enzymatic reactions catalyzed by 11 β -hydroxysteroid dehydrogenase (11 β -HSD).

Interconversion of cortisol and cortisone by 11 β -HSD1 and 11 β -HSD2, respectively. Under certain circumstances and in cell types, 11 β -HSD1 may acts as an NADP-dependent dehydrogenase, inactivating cortisol, while 11 β -HSD2 catalyzes the NAD⁺-dependent inactivation of cortisol, converting it to cortisone (Chapman et al., 2013).

Even despite the avid support of the eminent researcher Dr. John W. Funder advocating the MR promiscuity in cardiomyocytes, a bulk of evidence has shown aldosterone binding to cardiac MR, even from Pr. Funder (Arriza et al., 1987; Barnett and Pritchett, 1988; Pearce and Funder, 1987; Yang and Young, 2009). For instance, *in vivo* injection of [³H]aldosterone in adrenalectomized rats demonstrated that aldosterone binds to MR in epithelial and non-epithelial tissue. In the heart, aldosterone is a 3-fold better binder to MR than a GC (Funder and Myles, 1996).

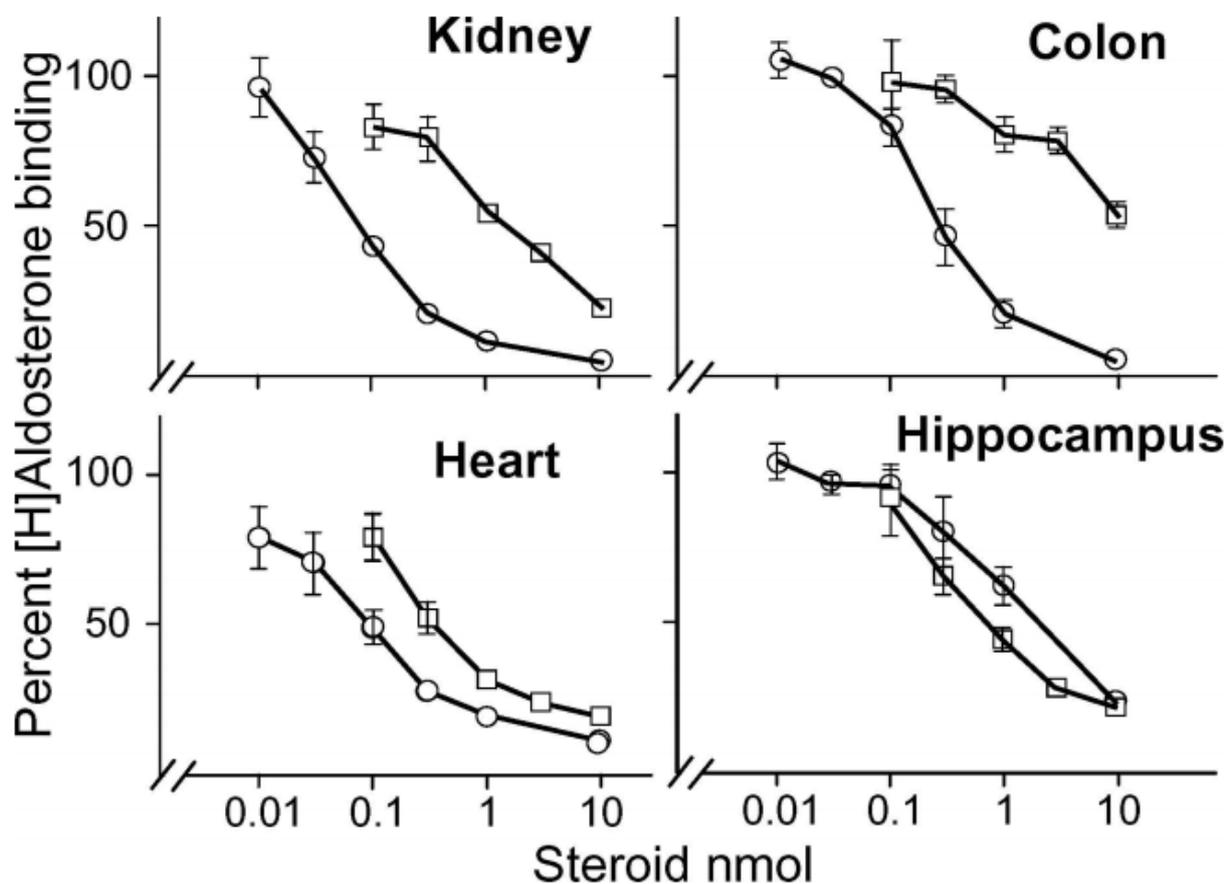


Figure 3. Binding of tracer [³H]aldosterone in kidney, colon, heart and hippocampal extracts 15 min after injection of tracer alone, or with half-logarithmic increasing doses of nonradioactive aldosterone (○) or corticosterone (□) (Funder and Myles, 1996).

Even though that mineralocorticoids and GC bind with equal affinity, there are compelling evidence showing that the off-rate of aldosterone from MR is 5 times lower than GCs (Hellal-Levy et al., 1999; Lombes et al., 1994) and, the MR activation by aldosterone reaches greater

transactivation compared to GCs (Farman and Rafestin-Oblin, 2001). Taken together, it has now become clear that distinct ligands may produce differential effects on gene transcription probably, due to conformational differences in the MR-ligand complex, which may lead to variable degrees of stability and DNA interactions (Farman and Rafestin-Oblin, 2001; Hellal-Levy et al., 2000; Huyet et al., 2012).

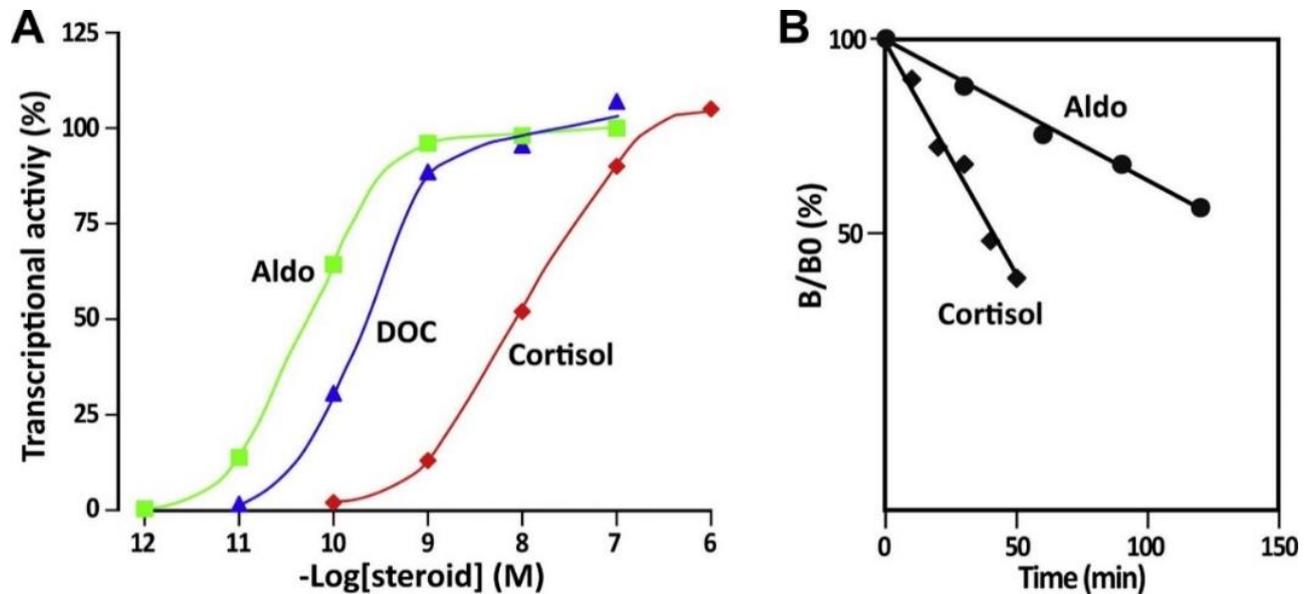


Figure 4. MR transcriptional activity in response to different ligands and MR-ligand complex stability. **A**, Transactivation activity of the human MR (hMR) in response to aldosterone (Aldo), deoxycorticosterone (DOC) and cortisol in COS-7 cells transiently and treated for 24 h with agonist. **B**, Kinetic dissociation of aldosterone and cortisol from hMR in response to 10^{-6} M aldosterone and cortisol (Huyet et al., 2012).

1.2 New pathological role of MR in non-classical target tissue

Aldosterone has been viewed as a hormone involved in the regulation of physiological responses such as ions transport, fluid homeostasis, and BP control (Booth et al., 2002; Chai and Danser, 2006). However, this traditional concept has been updated in the past decades. Experimental and clinical data indicate that aldosterone and its receptor, MR, play an important direct or indirect role in cardiovascular diseases. Notably, these findings suggest a detrimental association between high aldosterone levels and great incidence of cardiovascular events such as, inflammation, oxidative stress, endothelial dysfunction, hypertension, fibrosis, arrhythmias and HF, being some of them a salt-dependent dysfunction (Brilla and Weber, 1992; Funder, 2007; Marney and Brown, 2007; Pimenta et al., 2008; Rocha et al., 2002; Sato and Saruta, 2004). In accordance with the deleterious correlation involving plasmatic aldosterone levels, several studies have shown elevated plasma aldosterone concentration in patients with HF, even despite maximal renin-angiotensin system

blockade, which is associated with disease progression (Swedberg and Kjekshus, 1990), and higher prediction of mortality (Güder et al., 2007). However, in spite of effective blockade of ACE activity and beneficial effects, some biochemical anomalies still persist. In particular case, plasma aldosterone levels remained elevated in 40% of patients with symptomatic HF (MacFadyen et al., 1999), in 50% of patients with left ventricular hypertrophy (Sato and Saruta, 2001) and still progressively increasing in patients after-MI (Borghi et al., 1993) despite treatment with ACE inhibitor. This phenomenon has been termed as "aldosterone escape" (Struthers, 2004), being also found uncontrolled aldosterone levels even in a combination of ACE inhibitor plus angiotensin type-1 receptor antagonist (McKelvie et al., 1999). Therefore, this inability to decrease aldosterone concentrations may contribute to aggravate the disease and, might explain the poor outcomes obtained from the current and conventional therapies in patients with cardiovascular disease.

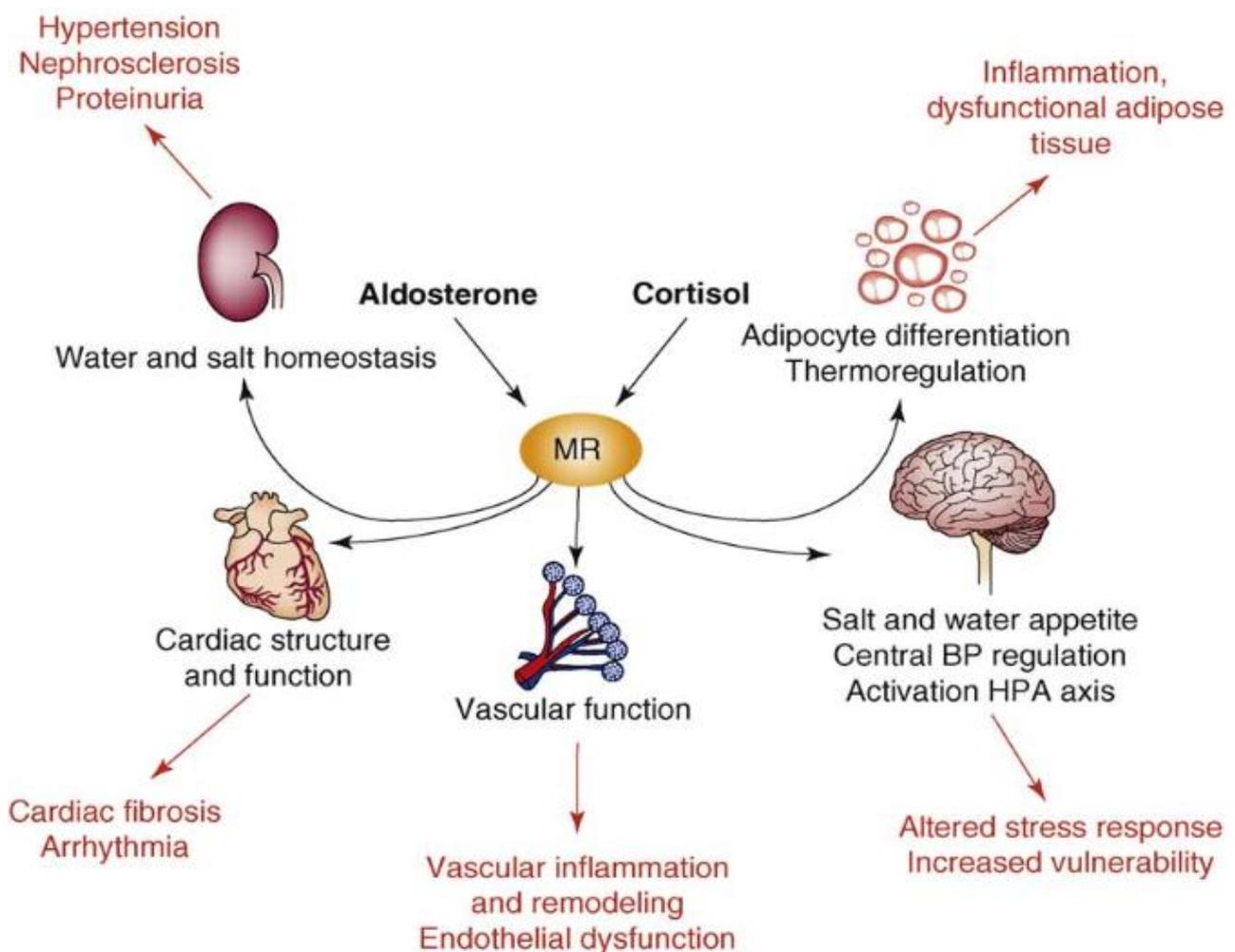


Figure 5. Inappropriate MR activation is linked with several physiological processes in various tissues (Zennaro et al., 2009).

1.2.1 Pathological role of MR in the heart

Since the pioneering work on the development of cardiac fibrosis induced by aldosterone in combination with high dietary sodium intake (Brilla and Weber, 1992), great attention has been given to aldosterone as an independent risk factor for the onset and /or progression of cardiovascular disease. Undoubtedly, aldosterone stimulates the expression of several pro-fibrotic factors that may contribute to the pathological cardiac remodeling. Later studies validated the findings of Weber, suggesting that cardiac fibrosis is an independent event of hypokalemia and hypertension (Young et al., 1995), being those deleterious effects blocked by MR antagonists (Brown et al., 1999; Zannad and Radauceanu, 2005). Altogether, these studies suggest that aldosterone is not simply a biomarker of disease, but a potent mediator of cardiovascular disease.

Intriguingly, a transgenic cardiac-specific overexpression of aldosterone synthase mice showed coronary endothelial dysfunction but not cardiac fibrosis (Garnier et al., 2004). Moreover, human MR overexpression mice in cardiomyocytes lead to cardiac arrhythmias but not cardiac fibrosis (Ouvrard-Pascaud et al., 2005). In contrast, transgenic cardiac-specific overexpression of 11β -HSD2 mice displayed cardiac hypertrophy, fibrosis, and HF under normal salt diet, while eplerenone, a MR antagonist, successfully reversed these effects (Qin et al., 2003). It has been shown that high aldosterone levels, when at appropriate physiological conditions such as during low-sodium intake, are not by themselves detrimental to the heart nor to develop evident cardiac hypertrophy and fibrosis. However, high plasmatic aldosterone concentrations associated with excessive salt loading leads to pathological cardiac remodeling (Wang et al., 2004). Altogether, these studies suggest that the cardiac hypertrophy and fibrosis as seen in DOCA- or aldosterone/salt-treated animal models may be due to a synergistic effect of salt loading and mineralocorticoid rather than to the elevated circulating aldosterone levels alone. Thus, whether aldosterone/salt loading induces cardiac fibrosis by directly increasing the expression of extracellular matrix component (Brilla et al., 1993, 1994; Robert et al., 1994; Sun et al., 2004; Wahed et al., 2005; Zhou et al., 1996) or, indirectly, by cross-talk with other signaling pathways it remains to be elucidated (Chander et al., 2003; Guarda et al., 1993; Rude et al., 2005; Tostes et al., 2002; Wong et al., 2007).

Severe vascular inflammation and augmented macrophage infiltration have been identified in the myocardium before the onset of fibrosis, suggesting macrophage as a key player in the initiation and progression of MR-mediated cardiac fibrosis (Fujisawa et al., 2001; Rocha et al., 2002; Young et al., 2003). It is worth to note that macrophages contain MR and GR but not 11β -HSD2, thereby the MR, in macrophage cell, is mainly occupied by GCs (Bienvenu et al., 2012; Lim et al., 2007). However, the relative contribution of activating MR- and GR-expressing macrophages in the context of cardiovascular disease is not fully understood.

Interestingly, it has been proposed that the macrophage MR signaling plays a critical role in the development of cardiac fibrosis under inappropriate mineralocorticoid-salt status (Rickard et al., 2009). This study showed that selective deletion of the MR in the monocyte/macrophage cells is protective against chronic mineralocorticoid/salt-induced cardiac fibrosis and increased systolic BP after deoxycorticosterone (DOC) administration for 8 weeks (Rickard et al., 2009). Moreover, the absence of recruited macrophages in *CCL2*-null mice after DOCA/salt was associated with attenuated macrophage-mediated proinflammatory gene expression, reduced cardiac fibroblast and significantly lower BP compared to WT mice (Shen et al., 2014). The gene *CCL2* encodes the monocyte chemoattractant protein-1, being considered as the major monocyte chemokine required for tissue macrophage recruitment and typically released by injured tissues. Recently, the activation of JNK pathway in macrophages has been related to cardiac injury and inflammation in DOC/salt model, suggesting the activation of MR-dependent transcription of downstream profibrotic factors in the pathological remodeling (Shen et al., 2016). Overall, these studies showed the pivotal macrophage-dependent recruitment in MR-dependent cardiac inflammation and remodeling.

Aldosterone has also been implicated in left ventricular remodeling after myocardial infarction (MI) (Hayashi et al., 2001; Udelson et al., 2003) and increased post-MI mortality and morbidity (Rouleau et al., 1994). Accordingly, acute post-MI led to an activation of cardiac aldosterone synthase correlating with 4-fold increase of aldosterone in the myocardium (Silvestre et al., 1999). Furthermore, higher susceptibility to develop MI has been shown in patients with primary aldosteronism than in hypertensive subjects with similar BO (Milliez et al., 2005). In accordance with the deleterious involvement of MR in the onset of the post-MI left ventricular dysfunction, spironolactone treatment, a MR antagonist, was able to decrease collagen deposition and interstitial fibrosis in the left ventricle (Silvestre et al., 1999). Similarly, long-term eplerenone treatment alleviates cardiac remodeling and progression of left ventricular dysfunction in dog failing hearts (Suzuki et al., 2002). Altogether, these studies support that increased aldosterone levels in post-MI patients are associated with adverse clinical outcomes, including higher mortality (Beygui et al., 2006).

As previously mentioned, our group has consistently established that activation cardiac MR plays a central role in the modulation of Ca^{2+} fluxes, which is involved in triggering after depolarization-related fatal ventricular tachyarrhythmia (Bénitah et al., 2001; Gómez et al., 2009; Lalevéé et al., 2005; Le Menuet et al., 2010; Ouvrard-Pascaud et al., 2005; Perrier et al., 2004, 2005; Bénitah and Vassort, 1999). For instance, fatal arrhythmias in experimental HF animal models (Bers, 2002; Pogwizd and Bers, 2004) are initiated by abnormal ventricular automaticity or triggered activity, in which the prolongation of action potential (AP) and aberrant Ca^{2+} fluxes are a recurrent theme

(Ruiz-Hurtado et al., 2012). Therefore, the upregulation of $Ca_v1.2$ and increased amplitude of $I_{Ca,L}$ in ventricular myocytes induced by aldosterone may represent a detrimental risk factor for the development of abnormal cardiac electrical activity and thus arrhythmias. Accordingly, transgenic mice overexpressing MR display moderate dilated cardiomyopathy associated with arrhythmias (Le Menuet et al., 2001). Moreover, conditional cardiac-specific overexpression of the MR led to fatal arrhythmias (Ouvrard-Pascaud et al., 2005). Indeed, aldosterone has been shown to be pro-arrhythmogenic in dogs after coronary ligation (Arora and Somani, 1962). In addition, the synthetic mineralocorticoid, fludrocortisone, exacerbates (Lim et al., 2001), while spironolactone improves, the electrophysiological parameters such as the QT interval dispersion (Akbulut et al., 2003; Yee et al., 2001). In contrast, spironolactone improves heart rate variability (MacFadyen et al., 1997; Yee et al., 2001) and decreases premature ventricular beats and no sustained ventricular tachycardia episode occurrence in patients with HF at rest and during exercise (Barr et al., 1995; Ramirez et al., 2000).

Within the first week after MI, aberrant electrical remodeling has been associated with prolonged AP duration due to an increase in $I_{Ca,L}$ and a decrease in transient outward potassium current, which were prevented by MR blocker (Perrier et al., 2004). After post-MI, the treatment with the active metabolite of spironolactone, canrenone, decreased the ventricular fibrillation threshold (Cittadini et al., 2003). These findings were later validated by the observation of the impressive reduction of ventricular extrasystoles and QT intervals after MR blockade (Shah et al., 2007). Interestingly, primary hyperaldosteronism (PA) patients, which commonly display severe hypertension and hypokalemia, show prolongation of QT interval and ventricular arrhythmias, being also reported higher incidence of sudden cardiac death (Matsumura et al., 2005; Maule et al., 2006; Reincke et al., 2012). Overall, these studies support the hypothesis that MR antagonist represents a beneficial therapy for life-threatening cardiac arrhythmias and sudden cardiac death.

Because of numerous experimental studies supporting the beneficial effects of MR antagonists on the cardiovascular pathophysiology. Two landmarks clinical trials, the Randomized Aldosterone Evaluation Study (RALES) (Pitt et al., 1999) and the Eplerenone Heart Failure and Survival Study (EPHESUS) (Pitt et al., 2003) have convincingly demonstrated that treatment with MR antagonist (such as spironolactone and eplerenone), significantly reduce mortality in patients with HF and systolic left ventricular dysfunction in post-MI patients.

The RALES trial study enrolled 3400 patients with severe HF in a randomized protocol of treatment with spironolactone in addition to the conventional therapy. Convincingly, these patients showed 30% of reduction in the mortality and 35% of reduction in the morbidity (Pitt et al., 1999). In addition, patients with high baseline serum levels of aldosterone were significantly associated with poor outcome and marked cardiac fibrosis, while spironolactone therapy, for 6 months, significantly

decreased the cardiac fibrosis. Thus, these findings suggest that the beneficial effects of MR antagonists on the cardiac remodeling may occur due extra-renal mechanisms (Sun et al., 2004; Zannad et al., 2000). Moreover, spironolactone treatment showed higher effectiveness to prevent left ventricle remodeling and to suppress markers of collagen synthesis than ACE inhibitors in 134 patients with acute MI (Hayashi et al., 2003).

The EPHESUS trial was a long-term clinical outcomes trial of MR antagonists in the setting of post-MI HF patients. This trial showed that treatment with eplerenone was associated with significant improvement in early and long-term mortality and morbidity in patients with MI and HF (Pitt et al., 2003). Importantly, EPHESUS concluded that eplerenone leads to additive benefits to the conventional therapy with ACE inhibitors and β -adrenergic blockers. Accordingly, experimental studies have shown that combination MR antagonist with ACE inhibitor potentiate the beneficial effects on the left ventricular remodeling in rats with extensive MI (Cohn and Colucci, 2006; Fraccarollo et al., 2003). Altogether, these findings suggest an important synergism and independent beneficial actions of MR antagonists in post-MI and HF patients.

A robust meta-analysis which included 7 trials and 8635 patients with HF or coronary artery disease examined the effects of spironolactone and eplerenone on ventricular arrhythmias (Wei et al., 2010). This meta-analysis demonstrated that MR antagonists reduced the rate of ectopic ventricular beats, the risk of ventricular tachycardia, and the risk of sudden cardiac death. Collectively, all these findings, suggest a detrimental association between aldosterone and life-threatening arrhythmia that may be prevented by MR blockade.

Overall, based on many experimental studies and clinical trials, the new guidelines of the European Society of Cardiology developed with the special contribution of the Heart Failure Association has been modified to recommend spironolactone or eplerenone. Nowadays, this class of drug is recommended as Class I (Levels of Recommendation: Class I, II, and III) based on Level A of evidence (Classified between A to C) in all symptomatic patients (despite treatment with an ACE inhibitors and a β -adrenergic blocker) with HF and reduced ejection function (HFrEF) patients (Left ventricular ejection function: $\leq 35\%$), to reduce mortality and HF hospitalization (Ponikowski et al., 2016). This current recommendation was previously supported by the clinical trial Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure (EMPHASIS-HF), which was the first randomized controlled trial to suggest that even patients with mild symptomatic HF are benefited from MR antagonist therapy (Zannad et al., 2011).

Recently launched, the clinical trial ALBATROSS treated patients with acute myocardial infarction (MI) with evidence ST-segment elevation MI (STEMI) or non-STEMI (NSTEMI) (Beygui et al., 2016). It is worth note that 92% of the patients had no evidence of HF. Thus, patients were

randomized to placebo group or treatment with 200 mg of intravenous canrenoate followed by oral spironolactone 25 mg/day in addition to standard therapy for 6 months. Despite the no significant reduction in the primary endpoint comparing MR antagonist regimen and standard therapy, the mortality risk was significantly reduced in patients with STEMI but not NSTEMI (Beygui et al., 2016). Therefore, the authors concluded that the overall study failed to show a benefit for early MR antagonist administration when added to standard therapy in non-failing patients with acute MI.

1.2.2 Pathological role of MR in the blood vessels

MR has been consistently found in endothelial cells and VSMC of blood vessels, being its activation at pathologic concentrations (10 nM) involved in many types of MR-induced vascular dysfunction, including atherosclerosis and hypertension (Barrett et al., 2013; Bender et al., 2015; Lombès et al., 2000; Pruthi et al., 2014). Classically, aldosterone regulates BP via activation of MR at renal epithelial cells in the distal nephron leading to sodium retention, which leads to increased blood volume (Soundararajan et al., 2010). In accordance, experimental studies and clinical trials have successfully shown that MR antagonists decrease BP (DuPont et al., 2014; McCurley et al., 2012; Mueller et al., 2015; Pitt et al., 1999, 2003). However, whether the actions of aldosterone on the BP controls occur directly, via vascular MR, or via indirect mechanisms (renal and central nervous system), remain a very active area of investigation due to its potential value for pharmacological interventions.

Rapid nongenomic aldosterone effects have been consistently reported in humans and *in vitro* studies. In normotensive patients, short-term systemic administration of aldosterone (12 pmol.min.kg for 4 h) results in endothelial dysfunction (Farquharson and Struthers, 2002). Rapid aldosterone infusion (500 ng/min for 8 min) into the brachial artery of healthy men causes increased forearm blood flow, whereas in the presence of a nitric oxide synthase inhibitor (L-NMMA) induces a greater vasoconstriction (Schmidt et al., 2003). Altogether, these results suggest that aldosterone acts through rapid nongenomic effects at the endothelium by increasing NO release and at VSMCs by promoting vasoconstriction. These findings are consistent with *in vitro* data showing an increase in intracellular Ca²⁺ influx in both cell types (Wehling et al., 1994, 1995). Furthermore, pulse wave velocity is greater in patients with primary hyperaldosteronism compared with patients with essential hypertension (Bernini et al., 2008). Accordingly, patients with essential hypertension show increased plasma levels of aldosterone, being associated with reduced arterial compliance (Blacher et al., 1997).

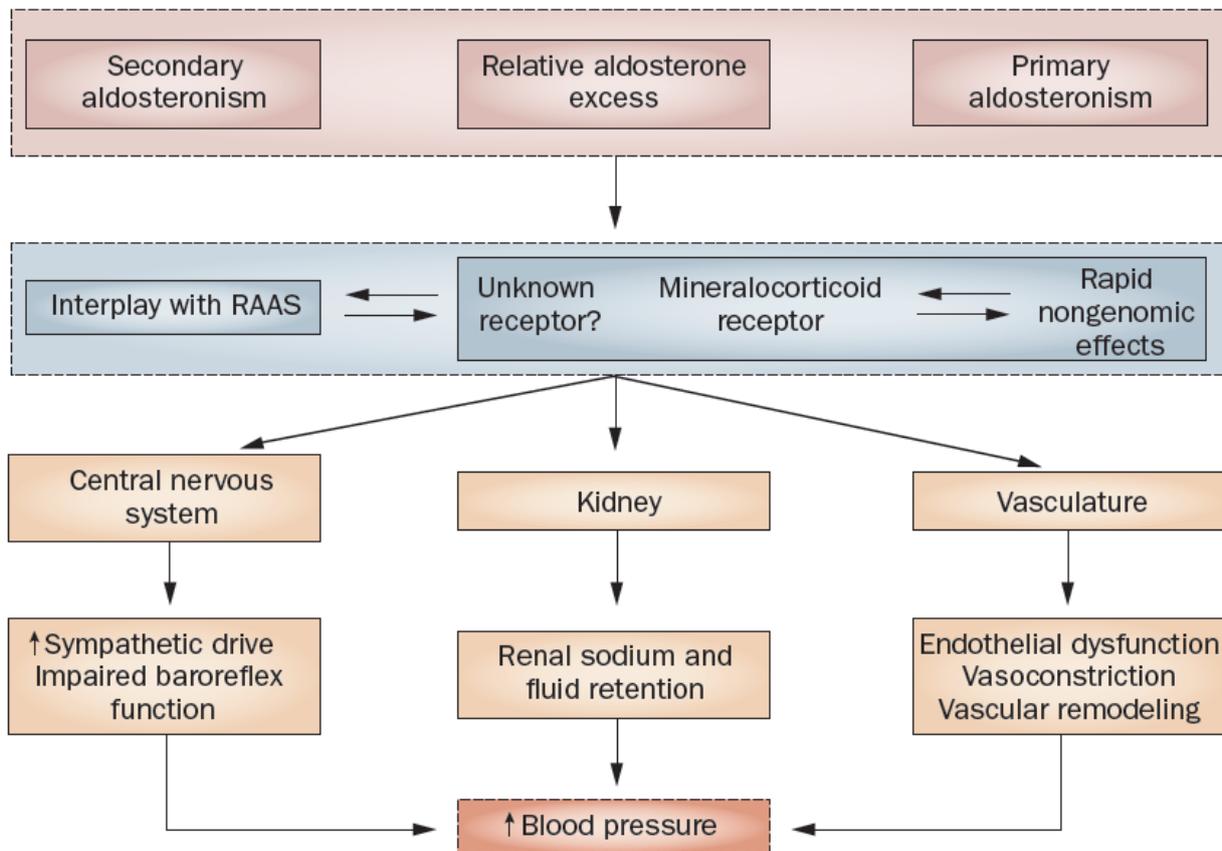


Figure 6. Mechanisms of aldosterone-mediated arterial hypertension (Tomaschitz et al., 2010).

A landmark study demonstrated that VSMC-specific MR knockout mice (VSMC-MR-KO) displayed a decrease in the age-associated elevation of BP without impairments in renal sodium handling (McCurley et al., 2012). Importantly, aged VSMC-MR-KO mice showed decreased myogenic tone, attenuated vasoconstriction and vascular oxidative stress induced by Ang II, important players of vascular dysfunction and hypertension. Moreover, the expression of $Ca_v1.2$ and contractile responses to an LTCC activator were attenuated in aged VSMC-MR-KO mice. However, just recently, Jaffe's group has discovered that during aging process, MR expression increases in resistance vessels along with a decline in the microRNA (miR)-155 and increased expression of predicted miR-155 targets, in which includes $Ca_v1.2$ and angiotensin type-1 receptor, genes that greatly contribute to vasoconstriction and oxidative stress in aging mice (DuPont et al., 2016). Altogether, these studies support the notion that VSMC-MR contributes to the age-associated rise of BP, at least in part, by regulating LTCC expression and activity.

Ca^{2+} -activated potassium channels (KCa), mainly, the large conductance KCa channels (BKCa) has been recognized as another important target of MR in blood vessels (Liu et al., 1995; Provencher et al., 2012), where it plays an important role in the regulation of vascular contraction by hyperpolarizing VSMC (Ledoux et al., 2006). Accordingly, impaired acetylcholine-mediated

relaxation in isolated coronary arteries has been shown in cardiac-specific overexpression of the aldosterone synthase (MAS mice) (Ambroisine et al., 2007). These findings correlate with decreased RNA and protein expression of BKCa α and β 1 subunits in the heart and coronary artery of MAS mice. Moreover, *in vitro* treatment of rat aortic VSMCs with increasing concentrations of aldosterone led to a reduced BKCa subunits expression in a concentration-dependent manner. Thus, these findings suggest that augmented local aldosterone production likely acts in a paracrine fashion way to suppress BKCa channel expression in the adjacent coronary VSMC, thereby contributing to the impaired endothelium-dependent VSMC relaxation.

Exacerbated generation of reactive oxygen species (ROS) have also been related to decreased nitric oxide bioavailability, which contributes to impaired endothelium-dependent relaxation of arteries and, hence, enhancing the vasoconstriction (Cooper et al., 2007). It is well-known that MR activation triggers a massive production of ROS via upregulation of NADPH oxidase causing oxidative stress and damage to target tissues, being also involved with proinflammatory actions and structural remodeling of blood vessels (Blasi et al., 2003; Gekle and Grossmann, 2009; Rocha et al., 2002). Moreover, aldosterone treatment of injured atherosclerotic vessels further enhances the expression of placental growth factor and vascular endothelial growth factors receptors, both involved in the proliferation of extracellular matrix that contributes to vascular thickening, fibrosis and stiffness (Jaffe et al., 2010; Newfell et al., 2011; Roy et al., 2006).

Although several clinical trials have shown that low-dose spironolactone (25 mg daily) substantially lowers BP in patients with of resistant hypertension, more evidence regarding the efficacy and safety were missing. In this context, the clinical trial ASPIRANT, a randomized, double-blind, placebo-controlled study reported that spironolactone, as an add-on treatment, induced a marked decreases in both office systolic BP (-14.6 mmHg) and 24-h systolic BP (-10.6 mmHg) than control groups with uncontrolled resistant hypertension (Václavík et al., 2011, 2014). Importantly, patients are diagnosed with resistant hypertension when BP remains above physiological range, even in spite of optimal doses of a triple regimen of antihypertensive drugs such as ACE inhibitor or angiotensin type-1 receptor antagonist, Ca²⁺ channel blocker, and thiazide diuretic, being the last one mandatory.

Despite several new therapeutic strategies have been developed to reduce BP, surgical and invasive interventions, such as renal denervation, have provided poor long-term benefits or unsatisfactory outcomes. Accordingly, a prospective, single-blind, randomized, sham-controlled trial (SYMPPLICITY HTN-3), showed no significant reduction of systolic BP in patients with resistant hypertension 6 months after renal artery denervation as compared with sham control group (Bhatt et al., 2014). However, as recently launched, the 2-year outcomes of the randomized PRAGUE-15 study, a randomized, multicentre study compared the efficacy of renal denervation versus spironolactone

addition (25 mg daily) in patients with true resistant hypertension (Rosa et al., 2017). This clinical trial reported that pharmacological spironolactone therapy was superior for BP lowering than renal denervation surgical intervention, over a period of 24 months (Rosa et al., 2017). Altogether, these results challenge the current belief that resistant hypertension patient cannot be adequately treated with drug therapies

Results of these trials are further supported by a landmark clinical trial (Williams et al., 2015). PATHWAY-2 was a 12-month randomized, double-blind and placebo- controlled clinical trial, which undoubtedly showed the overwhelming superiority of spironolactone compared to other antihypertensive agents including β -adrenergic blocker (bisoprolol) and α 1-adrenergic receptor antagonist (doxazosin) (Williams et al., 2015). Even the well-known side effects of spironolactone are common and are the major cause of nonadherence, these adverse effects occurred at a similar rate to placebo in the PATHWAY-2, being suggested that may reflect a response to a short-term therapy (Lobo, 2016; Williams et al., 2015).

1.3 L-type Ca^{2+} channel

Recognized as a universal second messenger in various cellular processes and cell types, Ca^{2+} signal plays a critical role in many cellular processes, including, but not limited to gene transcription, metabolism, apoptosis, synaptic transmission, neurosecretion and excitation-contraction coupling (Berridge et al., 2000, 2003; Bers, 2002; Clapham, 2007; Domínguez-Rodríguez et al., 2012). It is worth note that almost of all biological responses are mediated by Ca^{2+} -dependent and -controlled processes. However, Ca^{2+} signals need to be finely coordinated and precisely regulated. Thus, this incredible versatility faces various intracellular contexts including, space, time and amplitude to generate a proper Ca^{2+} response.

During cardiac excitation-contraction coupling, Ca^{2+} undergoes a fast cycling process. In summary, depolarization of the membrane activates the sarcolemmal Ca^{2+} channels (mainly LTCC, $\text{Ca}_v1.2$) leading to a slight increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is not enough to activate contraction. This highly localized rise in the $[\text{Ca}^{2+}]_i$, act as a powerful signal to activate ryanodine receptors (RYRs) on the sarcoplasmic reticulum (SR), a process known as a Ca^{2+} -induced Ca^{2+} release. Ca^{2+} released from SR increases global cytosolic Ca^{2+} concentrations, ultimately leading to contraction. The contraction ends by removal of Ca^{2+} from the cytosol via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which pumps Ca^{2+} into the extracellular space, while the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) moves the Ca^{2+} back into the SR, being all of these tightly controlled mechanisms regulated by multiple feedback regulators. Therefore, contractile dysfunction, arrhythmias, and many other pathologies may be a consequence of Ca^{2+} mishandling in

cardiomyocytes (Gómez et al., 1997, 2013).

In the heart, there are two types of voltage-gated Ca^{2+} channels, the L-type Ca^{2+} channels (LTCC) and the T-type Ca^{2+} channels (TTCC). Both isoforms of Ca^{2+} channels allow Ca^{2+} entry in response to changes in membrane potential. However, structural differences and biophysical properties differ both isoforms. For instance, LTCC activates at more positive membrane potentials, display slow inactivation, and are inactivated by intracellular Ca^{2+} compared to TTCC (Bers, 2008; Catterall, 2000; Ono and Iijima, 2010). Moreover, the abundance of L-type and T-type Ca^{2+} channels vary over time, being TTCC barely expressed, or even absent, in adult cardiomyocytes, while the LTCC is highly expressed (Larsen et al., 2002; Leuranguer et al., 2000).

The two main LTCC subtypes are $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ (encoded by the *Cacna1c* and *Cacna1d*, respectively). From neonatal stages, the LTCC $\text{Ca}_v1.2$ become the most predominant Ca^{2+} channel subtype in many tissues, being also found in ventricular cardiomyocytes, where acts as the major contributor of Ca^{2+} influx for excitation-contraction (EC) coupling (Bers, 2008; Bodi et al., 2005; Mikami et al., 1989). Despite the well-known contribution for EC coupling, essential for muscle contraction, Ca^{2+} entry through $\text{Ca}_v1.2$ acts also as a critical intracellular messenger that regulates a variety of cellular processes including: hormone secretion, neurotransmission, and gene expression (Catterall, 2011; Ma et al., 2011; Moosmang et al., 2007). Altogether, these pivotal Ca^{2+} -dependent processes, which depend on the activity of $\text{Ca}_v1.2$, highlight its critical physiological relevance.

As a heteromultimeric channel, LTCC is formed by four associated subunits, being the pore-forming region, named as $\text{Ca}_v1.2$ or $\alpha1C$ (encoded by the *Cacna1c*), the main subunit (Catterall et al., 2005; Dolphin, 2009, 2012; Moosmang et al., 2005). The $\alpha1C$ subunit is composed of four homologous domains (I-IV), each containing six membrane-spanning α -helices (termed S1 to S6) linked with cytoplasmic loops (linkers) and ended by NH_2 - and COOH -terminal intracellular segments. Importantly, $\alpha1C$ subunit contains the selectivity filter for Ca^{2+} , the voltage sensor (due positive charges at arginine and lysine residues within each S4), and gate machinery (Bodi et al., 2005; Catterall, 2000; Sather and McCleskey, 2003). Therefore, intracellular pathways that affect the expression and/or activity of the $\alpha1C$ subunit may represent a critical player in many types of physiological functions as well as a trigger of pathological response.

Coupled to the $\alpha1C$ subunit, three auxiliary subunits such as, the cytosolic β subunit regulates current kinetics and trafficking of $\alpha1C$ to the plasma membrane (Bichet et al., 2000; Brice et al., 1997; Catterall, 2000; Chien et al., 1995; Gao et al., 1999; Pragnell et al., 1994; Wei et al., 2000), while the $\alpha2\delta$ subunit modulates the voltage dependence and kinetics of $\text{Ca}_v1.2$ (Catterall, 2000; Gurnett et al., 1996; Hofmann et al., 1994). Last but not least, the γ subunit is involved in the anchorage and trafficking of $\text{Ca}_v1.2$ (Catterall, 2000; Hofmann et al., 1994; Moosmang et al., 2007).

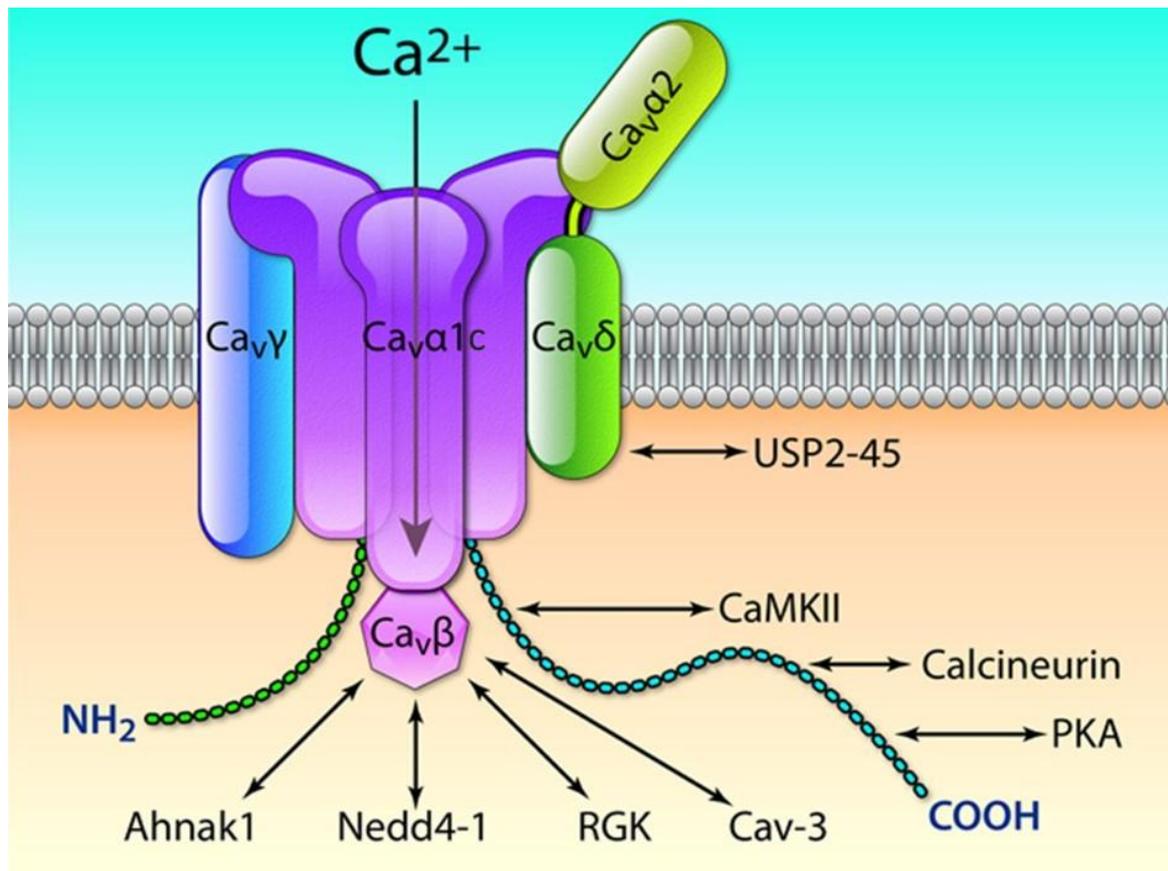


Figure 7. $Ca_v1.2$ channel subunits and their interacting proteins (Abriel et al., 2015).

As above mentioned, in spite of the multiple roles of $Ca_v1.2$, the Ca^{2+} current ($I_{Ca,L}$) provided by these channels is the main trigger of the cardiac EC coupling (Bers, 2002, 2008; Bodi et al., 2005). Thus, $I_{Ca,L}$ directly contributes to the inward depolarizing current of the cardiac action potential (AP), in which massively contributes to the plateau phase of the ventricular AP (Nerbonne and Kass, 2005). Moreover, multiple studies have suggested that enhanced Ca^{2+} influx via the LTCC is responsible for the longer AP during cardiac hypertrophy and pathological remodeling of ventricles, which could be pro-arrhythmogenic, favoring a higher incidence of early afterdepolarizations (Benitah et al., 2010; Keung, 1989; Mukherjee and Spinale, 1998; Richard et al., 1998). As expected, adult cardiomyocytes from $\alpha1C$ -knockdown mice ($\alpha1C^{-/+}$) show a decrease in the $I_{Ca,L}$ amplitude and a modest reduction in the cardiac contractility, which initially suggested the hypothesis that these mice would be cardioprotective against pathological stimuli. In contrast, $\alpha1C^{-/+}$ mice subjected to pressure overload stimulation (by transverse aortic constriction), chronic β -adrenergic infusion (by isoproterenol treatment), and forced physical activity (swimming) showed exacerbated cardiac hypertrophy, greater reductions in the ventricular contractile performance, and larger ventricular dilation than WT mice (Goonasekera et al., 2012). Taken together, these findings suggest that Ca^{2+} influx through LTCC is

tightly regulated in cardiomyocytes, being small increase or decrease involved in the development of cardiac dysfunction.

Ubiquitously expressed in whole body, $Ca_v1.2$ is also the main subunit in VSMC, where it comprises the primary pathway of Ca^{2+} entry, essential for vascular EC coupling and thus, the vascular myogenic tone (Murthy, 2006; Sanders, 2008; Somlyo and Somlyo, 2003). Although global knock-out mice of $Ca_v1.2$ are embryonic lethal (Seisenberger et al., 2000), VSMC-specific $Ca_v1.2$ knockout mice revealed the importance of $Ca_v1.2$ in the vasculature, displaying reduced mean arterial BP (~30 mmHg) and decreased phenylephrine-induced contraction (Moosmang et al., 2003). Recently, our knowledge regarding the dysfunctional consequences of $Ca_v1.2$ downregulation in the VSMC was expanded (Kudryavtseva et al., 2014). Using small interference RNA (siRNA) transfection *in vivo*, 3 days after siRNA transfection, rat mesenteric arteries showed reduced LTCC expression at mRNA and protein levels. Surprisingly, the authors showed increased resting $[Ca^{2+}]_i$, despite the attenuated contraction elicited by norepinephrine or depolarizing solution. Moreover, phenotypic and functional changes were associated with marked VSMC proliferation and, consequently, increased media thickness and area of the vessels. Altogether, these studies support the notion of the critical importance of $Ca_v1.2$ for the vascular function.

Vital for numerous physiological processes, Ca^{2+} signal is equally important for controlling arterial tone and BP. Functionally, the voltage-dependent Ca^{2+} influx allowed by a small fraction of LTCC is sufficient to mediate pressure-induced constriction in small arteries and thereby contribute to dynamic autoregulation in small vascular beds, commonly referred as myogenic tone (Goligorsky et al., 1995). Briefly, the vascular contraction is triggered by membrane depolarization allowing Ca^{2+} influx that quickly diffuses into the cell leading to an increased $[Ca^{2+}]_i$ associated with a small Ca^{2+} release from SR stores. As follow, Ca^{2+} /calmodulin complex activates several downstream cascades such as, the myosin light chain kinase (MLCK), which acts phosphorylating the myosin light chain (MLC) and thus, causing vascular contraction. Alternatively, activation of second messengers that inhibit myosin light chain phosphatase (MLCP) activity can thereby, sensitize the myofilaments to Ca^{2+} (Wray et al., 2005). Thus, sustained voltage-dependent Ca^{2+} influx through the LTCC maintains a tonic level of vasoconstriction and provides an excitatory template upon which endogenous vasoactive substances may act to modulate arterial diameter.

Importantly, the myogenic response may be viewed as a protective mechanism. In small arteries, which are primarily involved in the autoregulation of local blood flow and pressure, the loss of myogenic tone control appears to precede the occurrence of adverse events and predisposition to develop hypertension. Taking into account that LTCC plays a critical role in maintaining the diameter and tone of blood vessels, defects in Ca^{2+} signaling leads to increased vascular resistance, being considered as an important trigger for the development of hypertension. A central component of the

pathogenesis of hypertension is the rapid and persistent upregulation of Ca_v1.2 in the VSMC (Chen et al., 2004; Lozinskaya and Cox, 1997; Martens and Gelband, 1996; Simard et al., 1998; Wilde et al., 1994). Hence, upregulation of Ca_v1.2 channels may mediate the greater vasoconstriction, a hallmark in patients with hypertension (Sonkusare et al., 2006).

Several studies have consistently demonstrated that increased voltage-dependent Ca²⁺ current density in VSMCs is positively correlated with BP level. An increase in the expression of Ca_v1.2 channels, as well as Ca²⁺ current density, have been reported in arteries of both genetic and renal forms of experimental hypertension (Pesic et al., 2004; Pratt et al., 2002). Accordingly, the involvement of Ca_v1.2 in the pathogenesis of hypertension centers on evidence that Ca_v1.2 are markedly upregulated in blood vessels during the development of hypertension. Indeed, the expression Ca_v1.2 protein is higher in mesenteric, renal and skeletal muscle arteries of SHR and DOCA-salt hypertensive rats compared to their respective control rats (Pesic et al., 2004; Pratt et al., 2002; Sonkusare et al., 2006). These studies extended our understanding regarding the causative link between elevation of BP and vascular Ca_v1.2 expression, suggesting a molecular basis for the increased Ca_v1.2 channel-mediated vascular tone observed in hypertensive subjects. In line with this hypothesis, after aortic banding (48h), renal arteries exhibited an increased expression of Ca_v1.2 α 1C pore-forming, implying that even a short-term rise in the BP is able to trigger the expression of vascular Ca_v1.2 as an early event in the development of hypertension (Pesic et al., 2004).

1.3.1 Regulation of Ca_v1.2 expression

Although the mechanisms of regulation of Ca_v1.2 expression remain little known, certain intracellular pathways appear as potential candidates such as Ca²⁺ signaling and hormonal pathways (Hofmann et al., 2014; Satin et al., 2011). Ca²⁺ is able to regulate the expression of Ca_v1.2 via Ca²⁺-activated calmodulin pathway (Morad and Soldatov, 2005). Moreover, isolated adult rat cardiomyocytes exposed to Ca²⁺-rich medium (4.8 mM) for 72h showed an increase in the expression of Ca_v1.2 and amplitude of *I*_{Ca,L} (Davidoff et al., 1997). Interestingly, 48h of treatment with verapamil, Ca²⁺ channel blocker, also leads to an upregulation of Ca_v1.2 (Schroder et al., 2007). However, the molecular pathways involved in the upregulation of Ca_v1.2 by high Ca²⁺ and LTCC blocker are still not defined.

Ca²⁺-activated calmodulin kinase II (CaMKII) also plays a role in the regulation of Ca_v1.2 expression. Accordingly, CaMKII δ -overexpressing HL-1 cells showed a decrease Ca_v1.2 expression, whereas treatment with KN-93, a CaMKII inhibitor, increased Ca_v1.2 expression at mRNA and protein levels. These results are in accordance with increased peak of *I*_{Ca,L} reported on a cardiac-specific CaMKII δ KO mice (Xu et al., 2010). Mechanistically, it has been shown that CaMKII

activates the downstream regulatory element antagonist modulator (DREAM), described as Ca²⁺-binding transcriptional repressors (Carrión et al., 1999), by direct action on the *Cacna1c* promoter (Ronkainen et al., 2011).

In cardiac myocytes, VSMCs, and neurons, the intracellular C-terminus of Ca_v1.2 is susceptible to proteolytic cleavage, yielding a truncated and short subunit (~190 kDa) and a cleaved C-terminal fragment (CCt, ~50 kDa) (De Jongh et al., 1991; Gomez-Ospina et al., 2006; Schroder et al., 2009). Accumulating evidence have shown that the role of Ca_v1.2 CCt is not limited to the regulation of Ca_v1.2 function, but it could have a role as a transcription factor, in particular, by regulating its own expression (Gomez-Ospina et al., 2006; Satin et al., 2011; Schroder et al., 2009). CCt is localized in the nucleus and plasma membrane of cardiac myocytes, VSMCs, and neurons (Bannister et al., 2013; Gomez-Ospina et al., 2006; Schroder et al., 2009). In cardiomyocytes, CCt represses the transcriptional activity of Ca_v1.2 (Schroder et al., 2009). Similarly, in VSMC CCt inhibits Ca_v1.2 transcription and directly reduces voltage-dependent current activation, attenuating the myogenic tone in pressurized arteries. Thus, suggesting that CCt fragment acts as a bi-modal Ca_v1.2 channel inhibitor and vasodilator.

The positive inotropic effect of catecholamines is conferred by upregulation of LTCC expression and/or activity. Stimulation of β-adrenergic signaling pathway in neonatal rat cardiac myocytes for 24h to isoproterenol (10 nM) induces an increase in the peak of *I*_{Ca,L} and number of functional Ca²⁺ channels (Maki et al., 1996). Moreover, sustained β-adrenergic stimulation increases LTCC expression in cultured rabbit ventricular myocytes treated 72h with isoproterenol (2 μM). The mechanism by which adrenergic pathway regulates the expression of Ca_v1.2 mainly involves the stimulation of PKA signaling, which in turns activates cyclic AMP response element-binding protein (CREB), an important transcriptional factor involved in the regulation of several genes (Bodi et al., 2005; Kamp and Hell, 2000). However, these findings are not consistently reported in animals treated for 24 to 48 hours, or 7 days with isoproterenol (Golden et al., 2002; Schroder et al., 2007). Thus, underlying signaling pathways of this important physiological process have been investigated by many laboratories over the last two decades, but is still not completely understood.

A wide variety of hormones is also involved in the regulation of LTCC. Accordingly, angiotensin II treatment of atrial cardiomyocytes (HL-1) increased the expression of Ca_v1.2 and *I*_{Ca,L}, while the co-treatment with losartan, an angiotensin receptor antagonist, did not restore these effects (Tsai et al., 2007). Upregulation of Ca_v1.2 in response to GCs has also been consistently shown in cardiomyocytes and aortic VSMC (A7r5 cells) (Hayashi et al., 1991; Obejero-Paz et al., 1993; Rougier et al., 2008; Takimoto et al., 1997). Isolated ventricular cardiomyocytes treated for 48h with dexamethasone, a synthetic GC, induced an increase in the amplitude of *I*_{Ca,L} associated with

upregulation of $Ca_v1.2$ (Whitehurst et al., 1999). *In vivo* treatment with dexamethasone also induces a time-dependent $Ca_v1.2$ increase (5-to-15 days) at mRNA and protein levels as well as, in neonatal rat cardiomyocytes exposed for 24h (De et al., 2011). Moreover, adrenalectomized rats treated with dexamethasone displayed an increase in the transcript of $Ca_v1.2$ after 24h, while the protein levels increased after 3 days, even despite the mRNA levels returned to basal levels (Takimoto et al., 1997). A positive correlation between $Ca_v1.2$ expression and circulating cortisol levels has been demonstrated in patients with adrenal adenoma (Felizola et al., 2014). Similarly, rats subjected to a model of chronic stress display increased mRNA levels and protein expression of $Ca_v1.2$ (Zhao et al., 2009).

Our group has previously shown an increased $I_{Ca,L}$ amplitude and expression of $Ca_v1.2$ in rat cardiomyocytes treated with aldosterone for 24-48h (Bénitah et al., 2001; Lalevée et al., 2005; Bénitah and Vassort, 1999). Moreover, these findings correlate with *in vivo* evidence that lifelong exposure to aldosterone induces a upregulation of $Ca_v1.2$ and $I_{Ca,L}$ in cardiomyocytes (Martin-Fernandez et al., 2009; Perrier et al., 2005), as well as in a cardiac-specific MR overexpressing mice (Ouvrard-Pascaud et al., 2005). Interestingly, the modulation of $I_{Ca,L}$ amplitude by aldosterone described above is consistent with a genuine long-term physiological regulation of Ca^{2+} channels. However, the absence of changes in the kinetics and voltage-dependent properties of $I_{Ca,L}$ suggest a specific and genomic regulation. Moreover, in rat neonatal cardiomyocytes, aldosterone treatment (1 μ M for 24h) significantly increased mRNA levels of the auxiliary $Ca_v\beta_2$ subunit (Lalevée et al., 2005).

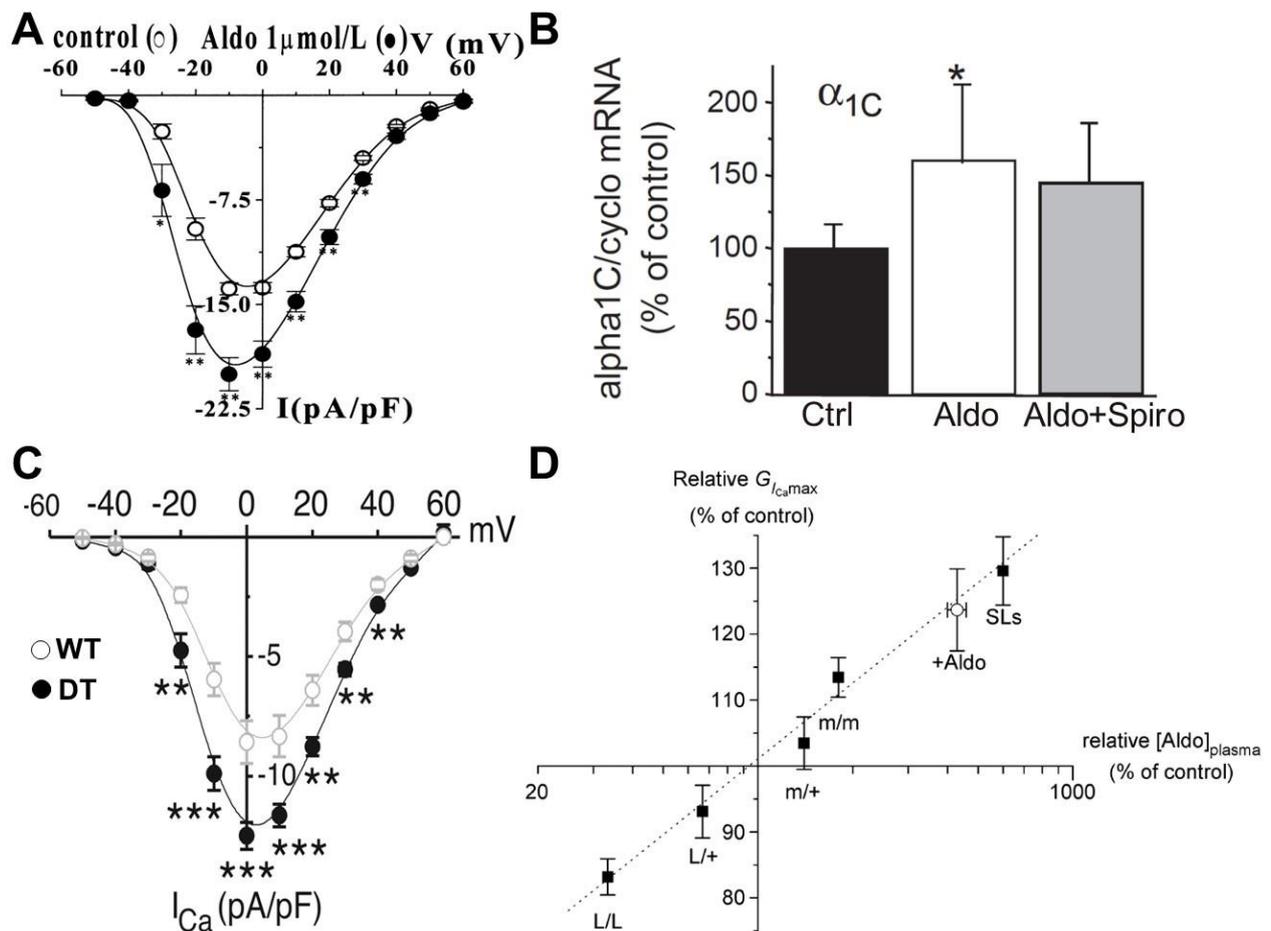


Figure 8. The long-term effect of aldosterone treatment on cardiac $I_{Ca,L}$ and expression of the $Ca_v1.2$ subunit. **A**, $I_{Ca,L}$ measured on adult rat ventricular myocytes treated with aldosterone (Aldo) for 24 h (Bénitah and Vassort, 1999). **B**, Expression of $Ca_v1.2$ subunit on neonatal rat ventricular myocytes treated with aldosterone (1 μM) for 24 h in the presence or absence spironolactone (Spiro, 10 μM) (Lalevéé et al., 2005). **C**, $I_{Ca,L}$ measured on adult ventricular myocytes from wild-type (WT) and double-transgenic (DT) mice overexpressing hMR under control of a cardiac-specific transactivator (Ouvrard-Pascaud et al., 2005). **D**, $I_{Ca,L}$ measured on ventricular myocytes from transgenic mice with a mutation on the ENaC, in which display increased aldosterone levels according to the degree of salt-losing phenotype, being classified as severe (SLs) or mild (m/+; heterozygous and m/m; homozygous mice). Mice with Liddle syndrome, which display salt-retaining phenotype (L/+; heterozygous and L/L; homozygous mice) and, consequently, low aldosterone levels. +Aldo, *in vivo* treatment with aldosterone (50 μg.day) for 3 weeks (Perrier et al., 2005).

1.3.2 $Ca_v1.2$ promoters and alternative splice variants

Although the major LTCC expressed in the cardiac and VSMC is the $Ca_v1.2$, there are remarkable molecular distinctions that influence the protein diversity and electrophysiological properties of both types of cells. *CACNA1C* is composed by 50 exons in human (Soldatov, 1994), whereas mouse and rat *Cacna1c* have been reported to lack the exon 45 resulting in 49 exons (Link

et al., 2009; Wellman and Nelson, 2003). Differential *Cacna1c* promoter usage has been associated with the cell-specific regulator of *Cacna1c* and responsible, at least in part, for these changes (Dai et al., 2002; Pang et al., 2003).

Indeed, it has been described two different alternative promoters for the $Ca_v1.2$ (Cheng et al., 2007; Pang et al., 2003; Saada et al., 2003, 2005). In the heart, the expression of $Ca_v1.2$ is primarily driven by the most distal 5' promoter, while the expression in other tissues is controlled by a second and most proximal promoter. The initial work was carried out on cardiac cells contributing to the description of the 'cardiac' *Cacna1c* promoter, first called as promoter A, which controls the transcription of exon 1a in rat (Liu et al., 2000; Pang et al., 2003) and human (Dai et al., 2002). The second *Cacna1c* promoter, named as promoter B, was first described in humans (Pang et al., 2003) and rat (Saada et al., 2005), controlling the transcription of the alternative first exon 1b, which is shorter in terms of size and found more ubiquitously.

Interestingly, the activity of these promoters seems to be regulated in a tissue-specific manner (Pang et al., 2003), being the promoter A being a cardiac-specific *Cacna1c* promoter, whereas promoter B is more active non-cardiac tissues (Pang et al., 2003; Saada et al., 2005). Accordingly, the promoter A is constitutive active in neonatal rat cardiomyocytes but not in cardiac fibroblasts, H9C2 cells, human vascular smooth muscle and HEK293 cells (Pang et al., 2003), while the promoter B is active fibroblasts (Soldatov, 1992), mouse brain (Snutch et al., 1991), vascular tissues (Saada et al., 2003), bone (Wang et al., 2000), and rabbit lungs (Biel et al., 1990). In summary, $Ca_v1.2$ channels are expressed as two distinct tissue-specific transcripts of *Cacna1c*, which encode, respectively, a long 'cardiac' ($Ca_v1.2$ -LNT) and a short 'vascular/brain' ($Ca_v1.2$ -SNT) NH₂-terminal region, regulated by two alternative promoters, hereinafter called as the P1 and P2, respectively (Hofmann et al., 2014). Thus, although these studies support the notion of an intrinsic cell-type factor that specifically modulates the activity of these promoters, the mechanism and/or factors are not fully understood.

Besides the undoubtedly importance of alternative promoter usage, differential exon-containing splices of $Ca_v1.2$ between cardiac and vascular tissue also contribute to gene regulation and protein diversity, which may affect the membrane expression of $Ca_v1.2$, voltage dependence, Ca^{2+} sensitivity, Ca^{2+} -dependent inactivation, β -subunit binding and sensitivity to dihydropyridine (DHP) blockers (Abernethy and Soldatov, 2002; Bannister et al., 2011; Perez-Reyes et al., 1990; Snutch et al., 1991; Soldatov, 1992, 1994; Tang et al., 2004; Yang et al., 2000). As shown in figure 7, at least 20 exons of the *Cacna1c* undergo alternative splicing in the NH₂- and COOH-terminus (Abernethy and Soldatov, 2002; Cheng et al., 2007; Soldatov, 1994).

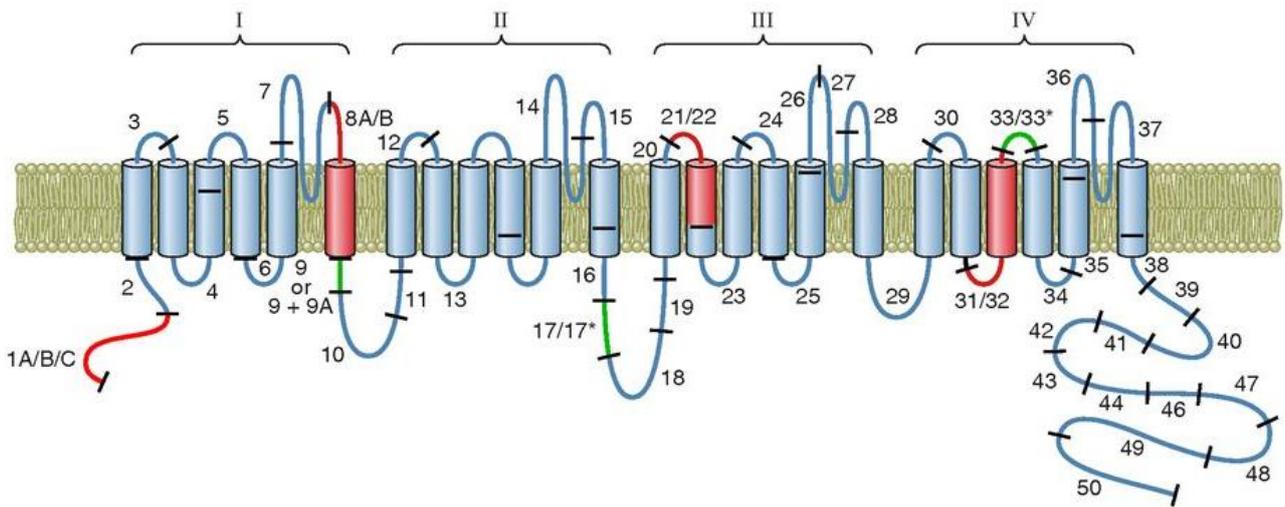


Figure 9. Composition of exons in Ca_v1.2 (*ca_v1c*) channel. Ca_v1.2 with exon numbering according to the nomenclature used for the human Ca_v1.2 gene (Soldatov, 1994). Worthwhile note the lack of exon 45 in rabbit, rat, and mouse Ca_v1.2. Alternative exons indicated in red (Hofmann et al., 2014).

As mentioned above, the regulation of temporal and spatial Ca²⁺ influx through LTCC has wide physiological and pathological implications. Despite the extensive alternative splices that can affect the activity and interaction of Ca_v1.2 with other modulatory proteins, the functional heterogeneity allows a highly specialized Ca²⁺ signals to certain cell types and cellular requirements, and therefore providing a novel fine-tuning Ca_v1.2 regulation (Hofmann et al., 2014; Liao et al., 2005; Shaw and Colecraft, 2013; Zuccotti et al., 2011). A detailed analysis of Ca_v1.2 splice variants in the heart and vascular cells revealed a great predominance of DHP-sensitive splice variants in arterial VSMC, resulting in a greater DHP sensitivity of LTCC currents in arterial VSMC than in cardiomyocytes. Importantly, some of these splices are associated with Ca_v1.2 activation at more negative voltages (Cheng et al., 2009; Liao et al., 2004) and thus, contributing to more hyperpolarized steady-state Ca²⁺ inward current (window current), which is close to the VSMC resting potential that controls myogenic tone (Fleischmann et al., 1994; Liao et al., 2007). Therefore, VSMC shows a more depolarized resting membrane potential (~-60 mV) compared to cardiomyocytes (~-90 mV) favors channel inactivation by DHPs. Splice-containing Ca_v1.2 is also prone to show pronounced steady-state inactivation, which also enhances DHP sensitivity (Liao et al., 2007). Moreover, alternative splicing of Ca_v1.2 also affects the molecular structure of the channel and the accessibility of DHP binding domain (Welling et al., 1993).

Intriguingly, a large number of studies in heart and arterial VSMC indicate that Ca_v1.2 displays higher susceptibility to undergoes splice variants under pathological conditions than in healthy state (Abernethy and Soldatov, 2002; Cheng et al., 2007; Gidh-Jain et al., 1995; Liao et al., 2004, 2005, 2007, 2009a, 2009b; Tang et al., 2008; Yang et al., 2000). Therefore, altered splicing

patterns of alternative exons have been linked to a number of diseases (Abernethy and Soldatov, 2002; Liao et al., 2009a, 2009b). However, in spite of many exons of $Ca_v1.2$ channel were found to be alternatively spliced (Abernethy and Soldatov, 2002; Liao et al., 2005), few major sites segregate $Ca_v1.2$ variants in cardiac and vascular cells (Cheng et al., 2007; Liao et al., 2004, 2007; Welling et al., 1997a).

One major splice locus is the mutually exclusive exons 8/8a. The rat cardiac cells express almost exclusively the cardiac exon 8a (Splawski et al., 2004). The first described Timothy syndrome (TS) was characterized by a recurrent *de novo* *Cacna1c* mutation (G406R) at the cardiac exon 8a (Splawski et al., 2004). Later, the same group identified an atypical form of TS, named as TS2, which is caused by two mutations (G406R and G402S) at the exon 8. TS mutations are located in the IS6 segment of $Ca_v1.2$, a region that largely contributes to the regulation of gate properties. This autosomal dominant disease caused by *de novo* gain-of-function mutations of $Ca_v1.2$ subunit is a multiorgan disease characterized by both cardiac and extracardiac symptoms associated with remarkable slow in the voltage-dependent and Ca^{2+} -dependent inactivation resulting in sustained depolarization (Barrett and Tsien, 2008; Splawski et al., 2004). Therefore, this enhanced Ca^{2+} influx delays cardiomyocyte repolarization, which substantially increase the risk of lethal arrhythmias, being more severe in TS2 patients (Splawski et al., 2004, 2005). The location of TS and TS2 mutations on two mutually exclusive exons determine the severity of cardiac phenotypes mainly, due to the differential expression levels of exons 8/8a in the heart. Functional studies comparing exon 8 and 8a $Ca_v1.2$ variants indicate similar electrophysiological properties but different sensitivities to DHP compounds (Welling et al., 1997a; Zühlke et al., 1998). Exon 8 and 8a variants are expressed in different tissues, and the ratio of exon 8/8a inclusion has been found to change in hypertrophied heart of spontaneously hypertensive rats (Tang et al., 2008). Moreover, such differential distributions of exon 8/8a would also affect other tissues beyond the cardiovascular system.

Mutually exclusive exon splices of the cardiac $Ca_v1.2$ generates a pair of developmental regulated exons 31/32 (Diebold et al., 1992), being their relative proportion altered in a rat model of MI (Gidh-Jain et al., 1995), suggesting that the expression of fetal cardiac isoform re-emerged in adult diseased hearts, such as in humans failing hearts (Yang et al., 2000). In hearts from spontaneously hypertensive rats, complex dynamic changes in the alternative splicing of $Ca_v1.2$ have been described both at single loci and in their combinatorial profile when compared with Wistar-Kyoto rats (Tang et al., 2008). In a recent study, exclusion of the alternative exon 33 in $Ca_v1.2$ has been reported to shift the activation potential -10.4 mV to the hyperpolarized direction associated with greater $I_{Ca,L}$ density. Moreover, increased Ca^{2+} current induced a prolongation in the AP duration in ventricular cardiomyocytes from $Ca_v1.2$ exon 33-specific knockout mice, which explains the increased early afterdepolarizations. Notably, *in vivo* analysis revealed that exon 33^{-/-} mice show

increased occurrences of premature ventricular contractions, tachycardia, and lengthened QT interval, suggesting that exclusion of exon 33 of the $Ca_v1.2$ is pro-arrhythmogenic (Li et al., 2017). The expression of exon 33 of the $Ca_v1.2$ is ~60% in human heart and ~90% in rodents (Tang et al., 2004). Unexpectedly, the proportion of $Ca_v1.2$ transcripts containing exon 33 was increased from ~60% in nonfailing hearts to ~80% in human failing hearts (Li et al., 2017). However, the precise role of $Ca_v1.2$ exon 33 inclusion in the pathogenesis of human heart failure remains unclear.

In VSMC from nonatherosclerotic human samples, RT-PCR analysis revealed an extensive repertoire of $Ca_v1.2$ transcripts characterized by the presence of exons 21 and 41A. In VSMC affected by atherosclerosis, despite decreased expression, it was found a replacement of the exon 21 by a unique exon 22 variant of $Ca_v1.2$, and lack of exon 41A. Interestingly, alternatively spliced exon 22 was related with altered electrophysiological properties and a potential association with VSMC proliferation (Tiwari et al., 2006). Importantly, the assortment of alternatively spliced $Ca_v1.2$ exons is not random and therefore, suggest that alternative splicing may serve as a modulatory mechanism that contributes to the dynamic changes in the expression and function of $Ca_v1.2$ in order to re-establish the physiological state. Altogether, these studies provide a compressive knowledge of signature combinatorial splicing pattern in several cardiovascular diseases, which certainly provide valuable molecular targets for the development of new therapeutic methods to manage the disease.

1.3.3 Tissue-specific regulation of $Ca_v1.2$ -LNT and $Ca_v1.2$ -SNT

A hallmark distinction of $Ca_v1.2$ -SNT and $Ca_v1.2$ -LNT is the exon-containing splices of $Ca_v1.2$. While $Ca_v1.2$ -SNT contains exons 1b/8/9*/32/33 (Biel et al., 1990; Liao et al., 2007), $Ca_v1.2$ -LNT contains the combination of exons 1a/8a/-9*/31/33 (Liao et al., 2005, 2007; Mikami et al., 1989), in which has been associated with the remarkable modulation of DHP sensitive (Liao et al., 2007). As previously reported, the transmembrane segment IS6 of $Ca_v1.2$ contains splices, in which confers the tissue-specific modulator site of DHP sensitivity (Welling et al., 1993, 1997b).

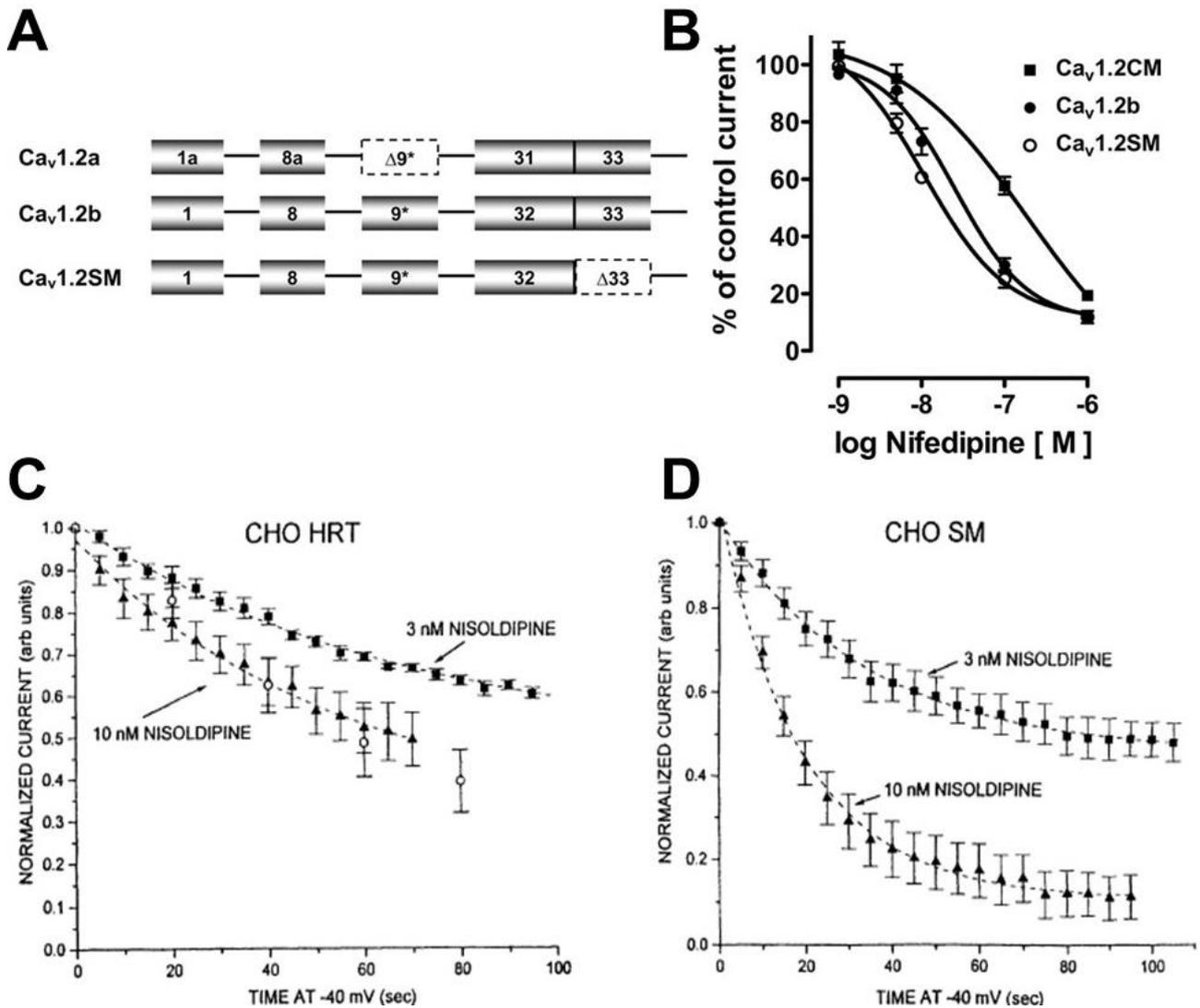


Figure 10. Differential DHP sensitivity between Ca_v1.2-LNT (Ca_v1.2a or Ca_v1.2CM) and Ca_v1.2-SNT (Ca_v1.2b or Ca_v1.2SM). **A**, Combinatorial Ca_v1.2-containing exons of Ca_v1.2a, Ca_v1.2b, and Ca_v1.2SM. Dashed boxes indicate the exclusion of exon 9* (Δ9*) or exon 33 (Δ33). **B**, dose-response of nifedipine in Ca_v1.2b, Ca_v1.2SM, and Ca_v1.2CM channels. **A and B** from (Liao et al., 2007). Nisoldipine sensitivity in Chinese hamster ovary cells expressing Ca_v1.2-LNT (**C**, CHO HRT – heart) or Ca_v1.2-SNT (**D**, CHO SM – smooth muscle). **C and D** from (Welling et al., 1993).

The ubiquitous Ca²⁺-sensing protein, calmodulin (CaM), is the Ca²⁺ sensor for both Ca_v1.2 Ca²⁺-dependent inactivation (CDI) and Ca²⁺-dependent facilitation (CDF) (Peterson et al., 1999; Zühlke et al., 1999). This important regulation involves a direct association of calmodulin (CaM) to the Ca_v1.2 channel (Anderson, 2001; Halling et al., 2006; Peterson et al., 1999; Zühlke et al., 1999). The CDI may serve as a negative feedback for regulating Ca²⁺ entry into the cell, and it may be a physiological safety mechanism against a harmful Ca²⁺ overload in the cell (Anderson, 2001; Benitah et al., 2010). CDI is mediated by Ca²⁺ binding to CaM tethered to the C-terminus of Ca_v1.2, on a classical IQ domain located at amino acids 1624–1635 (exon 42) (Halling et al., 2006; Peterson et

al., 1999; Zühlke et al., 1999). Accordingly, disruption of this domain or overexpression of mutant causes the abolishment of CDI (Ohrtman et al., 2008; Peterson et al., 1999). In adult ventricular myocytes, elimination of LTCC CDI with CaM mutants profoundly prolonged the AP duration, demonstrating its physiological importance (Alseikhan et al., 2002). Moreover, mice expressing mutation of Ile to Glu (Ile1624Glu) in the IQ motif abolished the regulation of Ca_v1.2 channel by CDI and CDF, leading to dilated cardiomyopathy and death (Blaich et al., 2012).

Despite many studies on the CDI has focused on the C-terminal tail of the pore-forming Ca_v1.2 subunit, a CaM-interacting domain in the N-terminal domain of Ca_v1.2 channels has also been implicated in the process that mediates CDI in response to local rather than global Ca²⁺ (Dick et al., 2008; Zhou et al., 2005), while the N-lobe CDI is mainly involved in the low-affinity Ca²⁺-binding domain of CaM (Dick et al., 2008). Furthermore, a novel second N-terminal CaM interaction site at residue C106 that contributes to the N-terminal lobe CDI has been recognized, being also identified a CamKII interaction domain adjacent to this CaM interaction (Simms et al., 2014).

Cardiac and VSMC Ca_v1.2 are tightly regulated by hormonal and neuronal signals via G-proteins and protein kinases (Trautwein and Hescheler, 1990; Wickman and Clapham, 1995). The N-terminus of Ca_v1.2 and Ca_vβ subunits appear to be modulated by protein kinases A and C (PKA and PKC, respectively) (Kamp and Hell, 2000; Puri et al., 1997). Although PKA and PKC have been involved in the activation of the channel (Trautwein and Hescheler, 1990; Wickman and Clapham, 1995). PKC-mediated effects seem to enhance LTCC current by several stimuli such as, intracellular ATP (McHugh and Beech, 1997), α-adrenergic agonists (Woo and Lee, 1999), angiotensin II (Dösemeci et al., 1988), GCs (Kato et al., 1992), vasopressin (Zhang et al., 1995). PKC activators, such as phorbol esters and diacylglycerols, also increase Ca²⁺ channel currents in cardiac and VSMC of various mammals (Chik et al., 1996; Dösemeci et al., 1988; Kato et al., 1992; Lacerda et al., 1988; Marinissen et al., 1994; Schuhmann and Groschner, 1994; Tseng and Boyden, 1991; Zhang et al., 1995). However, discrepancies regarding PKC-activating phorbol esters effects have been reported, such as initial enhancement (Boixel et al., 2000; Lacerda et al., 1988; Tseng and Boyden, 1991), inhibition (Satoh, 1992, 1995; Zhang et al., 1997) or a biphasic change in the Ca²⁺ current amplitude (Boixel et al., 2000; Lacerda et al., 1988; Tseng and Boyden, 1991).

In rabbit cardiac Ca_v1.2, the initial 46 amino acids are crucial for PKC modulation (Shistik et al., 1998, 1999), being experimentally demonstrated that disruption of the first 40 amino acids causes a 5–10-fold increase in the LTCC current (Shistik et al., 1998; Wei et al., 1996). In spite of the initial increase in a single channel open probability of LTCC (Shistik et al., 1998), these findings proposed that N-terminus of Ca_v1.2 acts as an inhibitory gate, since its removal enhances channel activation. Therefore, it has been suggested that PKC increases LTCC current by attenuating the inhibitory action of the N-terminus Ca_v1.2 (Shistik et al., 1998). Moreover, PKC is one of such regulators that its

actions appear to be tissue- and species-specific (Shistik et al., 1998). Supporting this hypothesis, studies using heterologous expressing systems in *Xenopus oocytes* demonstrated that reconstitution of LNT- and SNT-Cav1.2 is differently regulated by PKC (Blumenstein et al., 2002; Shistik et al., 1998; Weiss et al., 2004). In human Cav1.2-LNT PKC activator produced Ca²⁺ channel enhancement (Weiss et al., 2004), whereas the Cav1.2-SNT was inhibited (Blumenstein et al., 2002). For instance, the exon 1b-containing vascular Cav1.2-SNT lacks four amino acids, being two of them putative PKC phosphorylation sites (threonine 27 and 31), while the exon 1a-containing cardiac Cav1.2-LNT contains the first 20 amino acids that play the inhibitory gating role (Blumenstein et al., 2002; Liao and Soong, 2010; McHugh and Beech, 1997; Shistik et al., 1998, 1999).

It is known that the proper assembly of Cav1.2 subunits has effects on its expression and on the characteristics of Cav1.2 currents. Numerous studies have proposed that single Cav1.2 subunits may differentially modulate Cav1.2 currents in the heart and VSMC. From these findings, biochemical studies suggested that a single Cav1.2 contains a single Cav β subunit (Buraei and Yang, 2013). Studies indicate the case that the 1:1 stoichiometry is determined by the α -interaction-guanylate kinase (AID-GK) domain interaction. Thus, the transcripts encoding all four Cav β subunits (β 1, β 2, β 3, β 4) have been detected in arteries, although only Cav β 2 and Cav β 3 were detected at the protein level (Held et al., 2007; Hullin et al., 1992; Murakami et al., 2003). Cav β 2 is predominantly expressed in heart, aorta, and brain, whereas Cav β 3 is most abundant in the brain but also present in aorta, trachea, lung, heart and skeletal muscle (Hullin et al., 1992). Cav β 2 gene also undergoes alternative splice, resulting in at least five variants of the Cav β 2 subunit that differ in N-terminus. Because global knockouts of single Cav β 2 splice variants are not available, the delineation of specific functions to Cav β 2-N3 or of other Cav β 2 splice variants remains to be determined. Only a single variant of the Cav β 3 subunit (Cav β 3a) has been reported to be present in human myocardium (Hullin et al., 2003).

The role of Cav β 2 in VSMC have yet to be determined since the global deletion of Cav β 2 is embryonic lethal because of severe cardiovascular defects (Weissgerber et al., 2006). However, cardiac-specific inactivation of the Cav β 2 gene in adult mice resulted in viable animals and a reduction of Cav1.2 current (-25%) (Meissner et al., 2011). Despite the unnoticed cardiovascular phenotype, Cav β 3-deficient mice show reduced DHP-sensitive (-30%) and LTCC current in aortic SMC, while the BP remained unchanged (Murakami et al., 2003). Upon high-salt diet, Cav β 3-deficient mice display elevated BP and cardiac hypertrophy compared to WT mice (Murakami et al., 2003).

In addition to the Cav β subunits, the accessory Cav α 2 δ subunits have been shown to modulate channel gating and play an important role in the expression and function of Cav1.2. Genetically, four distinct Cav α 2 δ subunits (α 2 δ 1, α 2 δ 2, α 2 δ 3, α 2 δ 4) have been reported to arise from alternative gene splicing (Angelotti and Hofmann, 1996). Although knowledge related to the expression and

physiological functions of $\text{Ca}_v\alpha_2\delta$ subunits, mainly $\text{Ca}_v\alpha_2\delta-1$ and $\text{Ca}_v\alpha_2\delta-2$, is still limited. The mRNA for $\text{Ca}_v\alpha_2\delta-1$ and $\text{Ca}_v\alpha_2\delta-2$ have been detected in heart, while $\text{Ca}_v\alpha_2\delta-3$ is exclusively expressed in rat atria, and $\text{Ca}_v\alpha_2\delta-4$ is not present in the heart. In rat cerebral arteries, the $\text{Ca}_v\alpha_2\delta-1$ is the primarily isoform expressed, being essential for plasma membrane expression of VSMCs $\text{Ca}_v1.2$ (Bannister et al., 2009). In contrast, in cardiac myocytes, $\alpha_2\delta-1$ knockdown did not alter $\text{Ca}_v1.2$ membrane expression, but significantly reduces $I_{\text{Ca,L}}$ (45% decrease) and inhibited EC coupling (Fuller-Bicer et al., 2009; Tuluc et al., 2007).

Despite the eight distinct $\text{Ca}_v\gamma$ isoforms ($\text{Ca}_v\gamma 1-8$) (Chu et al., 2001), cDNA of four γ subunit isoforms ($\text{Ca}_v\gamma 4, \gamma 6, \gamma 7,$ and $\gamma 8$) has been detected in human heart, although only $\text{Ca}_v\gamma 6$ expression has been confirmed at the protein level in rat heart (Yang et al., 2011). In heterologous expression systems, cardiac $\text{Ca}_v\gamma$ forms a macromolecular complex with whole $\text{Ca}_v1.2$ channel, being its functional effects dependent on the presence of $\text{Ca}_v\beta$ and $\text{Ca}_v\alpha_2\delta$ subunits (Yang et al., 2011). However, it has not been reported whether VSMCs express multiple $\text{Ca}_v\gamma$ subunits and is still unknown whether Ca^{2+} current is affected by the $\text{Ca}_v\gamma$ subunits.

1.4 Rationale and Aim

Compelling evidence indicates that MR antagonists prevent aldosterone-mediated cardiovascular diseases. Thus, taking into account that aberrant Ca^{2+} fluxes through $\text{Ca}_v1.2$ is involved with the deleterious MR actions in cardiac and vascular cells, the precise molecular mechanisms whereby MR modulates $\text{Ca}_v1.2$ expression and function have yet to be identified.

Therefore, the overall and specific aims of this study were:

1. To characterize the molecular mechanisms by which the MR activation regulates $\text{Ca}_v1.2$ expression in cardiomyocytes.
 - a. Identifying whether MR activation changes the expression of the 'cardiac' $\text{Ca}_v1.2$ -LNT and/or the 'vascular/brain' $\text{Ca}_v1.2$ -SNT.
2. To identify targets regions of MR into the genomic DNA on the cardiac *Cacna1c* promoter.
 - a. Characterizing the functional role of GREs in the direct MR regulation of the cardiac *Cacna1c* promoter activation.

- 3.** To examine the effects of vascular MR activation on the expression and activity of $Ca_v1.2$, as well as its repercussion in the vascular contractility.
 - a.** Evaluating whether MR activation changes the expression of the native 'vascular/brain' $Ca_v1.2$ -SNT and/or the 'cardiac' $Ca_v1.2$ -LNT.
 - b.** Assessing whether the upregulation of $Ca_v1.2$ induced by aldosterone changes the responsiveness to DHP antagonist.

Chapter 2 Materials and Methods

All experiments were carried out according to the ethical principles laid down by the French Ministry of Agriculture (authorization 02023.02) and were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals.

2.1 Neonatal Rat Ventricular Myocytes (NRVM) primary cultures

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old Wistar rats, as previously described (Sabourin et al., 2016). Hearts were removed, ventricles were pooled, and cells were dispersed by successive enzymatic digestion with collagenase A (0.4 mg/ml, Roche, Boulogne-Billancourt, France) and pancreatin (0.5 mg/ml, Sigma-Aldrich). Cell suspension was thereafter purified by centrifugation through a discontinuous Percoll gradient to obtain myocardial cell cultures with 99 % myocytes. After seeding on either plastic dishes coated with gelatin (0.2% in PBS, Sigma-Aldrich) or, for confocal microscopy, glass coverslips coated with Poly-D-Lysine (0.1 mg/ml, Sigma-Aldrich) in 30 mm plastic wells, NRVMs were cultured in Dulbecco's Modified Eagle Medium DMEM/medium 199 (4:1) supplemented with 10 % horse serum, 5 % calf serum, 1 % glutamine and antibiotics and placed in 37°C - 5 % CO₂ atmosphere for 20 h. Approximately 95 % of the cells displayed spontaneous contractile activity in culture. Then, cells, transiently transfected if necessary, were cultured in serum free media for 24 h before treatment.

2.2 Cell transfection

NRVMs were transiently transfected in triplicate with plasmids or siRNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The control luciferase reporter pXp1 vector as well as the 2.3 kb 5' flanking region of rat *Cacna1c* cardiac gene inserted into the firefly luciferase reporter pXp1 construct were a kind gift from JD Marsh (Liu et al., 2000). This plasmid construct was used to generate by PCR (GenScript) mutant promoter in which the putative GRE sequences of the *Cacna1c* promoter were replaced by scrambled sequences. The phRL-null Vector (Promega), a vector that lacks eukaryotic promoter and enhancer elements upstream of a synthetic Renilla luciferase gene, was used as a control for transfection efficiency. The hMRA_{5,6}, a construct subcloned in the pCMV6 vector, contains a constitutively active human MR that results from an alternative splicing event, skipping exons 5 and 6, and acts a ligand-independent transactivator (Zennaro et al., 2001). Highly specific MR SMART pool siGENOME Nr3c2 siRNA (M-089538-01-0005) from GE HealthCare Dharmacon were used for knockdown of rat MR and siGENOME Non-Targeting siRNA (D-001210-01-05) were used as negative control. The MR-GFP

plasmid contains sequence encoding fluorescence-enhanced jellyfish GFP fused to the full-length human MR (Deppe et al., 2002). 0.1-1 µg of plasmid or 50 10⁻⁸ M of siRNA and Lipofectamine transfection reagent (twice of the total DNA quantity) were prepared separately in serum-free medium (Opti-MEM). Lipofectamine solution was added drop by drop on DNA solution and the mixture was incubated for 30 min at 37 °C before gently added in each cell culture dish. After 4 h, transfection medium was replaced with serum-free medium. After overnight recovery, cells were incubated with hormones for indicated time. Transient transfections were performed in triplicate wells in NRVMs and repeated n times.

2.3 Western Blot

10 µg for Cav1.2 or 30 µg for MR of total protein extracts from cultured NRVM lysates were subjected to SDS-PAGE (8% gel), then transferred to PVDF membranes, and incubated for 1 h at room temperature in a blocking buffer (5% non-fat milk in Tris-0.1% Tween 20) before overnight incubation at 4°C with antibodies. A rabbit anti-MR 39N (1:1,000) (Viengchareun et al., 2009) or a rabbit anti- Cav1.2 (Millipore, AB10515; 1:1,000) was used. Membranes were incubated with a peroxidase-conjugate goat anti-rabbit IgG second antibody (Santa Cruz, sc-2004; 1:10,000), and proteins visualized with ECL+ MilliporeTM in a ChemiDocXRSBioRad®. β-actin (mouse anti-rat β-actin - Santa Cruz, sc-47778; 1:30,000) or GAPDH (mouse monoclonal anti-GAPDH - Ambion Life Technologies Corp, AM4300; 1:100,000) were used for loading normalization and the results were analyzed with Image J software. For arteries, tissues of 3 to 6 rats were homogenized and lysed using Precellys (Bertin Technologies) according to the manufacturer's instructions and rabbit anti-Cav1.2 were used at 1:200 dilution.

2.4 Quantitative RT-PCR

Total RNA was extracted with TRIzol (Euromedex) according to the manufacturer's instructions and integrity was assayed by gel agarose electrophoresis. First-strand cDNA was synthesized with 1 µg of total RNA using iScriptTM cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions in a total volume of 20 µL. Transcript levels of cDNA were analyzed in duplicate by real-time polymerase chain reaction performed with CFX96 RT-PCR system (Bio-Rad) using SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad) that contained 500 10⁻⁹ M of specific primers (Eurofins MWG Operon, Table 1). Serial dilutions of pooled cDNA were used in each experiment to assess the efficiency of the PCR. Gene expression was quantified relative to the geometric means of housekeeping genes (Table 1) expression amplified in the same tube of

investigated genes and the $\Delta\Delta C_t$ method was used to determine the expression of genes of interest. For real-time RT-PCR analysis of mRNA decay, NRVMs were treated for 24 h with or without 10^{-6} M aldosterone and then with 5 $\mu\text{g/L}$ actinomycin D for different times. Decay of *Cacna1c* transcript is compared to cells before actinomycin D treatment in each condition (t_0). The relative quantities of exon 1 isoforms in coronary artery over the supposed common invariant region spanning exons 14 and 15 were estimated according to Vidal-Petiot et al. {Vidal-Petiot, 2012 #255} The calculated correction factors were 0.2 for exon 1a and 0.4 for exon 1b. For miRNA qRT-PCR, cDNA from total mRNA was generated with the miScript II RT Kit (QIAGEN, France) and the qRT-PCR was done by using the miScript SYBR Green PCR Kit (QIAGEN) following the manufacturer's instructions.

Table 1. List of primer sequences

Target	Sequence
<i>Cacna1c</i> (spanning exon 14- exon 15)	fwd 5'-CATCATCATCTTCTCCCTCCTG-3' rev 5'-CATCACCGAATTCCAGTCCT-3'
<i>Cacnb2</i>	fwd 5'-GCTCAACTCAGTAAGACCTC-3' rev 5'-CCTTTGTAAAACCTGTAAAA-3'
<i>Cacna2d2</i>	fwd 5'-GCGTCCTATAACGCCATCAT-3' rev 5'-GTCTCCTTTTGTAGCAGCCG-3'
<i>Cacna1c</i> exon 1a	fwd 5'-TGTCCTCAGACAGGCAACTG-3' rev 5'-CGAGCACATCCCTACTCCA-3'
<i>Cacna1c</i> exon 1b	fwd 5'-GCCAAAAACTGCTTGGAAG-3' rev 5'-CGAGCACATCCCTACTCCA-3'
<i>Cacna1c</i> exon 8a	fwd 5'-ACGCTATGGGCTATGAGTTGCC-3' rev 5'-GGCTTTCTCCCTCTCTTTG-3'
<i>Cacna1c</i> exon 8	fwd 5'-TTCGACAACTTCGCCTTCGC-3' rev 5'-TCCCTTCCTACGGCATCATT-3'
miR-155	UUAAUGC UAAUUGUGAUAGGGGU
<i>Housekeeping genes</i> YWHaz 14-3-3 Protein zeta/delta	fwd 5'-AGACGGAAGGTGCTGAGAAA-3' rev 5'-GAAGCATTGGGGATCAAGAA-3'
TATA box Binding Protein	fwd 5'-AAAGACCATTGCACTTCGTG-3' rev 5'-GCTCCTGTGCACACCATTTT-3'
Ribosomal Protein L 32	fwd 5'-GCTGCTGATGTGCAACAAA-3' rev 5'-GGGATTGGTGA CTCTGATGG-3'
Smooth muscle cell 22 alpha	fwd 5'-GTTTGGCCGTGACCAAGAAC-3' rev 5'-GAAGGCCAATGACGTGCTTC-3'

PCa.luc genotype	fwd 5'-ATTGGGGAAGGGTTTGTCTC-3' rev 5'-AGGAACCAGGGCGTATCTCT-3'
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2.5 Luciferase Assays

NRVMs were plated in 24-well plates at 5×10^5 cells/well in growth medium. The next day, cells were transfected with 1 μg /well of plasmid constructs (1 μg /well of pXp1 constructs or 0.5 μg /well of hMR $\Delta_{5,6}$ constructs plus 0.5 μg /well of control pXp1 vector) and 0.1 μg /well of the phRL-null Vector. After overnight recovery and eventually 24 h treatment with drugs, cell lysates were prepared and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Activity tests were performed and luminescence measured using a MiniLumat luminometer (Berthold). The luciferase activities were expressed as the relative Firefly/Renilla activities, normalized to those of control transfections in each case.

2.6 Promoter analysis

Putative glucocorticoid responsive element (GRE) sites within the regulatory regions of *Cacna1c* genes from rat (Chromosome 4, NC_005103 Region: 217181007..217191006), mouse (Chromosome 6 NC_000072 Region: 119192407..119202406) and human (Chromosome 12 NC_000012 Region: 2798392..2808391) were analyzed by MatInspector software algorithms (<https://www.genomatix.de/>). The core similarity was set to 75%, while matrix similarity was set to ≥ 0.85 (a perfect match to the matrix gets a score of 1).

2.7 Mutagenesis

Mutagenesis reactions were carried out by GenScript (Piscataway, NJ, USA) and confirmed by sequencing of the entire insert.

2.8 Immunostaining and fluorescence microscopy

4×10^5 NRVMs were plated in 35 mm Petri dishes coated with poly-D-lysine and cultivated for 20 h. After 24 h serum starvation, cells were incubated 30, 60 and 90 min with or without aldosterone. Cells were quickly rinsed with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min. Paraformaldehyde was then neutralized with NH_4Cl for 15 min before cells were permeabilized with 0.5% Triton X-100 for 5 min, washed three times with PBS, rinsed with PBS 5% BSA for 40 min, then incubated overnight at 4°C with anti-MR 39N polyclonal antibody (4 $\mu\text{g}/\text{ml}$) in PBS-0.25%-

Triton-10% goat serum (Santa Cruz), followed by washes and incubation with Alexa Fluor[®] 633 goat anti-rabbit IgG (H+L) (Molecular Probes, A-21071; 1:1,000) for 1 h at room temperature. Cells were next rinsed with PBS, incubated with 5 µg/ml Hoechst 33342 (Sigma) for 5 min, washed again with PBS and mounted with 15 µl Mowiol[®] 4-88 (Calbiochem). Cells were examined with a confocal microscope (Leica TCS-SP8 gated STED) equipped with a HC PL APO CS2 63x/1.40 oil-immersion objective. Alexa Fluor 633 was excited at 633 nm with a White Light Laser, and emission measured at 640-800 nm with a hybrid detector. Hoechst 33342 was excited by a 405 nm diode and emission measured at 420-460 nm. The pinhole was set at one Airy Unit. Co-localization maps were generated with LAF AS Lite software. Quantitative measurements of MR-nuclear translocation were performed using ImageJ software as previously described (Noursadeghi et al., 2008).

Life imaging of the hMR-GFP was performed at 37°C in DMEM without phenol red 48 h after transfection. GFP fluorescence was excited at 488 nm and detected with a 510–615 nm bandpass hybrid detector Leica HyD.

2.9 PCa.luc transgenic mice

The 4939 bp-linearized BamH1-Oli1 fragment of the pXp1 constructs (Liu et al., 2000) containing 2.3 Kb rat *Cacna1c* P1-promoter and full-length luciferase coding sequence was purified using Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, 28-9034-08). Transgenic mice were obtained after pronuclear injection into fertilized eggs by the SEAT laboratory (Service d'Experimentation Animale et de Trangénèse, Villejuif, France). We obtained 5 founders on a B6/CBA background, which were backcrossed onto a C57BL/6 outbreed strain for at least 5 generations, and 2 on a C57BL/6 background. Genomic DNA was extracted from the tail tip and used for genotyping using specific primers (Table 1) spanning the 293 bp region covering the end of rat *Cacna1c* P1-promoter and the beginning of the luciferase gene. Three-independent mouse strains, with the integrated transgene copy number varying between 4 and 20, were used for analysis (from 3 to 6-month old). These animals were viable and fertile, and no overmorbidity or major abnormality was noted.

Adult ventricular myocyte, isolated as previously described (Leroy et al., 2011), were plated on laminin (10 µg/ml, Millipore) at 5×10^4 cells/well in Tyrode's solution (in 10^{-3} M: NaCl 113, KCl 4.7, CaCl₂ 1, MgSO₄ 1.2, KH₂PO₄ 0.6, NaH₂PO₄ 0.6, NaHCO₃ 1.6, HEPES 10, Taurine 30, D-glucose 20, adjusted to pH 7.4) and kept 24 h in 37°C-5 % CO₂ atmosphere with or without aldosterone. Cell lysis was performed as for NRVMs, protein content was determined by BCA protein assay and luminescence measured using EnVision Xcite (Perkin Elmer). Luciferase activities were normalized to relative light units per microgram of total proteins in the lysate. For aorta, after explantation and

culture as described below, the tissue was homogenized and lysed using Precellys (Bertin Technologies) according to the manufacturer's instructions.

2.10 Artery harvest and organ culture

Male Wistar rats (250–320 g; 8–12 wks old) were anesthetized with sodium pentobarbital (100 mg.kg⁻¹, i.p.) and hearts were immediately removed and immersed into ice-cold physiological salt solution (PSS in 10⁻³ M, 118 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.18 MgSO₄, 2.5 CaCl₂, 25 NaHCO₃, 10 Glucose; pH 7.4). The left anterior descending coronary arteries (LADCA) were gently dissected from the myocardium free of adjacent and connective tissues. The aorta and the 6-7 order mesenteric arteries were explanted, cleaned of fat and connective tissue and placed in PSS. Then, arteries with intact or denuded endothelium (by gentle lumen rubbing) were transferred to free-serum DMEM supplemented with 0.5 U.mL⁻¹ penicillin-streptomycin. Vessels were cultured in a humidified atmosphere of 5% CO₂ at 37°C for 24 h in the presence or absence of aldosterone (10⁻⁸ M).

2.11 Vascular reactivity

After culture, the third or fourth order of LADCAs were cut into 1.5-2.0-mm long rings and then, mounted in a wire myograph (Danysh MyoTechnology) containing warmed (37°C) and aerated (95% O₂, 5% CO₂) PSS solution for isometric tension recordings using PowerLab software (AD Instruments). Rings were subjected to a transmural pressure equivalent to 100 mmHg and allowed to stabilize at resting tension for 45 min before experimentation. To evaluate the vessel viability and to achieve the maximal contractile response, LADCA rings were challenged with 60 10⁻³ M KCl-containing solution (equimolar substitution with NaCl to retain constant ionic strength) twice before concentration-contractile response curve with depolarizing solution (KCl, 10 to 60 10⁻³ M). Contraction in response to BayK 8644 (10⁻⁷ M, 10⁻⁶ M and 5 10⁻⁶ M) was also assessed. Functional removal of the endothelium was verified if the relaxant response of acetylcholine (ACh, 10⁻⁵ M) was absent. Concentration-response vasoconstriction and vasorelaxation data are shown as percentage of maximal developed tension triggered by high-K⁺ solution (60 10⁻³ M).

2.12 Rat coronary artery SMC isolation and electrophysiological recordings

Coronary artery SMCs were enzymatically isolated from full branches of LADCAs incubated 24 h with or without 10⁻⁸ M aldosterone using a previously standardized method (Rueda et al., 2013). Briefly, arteries were incubated in dissociation solution containing (in 10⁻³ M): 55 NaCl, 6 KCl, 80

Na⁺-glutamate, 2 MgCl₂, 10 glucose and 10 HEPES, pH 7.4, supplemented with 1.5 mg/ml papain, 1 mg/ml BSA, and 1 mg/ml dithiothreitol for 15 min at 37°C. Then, the tissue was transferred to a collagenase solution (mixture of collagenase type F and H, 7:3, Sigma) plus 100 10⁻⁶ M CaCl₂ and incubated for further 8 min at 37°C. Enzymatic digestion was stopped by repeated washing the tissue with dissociation solution. Digested tissue was triturated with a fire-polished glass Pasteur pipette to yield single VSMCs. Myocytes were kept at 4°C and used within the same day.

Patch-clamp recordings were performed in the whole-cell configuration using an Axopatch 1D amplifier (Axon Instruments). Currents were filtered at 1 kHz, digitized and sampled at 10 kHz using pClamp 8 software (Axon Instruments). VSMCs were superfused with physiological saline solution containing (in 10⁻³ M): 122 NaCl, 1.1 MgCl₂, 20 CaCl₂ or BaCl₂, 4 CsCl, 10 HEPES, 10 Glucose, pH 7.4 with LiOH. 2.5–3 MΩ resistance pipettes were filled with internal solution containing (in 10⁻³ M): 135 CsCl, 4 MgCl₂, 5 EGTA, 10 HEPES, 10 Glucose, 5 Na₂-ATP, 3 Na₂-CP, pH 7.2 with LiOH. Cells were clamped at holding potential of -60 mV and whole cell currents were evoked by 300-ms depolarization steps from -70 to +60 mV with +10 mV of increments followed by single step to +10 mV. Current values were normalized to cell capacitance and expressed as current density (pA/pF). The voltage-dependent availability (activation and inactivation) and the kinetics of the current were analyzed as previously described (Martin-Fernandez et al., 2009).

2.13 Aldosterone-salt model

Littermates PCa.luc male mice were randomly divided into 2 groups: in the control group (cont), mice received each day subcutaneous vehicle injection (0.5 μl/g injected, 0.9% NaCl, 1% DMSO) for 3 weeks. In aldosterone group (Aldo-salt), mice were treated for 3 weeks with intraperitoneal injection of aldosterone (1 mg/kg per day) dissolved in ethanol (0.9% NaCl, 1% DMSO) and received 1% NaCl as drinking water. The model and the dose of aldosterone were chosen from previous studies in which the treatment increased BP (Calvier et al., 2015). Tail cuff BP (CODA™, Kent Scientific) was monitored throughout the treatment. Transthoracic echocardiography was performed using a 15 MHz transducer (Vivid 9, General Electric Healthcare, GE Medical Systems) under 2% isoflurane gas anesthesia. Two-dimensional (2D)-guided M-mode echocardiography was used to determine wall thickness and left ventricular chamber volume at systole and diastole and contractile parameters, such as fractional shortening (FS) or ejection fraction (EF). Images were acquired and analyzed by an operator blinded to mouse treatment

2.14 Data analysis

Concentration-dependent effects were fitted (Origin® software) using Hill equation: $100+(E_{\max}-100)([X]^{n_H}/(EC_{50}^{n_H} + [X]^{n_H}))$, where E_{\max} is the maximal effect, $[X]$ is the used drug concentration, EC_{50} is the median effective concentration, and n_H the Hill coefficient. In the case of luciferase activities (Figures 15C and 15D), only experiments with full concentration range were individually analyzed and the presented values are the average of each individual fit. For nifedipine dose-response curves (Figure 16F), E_{\max} is fixed at 0.

All the data are presented as mean \pm s.e.m of n independent experiments. Statistic significance was analyzed using one-way ANOVA followed by post hoc Fisher LSD test (Origin® software). A P value of 0.05 was considered as statistically significant.

Chapter 3 Results

3.1 MR mediates aldosterone-induced expression of Ca_v1.2

In primary cultures of neonatal rat ventricular myocytes (NRVMs), aldosterone exposure for 24 h induced a dose-dependent increase in Ca_v1.2 expression at both protein (Figure 11A) and mRNA (Figure 11B) levels, with as little as 10⁻⁹ M aldosterone required to significantly activate expression, reaching maximum at supraphysiologic concentrations. The two main cardiac auxiliary subunits, the intracellular Ca_vβ2 subunit and the dimer Ca_vα2δ2 subunit, which modulate Ca_v1.2 gating, trafficking, and response to neurohormonal stimuli (Benitah et al., 2010), also displayed a coordinated increase of their mRNA levels (Figure 11C and D), emphasizing that the expression of all the LTCC subunits is under coordinated transcriptional control (Fan et al., 2000).

Aldosterone, a steroid hormone, freely diffuses from the source and crosses plasma membrane of a target cell to primarily interact with and activate MR. Aldosterone intracellular binding to MR triggers an allosteric change enabling the hormone-receptor complex to migrate into the nucleus and regulate target gene transcription (Pascual-Le Tallec and Lombès, 2005; Viengchareun et al., 2007). To determine whether MR could mediate aldosterone actions on Ca_v1.2 expression, we first examined cardiac MR cytonucleoplasmic trafficking upon aldosterone treatment on NRVMs. We observed that immunoreactive MR was evenly distributed throughout the NRVMs in the absence of hormone (Figure 11E top panels). Upon addition of 10⁻⁹ M aldosterone for 60 min, MR concentrated into the nuclei (Figure 11E bottom panels). The time-dependent increase in MR nuclear/cytoplasmic ratio was evident across the aldosterone dose range (Figure 11F). To dynamically validate the aldosterone-dependent MR subcellular trafficking on living cells, we transfected NRVMs with GFP-human MR chimeric construct (Deppe et al., 2002). Two days after transfection, while GFP fluorescence was diffuse in the cytoplasm under basal conditions, aldosterone exposure (10⁻⁹ M) induced a rapid nuclear MR translocation as early as after 5 min and was fully achieved after 1 h exposure (Figure 11G, top panels). The synthetic glucocorticoid (GC) agonist, dexamethasone, even at higher dose did not alter GFP-hMR cellular distribution (Figure 11G bottom panels). Moreover, aldosterone-upregulation of Ca_v1.2 expression at the mRNA and protein levels was prevented by RU28318 (Figure 11H and 11I), a selective MR antagonist (Kim et al., 1998), which did not modify NRVMs Ca_v1.2 expression by itself after 24-h treatment. Collectively, these results clearly demonstrated that aldosterone-activated MR mediates Ca_v1.2-LNT transactivation.

Increased mRNA levels may be assigned to stimulation of gene transcription and/or enhanced mRNA stability. On NRVMs incubated 24 h, upon exposure to the transcription inhibitor, actinomycin D (5 μg/ml, Figure 11J), Ca_v1.2 mRNA levels rapidly declined with a half-life of ~5h ($t_{1/2} = 5.0 \pm 0.7$ h, n=6), which was remarkably similar to that measured after aldosterone treatment ($t_{1/2} = 4.8 \pm 0.8$ h, n=6). This result demonstrates an aldosterone-dependent transcriptional activation accounting for enhanced Ca_v1.2 mRNA and proteins levels.

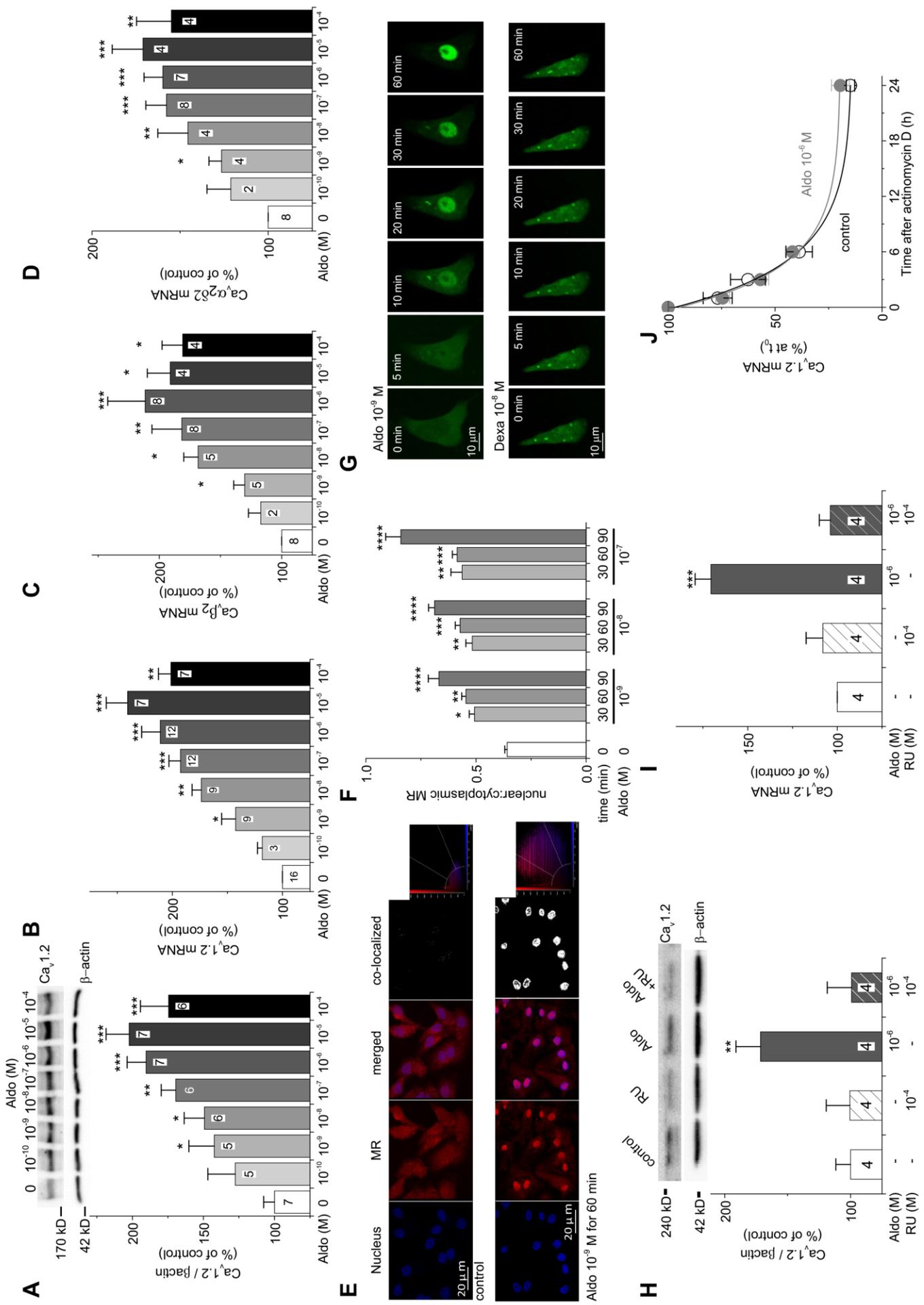


Figure 11. Aldosterone-activated MR mediates cardiac *Cacna1c* transactivation. **A**, Dose-dependent effects of 24-h aldosterone (Aldo) treatment of NRVMs on $Ca_v1.2$ expression levels. The top panel illustrates a typical western blot image of $Ca_v1.2$ and β -actin protein detections. The relative protein signals were quantified by densitometry using ImageJ software and the relative expression of $Ca_v1.2$ to β -actin are expressed as the % of the value in untreated cells for each n independent experiment. **B, C and D**, Dose-dependent effects of 24-h aldosterone treatment on relative mRNA levels of $Ca_v1.2$ (**B**), $Ca_v\beta 2$ (**C**) and $Ca_v\alpha 2\delta 2$ (**D**) determined by quantitative RT-PCR (RT-qPCR), normalized to geometric average of relative quantities for housekeeping genes and expressed as the ratio of the control group for each independent experiment (performed in triplicate). **E**, Representative immunofluorescence images after vehicle (control) or aldosterone exposure showing cells simultaneously immunostained with Hoechst DNA labels nuclei (blue) and an anti-MR antibody (red). Merged images are shown as well as co-localization overlays processed by the LAS AF software (Leica) after background correction and thresholds of the individual channel each set to 50%, meaning that any pixel lower than these percentages on the threshold scale is not considered co-localized. **F**, Quantification of nuclear/cytoplasmic ratios of MR staining in time course studies of MR nuclear translocation in 0 (control), 10^{-9} , 10^{-8} and 10^{-7} M aldosterone stimulated NRVMs. Analysis of 10 to 20 separate field images as shown in **C** for each group from 3 to 5 NRVM cultures. **G**, Representative time-lapse confocal fluorescence images of NRVMs transfected with GFP-hMR before and after treatment with 10^{-9} M aldosterone (top panels) or with 10^{-8} M dexamethasone (bottom panels). **H and I**, Effects of 24-h co-treatment with aldosterone and RU28318 (RU), a selective MR antagonist, on the $Ca_v1.2$ mRNA (**H**) and protein (**I**) expression. **J**, $Ca_v1.2$ mRNA levels were measured by RT-qPCR at the indicated times after actinomycin D application, presented as percent of transcript levels relative to time-point zero of each condition. Statistical significance was calculated by one-way ANOVA. The no-treatment condition was compared with each aldosterone condition using a Fisher LSD's comparison test: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ and **** $P < 0.00005$.

3.2 Aldosterone increases $Ca_v1.2$ -LNT expression through MR-dependent activation of the 'cardiac' *Cacna1c* P1-promoter

The $Ca_v1.2$ subunit is expressed as two distinct tissue-specific transcripts, which encode for the $Ca_v1.2$ -LNT and $Ca_v1.2$ -SNT isoforms, regulated by two alternative promoters (P1 and P2), located upstream of the mutually exclusive first exons (Figure 12A inset); the exon 1a being predominantly expressed in the heart and the exon 1b in smooth muscle cells and the brain (Hofmann et al., 2014). Twenty-four hours treatment of NRVMs with aldosterone induced a dose-dependent increase in exon 1a transcript levels, whereas exon 1b transcript levels were unaffected (Figure 12A). The 10^{-6} M aldosterone-induced increase of exon 1a expression was prevented by RU28318 (Figure 12B).

To investigate how aldosterone controls exon 1a transcription through MR, NRVMs were transiently transfected with a reporter gene plasmid containing the rat *Cacna1c* P1-promoter driving the firefly luciferase gene (Liu et al., 2000), and then were exposed to increasing aldosterone

concentrations for variable periods of time. Aldosterone enhanced the luciferase activity in a concentration- and time-dependent manner (Figures 12C and 12D). Remarkably, the dose-dependent changes in luciferase activity were superimposable (Figure 12C) to those of protein and mRNA levels (Figures 11A, 11B and Fig. 12A). In addition, the time-course showed that the aldosterone responses were rapid and sustained with similar rate at all concentrations used (Figure 12D). The MR-specificity of aldosterone-induced enhancement of rat *Cacna1c* P1-promoter activity was blunted by co-incubation with RU28318 at each concentration used (Figure 12E). Moreover, MR siRNA strategy, which completely knockdowns MR expression at the protein level (Figure 12F inset), prevented aldosterone-dependent increase of P1-promoter-driven luciferase activity at every aldosterone concentration tested (Figure 12F). On the other hand, co-transfection of NRVMs with a natural constitutively active splice variant of the human MR, hMR $\Delta_{5,6}$, which lacks the entire receptor hinge region and the ligand-binding domain of the MR (Zennaro et al., 2001), increased luciferase activity of the rat *Cacna1c* P1-promoter (see Figure 12J open bars).

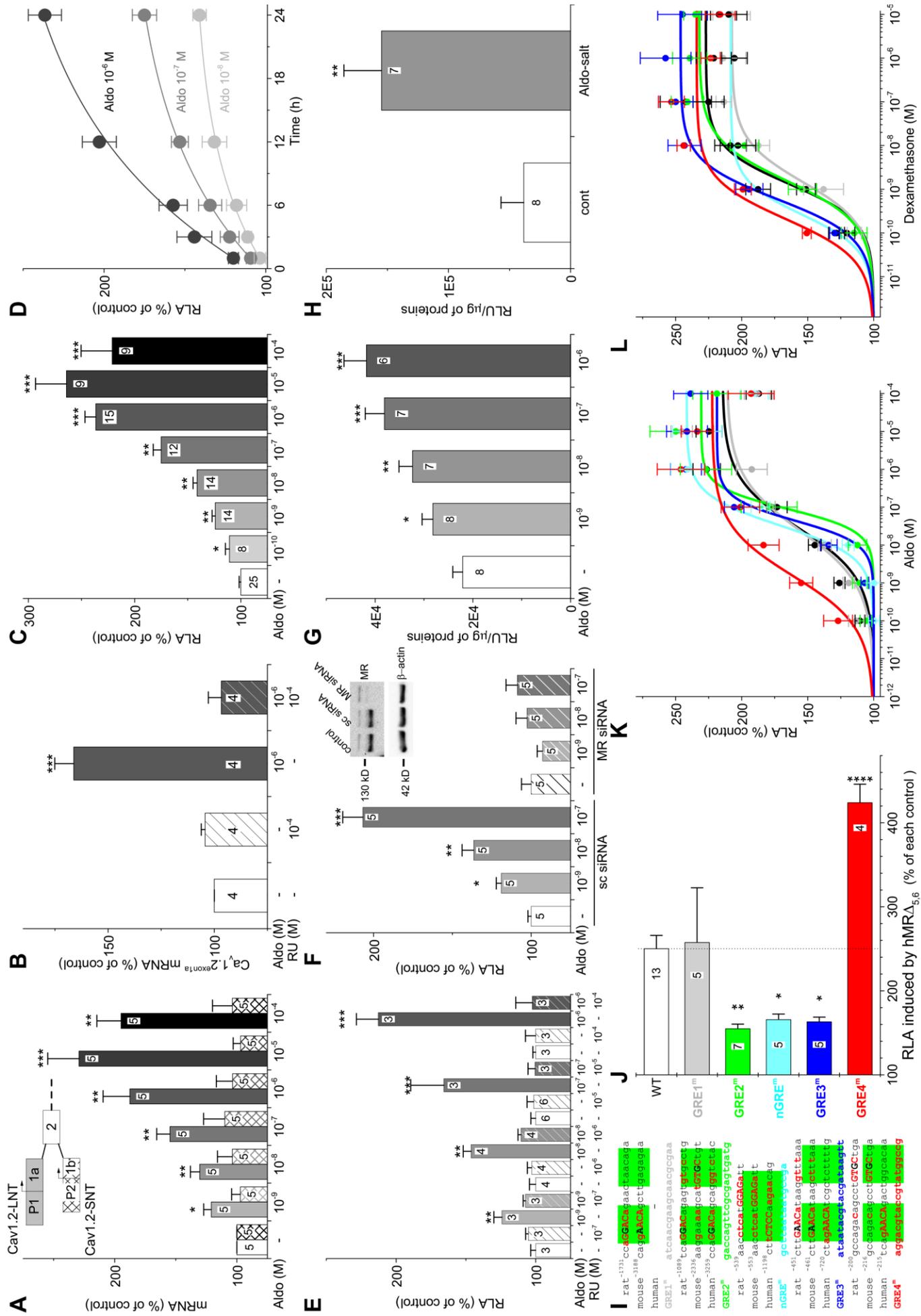


Figure 12. Aldosterone induces expression of the cardiac $Ca_v1.2$ through transactivation of cardiac *Cacna1c* P1-promoter. **A**, Dose-dependent effects of aldosterone (Aldo) in NRVMs on relative mRNA expression of the mutually exclusive exons 1a and 1b, which were transcriptionally regulated by two alternative promoters (P1 and P2) as illustrated in the inset. **B**, Effects of aldosterone and RU28318 co-treatment on the NRVM $Ca_v1.2$ exon 1a mRNA expression. **C and D**, Dose- and time-dependent effects of aldosterone (at indicated concentrations) on the rat *Cacna1c* P1-promoter activity as measured by luciferase assays 30 performed in triplicate. The relative luciferase activity (RLA) is expressed as the ratio of Firefly/Renilla activities, normalized to those of untreated NRVMs in each experiment (in **D**, n=7 for each condition). **E**, Effects of RU28318 (RU) on the aldosterone-mediated transcriptional activities of rat *Cacna1c* P1-promoter. **F**, Relative transcriptional activity of *Cacna1c* P1-promoter in NRVMs transfected with either MR siRNA or non-targeting siRNA (sc siRNA) subjected to 24-h aldosterone treatment. The inset illustrates the effective knockout of MR protein after MR siRNA treatment compared to control or sc siRNA transfected cells. **G**, Dose-dependent effects of 24-h aldosterone treatment on luciferase activities, expressed as relative light units (RLU) per μ g of protein, in isolated ventricular myocytes from transgenic mice expressing whole body luciferase reporter gene under the control of the rat P1-promoter sequence (PCa.luc). **H**, Heart luciferase activities, expressed as relative light units (RLU) per μ g of proteins, from aldosterone-salt-treated (Aldo-salt, 1 mg/kg per day + 1% NaCl) PCa.luc mice compared to vehicle-treated littermates (cont). **I**, Genomic sequences for the putative GRE sites in rat *Cacna1c* P1-promoter are indicated as well as their corresponding sites in the murine and human orthologues. The site locations are indicated relative to the transcription start site of exon 1a. Nucleotides in capital letters denote the core sequence and marked in red the ones with a consensus index vector value >60. Green boxes represent the GRE typical imperfect hexameric dyad symmetry. Mutated sequences of the corresponding GRE are also presented. **J**, Transcriptional activities were determined by luciferase reporter assays in NRVMs cotransfected with a constitutively active human MR, hMR Δ 5,6, subcloned into the pCMV6 vector. Luciferase activities are given relative to the value of 100 assigned to that of the promoter in cells transfected with a mock plasmid after correcting for transfection efficiency. **K and L**, Dose response effects of aldosterone (**K**) and dexamethasone (**L**) on WT and mutated *Cacna1c* P1- promoter activities after 24-h treatment. All the experiments were conducted after 24 h treatment. Data show mean \pm s.e.m of n independent experiments performed in triplicate for luciferase assays and RT-qPCR. Statistical significance was calculated by one-way ANOVA with post-hoc Fisher LSD test to compare with respective untreated conditions: *P < 0.05, **P < 0.005 and ***P < 0.0005.

To further explore the aldosterone-dependent upregulation of $Ca_v1.2$ -LNT in adulthood, we generated a transgenic mouse line harboring the luciferase reporter gene under the control of the rat P1-promoter sequence (PCa.luc). Similarly to NRVMs (Figure 12C), adult ventricular cardiomyocytes isolated from PCa.luc mice showed a dose-dependent increase of luciferase activities upon 24-h aldosterone treatment (Figure 12G). Next, we examined the *in vivo* effect of chronic aldosterone infusion. After 3 weeks aldosterone-treatment with high-salt diet, isolated hearts of PCa.luc mice exhibited increased luciferase activity (Figure 12H) associated with enhanced ventricular function in absence of structural abnormalities (Table 2) compared to vehicle-treated PCa.luc littermate mice. Collectively, these results clearly demonstrated that aldosterone-activated MR mediates $Ca_v1.2$ -LNT transactivation *in vivo*.

Table 2. Characteristics of aldosterone-salt-treated PCa.luc mice. BW, HW, LW and LVW: body, heart, lung and left ventricle weights, respectively; TL: tibia length; DBP, SBP and MBP: diastolic, systolic and mean blood pressures, respectively; HR, heart rate. FS, fractional shortening; EF, ejection fraction; EDV and ESV, end-diastolic and -systolic volumes; LV interventricular septal thicknesses (IVS), LV internal dimensions (LVID) and posterior wall thicknesses (PW) at diastole (d) and systole (s); SV, stroke volume. Statistical significance was calculated by ANOVA, *P<0.05 and **P<0.005 vs control.

	Control (n=8)	Aldosterone-salt (n=7)
<i>Anatomical parameters</i>		
BW (g)	33.3±1.5	31.5±1.3
HW (mg)	210.0±17.4	181.4±12.2
HW/BW (mg/g)	6.2±0.6	5.4±0.2
TL (cm)	1.86±0.01	1.84±0.01
HW/TL (mg/cm)	112.2±9.3	98.5±6.5
LW (mg)	238.6±37.7	215.7±28.2
<i>Tail-cuff BP parameters</i>		
DBP (mmHg)	96.2±2.4	110.1±2.9 **
SBP (mmHg)	124.1±2.4	139.8±2.4 ***
MBP (mmHg)	105.2±2.4	119.7±2.7 **
HR (beats/min)	657.0±13.1	627.2±32.6
<i>Echocardiographic parameters</i>		
FS (%)	30.4±7.5	36.7±8.3 ***
EF (%)	64.7±1.0	73.1±1.0 ***
EDV (ml)	0.15±0.01	0.13±0.005
ESV (ml)	0.054±0.005	0.035±0.002 **
IVSd (mm)	0.86±0.08	0.87±0.04
IVSs (mm)	1.38±0.10	1.37±0.05
LVIDd (mm)	3.88±0.14	3.7±0.05
LVIDs (mm)	2.69±0.10	2.44±0.08
LVPWd (mm)	0.88±0.08	0.99±0.05
LVPWs (mm)	1.27±0.16	1.50±0.08
SV (ml)	0.097±0.008	0.096±0.004
LVW (mg)	126.8±7.8	133.6±10.8
HR (beat/min)	472.9±21.9	471.2±11.7

Like other nuclear receptors, MR is a ligand-regulated transcription factor, which controls target gene transcription by its ability, *via* the DNA-Binding Domain (DBD), to recognize specific target DNA sequences (hormone response elements) or alternatively by acting indirectly through tethering to other DNA-bound transcription factors, without contacting DNA itself (*cis*-regulatory modules, CRMs). The MR DBD has 94% homology with the glucocorticoid receptor (GR), and thus it is generally accepted that MR and GR share similar mechanisms of transactivation including the glucocorticoid response elements (GRE) (Pascual-Le Tallec and Lombès, 2005). *In silico* analysis using the Genomatix “MatInspector” tool identified 4 putative GREs with typical imperfect hexameric dyad symmetry (Meijsing et al., 2009) and a "negative" GRE (Surjit et al., 2011) (nGRE) within the 2 kb upstream region of the transcription start site on the rat exon 1a of *Cacna1c* thus overlapping the P1-promoter sequence used herein (Figure 12I). Over the Genomatix library, no consensus GRE-related CRMs were detected. Matching GREs in the same sequential order were found in the upstream region of *Cacna1c* orthologues from mouse and human, except for GRE-1 that was not found in the human sequence (Figure 12I). To assess the functional importance of these sites

in the rat *Cacna1c* P1-promoter region, each putative GRE was substituted by a scrambled sequence in the luciferase reporter vector (Figure 12I). Mutated sequences were checked with Genomatix software to confirm disruption of the consensus sites. The GRE wild-type (WT) or mutated constructs together with the hMRA_{5,6} encoding plasmid were then transiently co-transfected into NRVMs. As shown in Figure 12J, the luciferase activity with the WT P1-promoter increased by 2.5-fold in the presence of hMRA_{5,6}. Mutation of the most distal GRE-1 did not alter responses elicited by hMRA_{5,6}. However, the GRE-2, -3 and nGRE mutated promoters showed reduced responses to hMRA_{5,6} compared to WT, albeit hMRA_{5,6} still significantly increased the luciferase activity compared to NRVMs transfected with mutated constructs in absence of hMRA_{5,6} (Figure 12J). In sharp contrast, mutation of the most proximal GRE-4 increased responses to hMRA_{5,6} by more than 400%, 1.7-fold higher than WT P1-promoter driven activity (Figure 12J). These observations were corroborated by aldosterone dose-responses generated with endogenous MR in NRVMs (Figure 12K). Compared to WT, GRE-1 mutant did not modify aldosterone responses. GRE-2 mutant profoundly affected aldosterone dose-responses: the luciferase activities of this mutant were not increased by low doses of aldosterone (10^{-9} to 10^{-8} M) whereas at higher concentrations the increased P1-promoter activities were maintained compared to WT promoter; This leads to a 3-fold increase in the median effective aldosterone concentration (EC₅₀) associated with a 2-fold increase in Hill coefficient (n_H, Table 3) suggesting modification of both the affinity and the extent of cooperativity among multiple binding sites. The increased activation threshold was also observed for mutants GRE-3 and nGRE (Figure 12K) leading to increased n_H values without significant alteration of EC₅₀ (Table 3). With respect to mutated GRE-4 promoter (Figure 12K), a drastic reduction of the EC₅₀ was observed with no modification of the n_H (Table 3), consistent with an increase in aldosterone sensitivity. For comparison, the mutant P1-promoter responses to dexamethasone were definitively different from those to aldosterone (Figure 12L and Table 3), while mutation of the GRE-1 and -2 did not modify dexamethasone sensitivity, mutation of either GRE-3, -4 or nGRE decreased the dexamethasone EC₅₀ without alteration of n_H. These reporter assays delineated specific aldosterone-dependent, MR-mediated activation of the P1-promoter to GRE-2, -3 and nGRE and repression to GRE-4.

Table 3. Estimated values (95% confidence interval) of the median effective concentration (EC_{50}), maximal effect (E_{max}) and Hill coefficient (n_H) for dose-responses of aldosterone and dexamethasone on WT and mutated P1-promoter activities. Statistical significance was calculated by ANOVA with post-hoc Fisher LSD test, * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$ vs WT promoter.

	Aldosterone			Dexamethasone		
	EC_{50} (10^{-9} M)	E_{max} (% of control)	n_H	EC_{50} (10^{-9} M)	E_{max} (% of control)	n_H
WT	28.6±8.5	214.6±8.1 (n=8)	0.6±0.1	1.3±0.1	227.1±13.6 (n=6)	1.0±0.1
GRE-1 ^{mut}	23.9±1.0	201.6±5.9 (n=3)	0.6±0.1	1.5±0.3	207.5±4.5 (n=3)	0.9±0.1
GRE-2 ^{mut}	90.0±2.3***	240.6±10.9 (n=6)	1.5±0.1*	1.5±0.1	232.1±12.1 (n=4)	0.9±0.1
nGRE ^{mut}	32.8±6.8	241.6±10.8 (n=5)	1.1±0.2*	0.2±0.04***	208.2±7.5 (n=4)	1.1±0.1
GRE-3 ^{mut}	43.9±5.2	218.7±10.2 (n=5)	1.5±0.2*	0.6±0.1**	246.3±17.7 (n=5)	1.0±0.1
GRE-4 ^{mut}	1.5±0.5**	222.4±10.4 (n=5)	0.6±0.1	0.2±0.03***	234.1±4.8 (n=4)	0.9±0.1

3.3 Aldosterone induced a MR-dependent 'cardiac' *Cacna1c* P1-promoter switch in vascular cells

Several studies have observed effects of aldosterone on VSMCs $Ca_v1.2$, but the mechanisms are unclear. Notably, VSMC-MR directly participates to the age-associated rise in BP, in part, by regulating the expression and activity of resistance vessel $Ca_v1.2$ (McCurley et al., 2012). We examined whether *ex vivo* aldosterone treatment modulates $Ca_v1.2$ expression in different rat vascular beds: left anterior descending coronary arteries (LADCA), aorta and mesenteric arteries. For all these vascular beds, 24-h exposition to 10^{-8} M aldosterone, roughly corresponding to the aldosterone EC_{50} in cardiomyocytes (Figures 11 & 12), $Ca_v1.2$ protein levels were increased by about 3-fold compared to 24-h cultured vessels in the absence of aldosterone (Figure 13A). This effect was associated with a MR-dependent increased expression of exon 1a mRNA, whereas exon 1b expression, the main transcript expressed in VSMC (Hofmann et al., 2014), was unaffected (Figure 13B). In LADCA, we estimated that exon 1a was expressed at low levels in control conditions (4% of total $Ca_v1.2$ transcript, 21 times lower than exon 1b). Upon aldosterone treatment, exon 1a expression reached 18% of that of the total $Ca_v1.2$ transcript, even though it remained 7 times lower than exon 1b. In line with these results, luciferase assays of 24-h cultured aorta from P $Ca_{1.2}$ mice showed barely detectable P1-promoter activities in control conditions, whereas luciferase activities dramatically increased in a dose-dependent manner after 24-h aldosterone treatment (Figure 13C) without reaching the levels observed in cardiomyocytes (see Figure 12G). This effect was prevented by co-treatment with RU (Figure 13C). On the other hand, the microRNA-155 (miR-155) expression, which has been involved

in MR regulated $Ca_v1.2$ -SNT expression in blood vessels (DuPont et al., 2016), was not significantly altered in rat aorta exposed 24 h to aldosterone (Figure 13D). The translation of the MR-mediated $Ca_v1.2$ isoform change to chronic *in vivo* conditions was then studied. Aorta samples from previously published hypertensive rat aldosterone-salt model (Calvier et al., 2015), showed a spironolactone sensitive increased of exon 1a transcript levels whereas exon 1b levels were not modified (Figure 13E). To further explore *in vivo* the promoter *switch*, PCa.luc mice were treated with aldosterone-salt or vehicle for 3 weeks. Aorta of aldosterone-salt PCa.luc mice, which presented higher BP pressure than littermates vehicle-treated PCa.luc (Table 2), showed an increased luciferase activity (Figure 13F), which positively correlated with BP (Spearman correlation coefficients of 0.77, 0.73 and 0.76 between luciferase activity vs diastolic, systolic and mean BP, respectively; $P < 0.005$ in each case).

Figure 13. Aldosterone-induced transactivation of 'cardiac' Ca_v1.2-LNT transcript in smooth vessels. A and B, Effects of aldosterone (Aldo, 10⁻⁸ M) with or without RU28318 (RU) on relative Ca_v1.2 protein (**A**) and exon 1a and 1b mRNA (**B**) levels after 24 h treatment of rat coronary arteries, mesenteric arteries and aorta. n represents pooled of 3 to 4 arteries from different animals. **C**, Luciferase activities, expressed as relative light units (RLU) per mg of proteins, from PCa.luc aorta treated 24 h with or without 10⁻⁸ M aldosterone. **D**, Relative miR-155 expression measured in rat aorta after 24 h treatment. **E**, Effects of on relative mRNA levels of the exons 1a and 1b in rat aorta after 3 weeks *in vivo* treatment with aldosterone-salt (Aldo, 1 mg/kg per day + 1% NaCl) in combination with spironolactone (Spiro, 200 mg/kg per day). **F**, Aorta luciferase activities, expressed as relative light units (RLU) per mg of proteins, from aldosterone-salt-treated PCa.luc mice compared to vehicle-treated littermates. Data are expressed as mean ± s.e.m. Statistical significance was calculated by one-way ANOVA with *post-hoc* Fisher LSD test to compare aldosterone treated group to control ones. **P* < 0.05, ***P* < 0.005 and ****P* < 0.0005.

To functionally substantiate these results, we analyzed the aldosterone impact in vessel contractility and excitability. The contractile responses of LADCAs to KCl depolarization (Figure 14A) or to the Ca_v1.2 channel agonist BayK8644 (Figure 14B) were significantly increased after aldosterone treatment, independently of the presence of the endothelium (Figures 15A and 15B). Moreover, aldosterone treatment impaired vascular relaxation by acetylcholine (Figure 14C) as previously reported (Leopold et al., 2007). Patch clamp studies on isolated VSMCs (Rueda et al., 2013) from LADCAs incubated 24 h with aldosterone showed higher Ca²⁺ current density compared to isolated cells from LADCAs incubated 24 h without aldosterone (Figure 14D). This effect, even observed using Ba²⁺ as charge carrier (Figure 15C), did not alter neither voltage- nor time-dependent properties of the currents (Figures 14E, 14F and 14G).

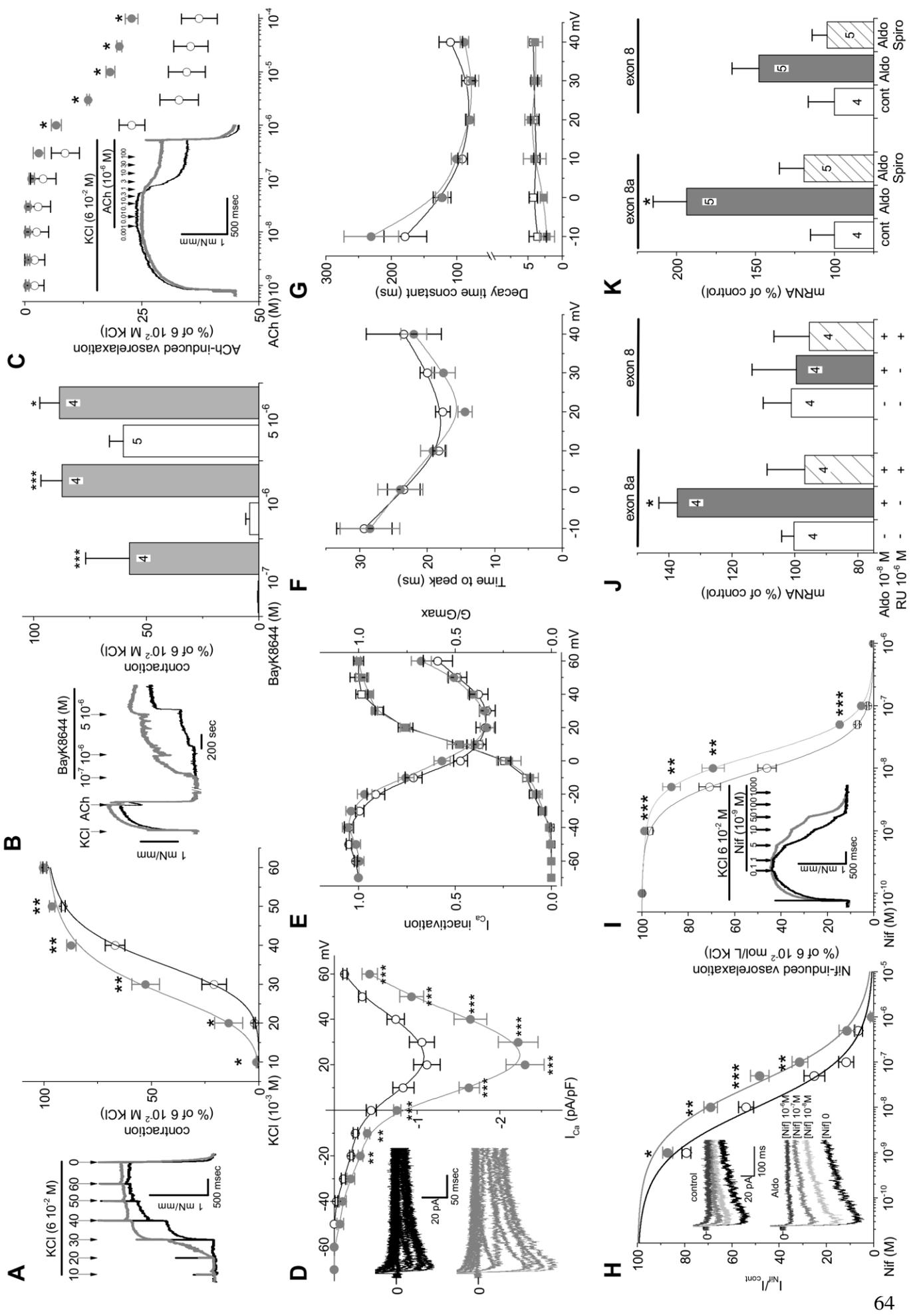


Figure 14. Effects of aldosterone on vascular function. **A**, Tracing on *left* shows the tension changes in response to the cumulative addition of KCl (10 to 60 10^{-3} M) of endothelium-intact rat coronary artery rings incubated 24 h with 10^{-8} M aldosterone (gray line) or without (Black line). Panel on *right* shows the dose-response curves for depolarization-induced constriction by KCl of endothelium-intact rat coronary arteries incubated 24 h with 10^{-8} M aldosterone (closed symbols, n=9) or without (open symbols, n=12). **B**, Representative tracings and summarized data of contractile responses to BayK8644, an L-type Ca^{2+} channel agonist, in rat coronary arteries treated 24 h with (gray) or without 10^{-8} M aldosterone. **C**, Representative tracings and summarized data (n=3 in each group) of relaxant effects of Acetylcholine (10^{-9} to 10^{-4} M) in rat isolated coronary arteries treated or not for 24 h with 10^{-8} M aldosterone and precontracted with 60 10^{-3} M KCl. **D**, Representative time courses of LTCC currents recorded in isolated VSMCs from rat coronary arteries cultured for 24 h with Aldosterone (gray lines) or without (black lines) together (bottom) with average peak current density–voltage relations in control (open symbols, n=18 in both case) and 10^{-8} M aldosterone treated (closed symbols, n=10) cells. **e**, Superimposed activation (square symbols) and inactivation curves (circle symbols) of Ca^{2+} currents from the rat coronary artery cells cultured with (closed symbols) or without (open symbols) 10^{-8} M aldosterone. **F**, Activation kinetics of Ca^{2+} currents in control (open symbols) and 10^{-8} M aldosterone treated (closed symbols) cells, expressed as the time from onset of voltage step to the peak of current amplitude are plotted *vs* voltage potential. **G**, Voltage dependence of the fast (square symbols) and slow (circle symbols) decay phases of the Ca^{2+} current fitted to a biexponential function. **H**, Dose–response curves of nifedipine inhibitory effects on LTCC currents, using Ba^{2+} as charge carrier, in isolated VSMCs (6 to 16 cells in each group) from rat coronary arteries incubated for 24 h without (open symbols) or with 10^{-8} M aldosterone (closed symbols). Inset, superimposed traces of whole-cell membrane currents recorded from a holding potential of -60 mV, which were evoked using 300 ms voltage steps to +10 mV in the presence of 0, 0.01, 0.1 and 1×10^{-6} M nifedipine (Nif). **i**, Concentration–response curves for effects of nifedipine on KCl-induced contraction of rat coronary artery rings incubated 24 h with 10^{-8} M aldosterone (closed symbols, n=5) or without (open symbols, n=5). Inset, representative traces of cumulative effects of nifedipine on the KCl induced tension developed in coronary arteries preincubated 24 h with 10^{-8} M aldosterone (gray line) or without (black line). **J and K**, Effects of on relative mRNA levels of the exons 8a and 8 in rat aorta (**J**) after 24 h *ex vivo* aldosterone-treatment (Aldo) in combination with RU28318 (RU) and (**K**) after 3 weeks *in vivo* treatment with aldosterone-salt (Aldo, 1 mg/kg per day + 1% NaCl) in combination with spironolactone (Spiro, 200 mg/kg per day). In a, b, c and i, experimental values were calculated relative to contractile response produced by 60 10^{-3} M KCl, which was taken as 100%. Data are expressed as mean \pm s.e.m. Statistical significance was calculated by one-way ANOVA with *post-hoc* Fisher LSD test to compare aldosterone treated group to control ones: * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$.

$Ca_v1.2$ isoforms differ notably in marked 1,4-dihydropyridines selectivity for VSMCs with respect to myocardium (Zamponi et al., 2015). Isolated VSMCs after 24 h aldosterone-treatment of rat

LADCAAs showed a less potent nifedipine inhibition of LTCC (Figure 14H), with half-maximum inhibitory concentration values of 9.8 ± 1.5 vs 33.3 ± 5.5 10^{-9} M ($P < 0.05$) and similar n_H values of 0.71 ± 0.07 vs 0.72 ± 0.08 , in untreated vs 10^{-9} M aldosterone-treated groups, respectively. Similarly, the blocking action of nifedipine on induced KCl contraction of rat LADCA rings is reduced after 24 h aldosterone treatment (Figure 14I, half-maximum inhibitory concentration values of 9.0 ± 0.15 vs 16.6 ± 0.48 10^{-9} M ($P < 0.05$) and similar n_H values of 1.51 ± 0.04 vs 1.59 ± 0.04 , in untreated vs 10^{-9} M aldosterone-treated groups, respectively). Structural differences of $Ca_v1.2$ isoforms impact at least partially the DHP sensitivities between heart and vessels, notably in N-terminus region and IS6 segments, encoded by mutually exclusive exons 8/8a (Welling et al., 1997b). In rat aorta exposed either *ex vivo* (Figure 14J) or *in vivo* (Figure 14K) to aldosterone, we observed an upregulation of the 'cardiac' exon 8a, which is blunted by MR antagonist, whereas expression of 'vascular' exon 8 was not significantly altered.

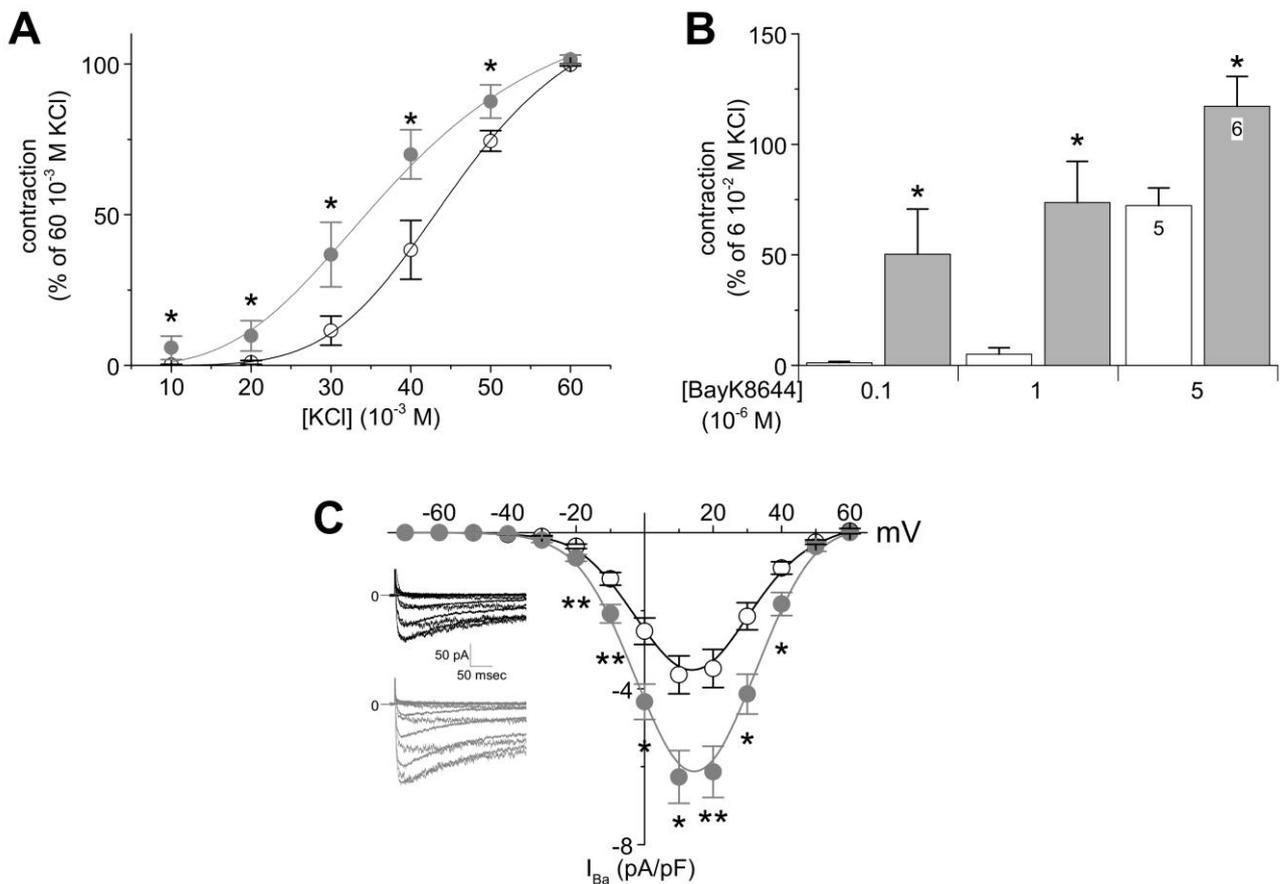


Figure 15. A and B, Depolarization-induced constriction by KCl (**A**, 6 arterial rings in each group) and by BayK8644 (**B**) of endothelium-denuded rat coronary arteries incubated 24 h with 10^{-8} M aldosterone (closed symbols) or without (open symbols). Experimental values were calculated relative to contractile response produced by 60 10^{-3} M KCl, which was taken as 100%. **C**, Average peak current density–voltage relations of LTCC currents with 20 10^{-3} M Ba^{2+} as the charge carrier recorded in isolated cells from rat coronary arteries cultured for 24 h in control conditions (open symbols, $n=18$) and with 10^{-8} M aldosterone (closed symbols, $n=14$). Data are expressed as mean \pm s.e.m. Statistical significance was calculated by one-way ANOVA with post-hoc Fisher LSD test to compare aldosterone treated group to control ones: * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$.

Chapter 4 Discussion

Collectively, our results establish a new mechanistic concept, identifying the Ca_v1.2-LNT as a critical molecular target of the mineralocorticoid (MC) pathway in the cardiovascular system, and extend our understanding of the mechanisms underlying the multiple biological processes known to be MR-regulated. This pathway emphasizes the major role of the transcription factor MR as a global regulator of ion channels (DuPont et al., 2014) and provides a conceptual framework for understanding how transcription factors regulate alternative promoter usage.

Deregulated aldosterone-MR signaling has been identified as the cause of multiple clinical diseases, suggesting the pathophysiological importance of this signaling pathway. Aldosterone is the primary MC involved in maintaining fluid and electrolyte balance through its control of Na⁺ and K⁺ transport in the distal renal tubules, thereby regulating BP under physiological conditions. In addition, the excessive production/secretion of this hormone not only results in hypertension but also likely contributes to HF and exacerbates the morbidity and mortality associated with these disorders. Basal plasma aldosterone levels in humans are around 10-500 10⁻¹² M, which might be drastically increased up to 2000% in individuals with HF (Weber, 2001). Thus, the significant increase in Ca_v1.2 expression, observed *in vitro* from 10⁻⁹ M, seems to be pathophysiological relevant for aldosterone levels prevailing *in vivo*, which might be further achieved taking into account that cardiac MR expression is upregulated in HF (Chai et al., 2010).

We show that aldosterone regulates Ca_v1.2-LNT by recruiting MR onto targeted genomic regions in 'cardiac' *Cacna1c* P1-promoter. *In silico* and mutational analysis functionally identified 3 GRE and 1 nGRE within the P1-promoter that is conserved between rats, mice and humans and have specific aldosterone-dependent enhancing and repressive capabilities. Of note, the GRE-2 has been already reported in the original sequence scanning (Liu et al., 2000). The combined presence of these GRE sets in mouse and human orthologues provides strong evidence for similar gene regulation (Michelson, 2002). Actually, promoter *cis*-regulatory elements are commonly conserved across orthologous genes and contribute to the specificity of transcription initiation (Smale and Kadonaga, 2003). In this regard, it may be significant that putative GREs are retained in similar locations in rat, mouse and human promoters. Individual deletion of the first three putative GREs, including nGRE, reduced, but did not completely abolish MC responsiveness. A parsimonious interpretation of these data is that these 3 sites are required to enhance P1-promoter activity through aldosterone-mediated MR in a cooperative manner, with GRE-2 being the most crucial functional binding site. Indeed, multiple GREs are often found in the regulatory sequences of the steroid receptor (SR)-controlled genes, leading to the concept of element cooperativity due to allosterically-induced receptor changes that may be essential for differential gene expression (Geserick et al., 2005). Moreover, distinct

regulatory complexes established at individual GREs may collaborate to control any single GR-regulated gene (Weikum et al., 2017). Consistent with the repressive nature of the nGRE for GC action (Surjit et al., 2011), our data pointed out the differential transcriptional activity of GR and MR as early reported. Some GREs can preferentially enhance transcription in response to MR than to GR (Kolla et al., 1999), or may only bind MR specifically (Meinel et al., 2013). Indeed, despite the conserved 3D structure shared among members of the SR family and their overlapping DNA binding preference, activation of individual SRs drive unique effects on gene expression (Hudson et al., 2014). Notably, the binding to and the transcriptional repression by nGREs is a property of the GR DBD not shared by other members of the SR family (Hudson et al., 2016). These facts may help explain why MR and GR elicit opposite effects, while they both bind to the same GRE. In addition, these results further demonstrate that GC actions are not identical to those of aldosterone and the MR (Mihailidou and Funder, 2005; Rossier et al., 2008)

Aldosterone plays a wide range of direct vascular effects, including those regulating vascular reactivity and growth or cell death. With respect to vascular reactivity, both NOS-related endothelial-dependent vasodilator (Leopold et al., 2007) and direct VSMC vasoconstrictor (McCurley et al., 2012; Romagni et al., 2003) effects have been described. Notably, aldosterone increased vascular tone directly by increasing ion influx, particularly Ca^{2+} , within VSMCs (DuPont et al., 2014). Our data support the central role of aldosterone-activated VSMC MR in the regulation of myogenic tone, leading to increased LTCC current density and greater vascular contraction, and thereby the pivotal role of MR pathway in the regulation of BP. Accordingly, $Ca_v1.2$ downregulation has been linked with attenuated age-related hypertension of VSMC-specific MR knockout mice (McCurley et al., 2012). Importantly, we showed that aldosterone effects through MR are related to an upregulation of $Ca_v1.2$ -LNT in consonance with a dramatic increase in 'cardiac' P1-activation and exon 1a levels in blood vessels. Blood vessels mainly express $Ca_v1.2$ -SNT due to overwhelming P2-promoter activity (Hofmann et al., 2014), as confirmed here by the major exon 1b abundance. Indeed, the P1-promoter activity is reduced (Liu et al., 2000) or absent (Pang et al., 2003) in VSMCs. Of note, several studies have reported during hypertension an unmatched upregulation of $Ca_v1.2$ protein expression with no change in $Ca_v1.2$ -SNT transcript levels (Joseph et al., 2013). Although we cannot exclude the possibility of other effects, the $Ca_v1.2$ -LNT transcript switch reported here in VSMCs might be involved in the mechanisms of resistant hypertension in patients receiving LTCC blockers. Indeed, these blockers are less effective on the cardiac isoform than on the VSMC one (Cataldi and Bruno, 2012). The $Ca_v1.2$ -LNT transcript switch might thus participate in the beneficial effect of MR antagonists in those patients (Williams et al., 2015). In addition to increased expression of 'cardiac' exon 1a, following aldosterone-treatment VSMC LTCC were less sensitive to the DHP antagonist, nifedipine. The high sensitivity of the VSMC LTCC for DHPs might be caused not only by the lower

membrane potential of VSMCs but also by structural differences between the cardiac and vascular LTCC. The two isoforms are alternatively spliced from the same *CaCna1c* gene, with 95% similarity of the two proteins. The 5% differences are mainly located at the exon 1a/1b, exon 8a/8, exon 31/32 and exon 9 (Hofmann et al., 2014). Although both isoforms do not differ in the putative DHP binding domain, as deduced by analogy with the bacterial Ca_v channel (Tang et al., 2016), mutually exclusive exon 8/8a (Welling et al., 1997b) and exon 31/32 (Zühlke et al., 1998) participate to the tissue-specific DHP sensitivity, as well as the N-terminus region (Blumenstein et al., 2002; Pang et al., 2003). Even if the complete alternative splicing profile changes with aldosterone treatment awaits further investigation, one might put forward that transcription factors binding to transcribed promoters modulate alternative splicing, supporting a physical and functional link between transcription and splicing (de Klerk and 't Hoen, 2015).

Gene expression is controlled by a variety of mechanisms, both at transcriptional and translational levels, including chromatin condensation, transcription initiation, DNA methylation, alternative RNA splicing, mRNA stability and others. During the biogenesis of mRNAs, regulation of transcription initiation represents the first level in the control of gene expression. This common phenomenon to generate protein diversity from one gene has been mainly recognized as an important transcriptional mechanism for both qualitative and quantitative regulation of developmental and/or tissue-specific gene expression (Davuluri et al., 2008; de Klerk and 't Hoen, 2015; Vacik and Raska, 2017). However, alternative promoter usage also leads to various diseases including cancer (Davuluri et al., 2008), neuropsychiatric disorders (Tan et al., 2007), and developmental disorders (Hill and Lettice, 2013). Yet, the molecular mechanisms of the selective promoter usage have not been well studied (Pal et al., 2011). Our results provide evidence that hormonal *cis*-regulation might lead to alternative promoter usage, as previously reported (Mahendroo et al., 1993; Ruike et al., 2007). Differential usage of alternative promoters affects the abundance and translational efficiency of transcripts, and also entails new molecular signature of target genes. Indeed, key determinants of LTCC function and regulation have been associated with the cytosolic localized N-terminus portion of the channel, which might encompass interactions with other proteins such as calmodulin, calmodulin kinase II, $Ca_v\beta$ subunits, and Rad/Rem/Rem2/Gem proteins (Ter Keurs and Boyden, 2007) and modulations of sensitivity to protein kinase C (Weiss and Dascal, 2015) and LTCC blockers (Blumenstein et al., 2002). Thus, a model for the MR action on $Ca_v1.2$ -LNT might be of prime importance to understand the new global pathophysiological action of MR. These results open interesting perspectives, taking into account that the ubiquitous LTCCs expression regulation is critically involved in the pathogenesis of serious neurological, retinal, cardiac, vascular and metabolic disorders (Benitah et al., 2010; Joseph et al., 2013; Zamponi et al., 2015), in which aldosterone *via*

MR plays pivotal but elusive roles (Gilbert and Brown, 2010; Jaisser and Farman, 2016; de Kloet et al., 2016; McCurley et al., 2012).

Chapter 5 Conclusion

In summary, our results identify the $Ca_v1.2$ -LNT as a critical player of MR pathway in the cardiovascular system, deciphering a complex and coordinated hormonal *cis*-regulation on the P1-promoter of *Cacna1c*. Moreover, the alternative promoter usage elicited by MR pathway in vascular tissue sheds new light into the understanding of alternative transcription initiation and protein diversity, in which explain, at least in part, the inefficiency of Ca^{2+} blockers to treat certain types of diseases.

Perspectives

As expected from a scientific study, this work deserves further exploration as well as open new questions to be addressed in future research. Here, we provided a detailed molecular study supporting that MR signaling has specific targets into the genomic DNA regulating the *Cacna1c* promoter. The understanding of this mechanism highlights the dysfunctional role of MR activation in the onset and progression of cardiovascular diseases (Brown, 2008; Jaisser and Farman, 2016; Weber, 2001; Zannad et al., 2011). Indeed, a close association of MR activation and increased Ca^{2+} influx through LTCC has been linked with deleterious cardiac actions favoring the occurrence of aberrant electrical activities and, ultimately, lethal arrhythmias (Benitah et al., 2010; Gómez et al., 2009; Ouvrard-Pascaud et al., 2005; Perrier et al., 2004; Ramirez et al., 2000). As already shown, our group recently generated a unique transgenic mouse model expressing luciferase reporter gene under the control of the promoter sequence of *Cacna1c*, which will allow a time-lapse *in vivo* analysis of the *Cacna1c* promoter. However, up to date, the knowledge regarding mechanisms responsible for the regulation of *Cacna1c* promoter is limited and require new studies to determine potential physiological regulators of *Cacna1c* promoter. Equally important, evaluation of pathological *in vivo* models certainly will add valuable insights into the understanding of the regulation *Cacna1c* promoter in cardiac and non-cardiac tissues. Therefore, further exploration of *in vivo* study surely will support that the MR response obtained through *in vitro* and *ex vivo* studies are overlapped by the same molecular mechanism. From a molecular point of view, *in silico* analysis reveals distinct GREs MR genomic targets on the promoter of *Cacna1c*. We successfully have shown that one of them gives the promoter a singular sensitivity in response to MR activation. However, to unequivocally validate these findings, characterization of MR recruitment on specific GRE sites of *Cacna1c* promoter through chromatin immunoprecipitation (ChIP) is certainly needed. Moreover, identification of other

molecular MR signaling targets emerges as a promising field that may explain the diverse physiological and pathological aldosterone actions.

Despite the complex etiology of hypertension, an elevated Ca^{2+} -dependent vascular tone is a shared feature between different forms of hypertension. The pore forming $\alpha_1\text{C}$ subunit of the $\text{Ca}_v1.2$ channels has binding sites for all three classes of clinical Ca^{2+} channel blockers (CCBs), which include the DHPs, phenylalkylamines and benzothiazepines. These drugs reduce elevated vascular tone by blocking the voltage-dependent influx of Ca^{2+} ions through $\text{Ca}_v1.2$ channels into VSMCs (Wray et al., 2005). An increased abundance of $\text{Ca}_v1.2$ subunit in VSMCs of small arteries and arterioles during hypertension may provide more binding sites for CCBs, thereby increasing the vasodilator effectiveness of these drugs proportional to the rise in blood pressure. Indeed, CCBs effectively lower BP in certain forms of hypertension in animals and humans (Godfraind et al., 1991; Miyamori et al., 1987; Narita et al., 1983; Takata and Hutchinson, 1983). However, even despite the well-established clinical usage of CCBs, effectiveness is unpredictable being physiological levels of BP achieved only in a subset of patients (Dustan, 1996; Mancia and Grassi, 2002). Therefore, we identified a potential molecular mechanism that may explain the failure of DHPs treatment in certain forms of hypertension. Thus, new studies are clearly needed to repositioning the clinical and therapeutic relevance of MR antagonists, as well as the development of selective $\text{Ca}_v1.2$ -LNT blockers, which emerges as a compelling pharmacological strategy. Alternatively, molecular therapeutic approach to treat hypertension based on reducing the expression of the $\text{Ca}_v1.2$ -LNT might be a more effective antihypertensive strategy than the classical pharmacological blocking of $\text{Ca}_v1.2$.

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Appendix

Academic publications

1. Gómez AM, **Mesquita TRR**, Mercadier JJ, Álvarez JL, Benitah JP. Calcium Microdomains in Cardiac Cells. In: *Microdomains in the Cardiovascular System*. Springer, 2017 (in press).

Book chapter

2. **Mesquita TRR**, Campos-Mota GP, Lemos VS, Cruz JS, Jesus ICG, Camargo EA, Pesquero JL, Pesquero JB, Capettini LSA, Lauton-Santos S. Vascular Kinin B1 and B2 Receptors Determine Endothelial Dysfunction through Neuronal Nitric Oxide Synthase. *Frontiers in Physiology*, 8, 228, 2017.
3. **Mesquita TRR**, Jesus ICG, Santos JF, Almeida GKM, Vasconcelos CML, Guatimosim S, Macedo FN, Santos RV, Menezes-Filho JER, Miguel-dos-Santos R, Matos PTD, Scalzo S, Santana-Filho VJ, Albuquerque-Júnior RLC, Pereira-Filho RN, Lauton-Santos S. Cardioprotective Action of Ginkgo biloba Extract against Sustained β -Adrenergic Stimulation Occurs via Activation of M2/NO Pathway. *Frontiers in Pharmacology*, 8, 1-13, 2017.
4. Mota MM, Silva TLTB, Macedo FN, **Mesquita TRR**, Quintans Junior LJ, Santana-Filho VJ, Lauton-Santos S, Santos MRV. Effects of a Single Bout of Resistance Exercise in Different Volumes on Endothelium Adjustments of Healthy Animals. *Arquivos Brasileiros de Cardiologia* (Impresso), 108(5):436-442, 2017.
5. Macedo FN, **Mesquita TRR**, Melo VU, Mota MM, Silva TLTB, Santana MN, Oliveira LR, Santos, RV, Miguel Dos Santos R, Lauton-Santos S, Santos MRV, Barreto AES, Santana-Filho VJ. Increased Nitric Oxide Bioavailability and Decreased Sympathetic Modulation Are Involved in Vascular Adjustments Induced by Low-Intensity Resistance Training. *Frontiers in Physiology*, 7, 265, 2016.
6. Rios L, Júnior MAC, Lauton-Santos S, Capettini LSA, **Mesquita TRR**, Cruz JS, NataliAJ, Prímola-Gomes TN. Óxido Nítrico e Dinâmica de Ca^{2+} em Cardiomiócitos: Influência da Capacidade de Exercício. *Revista Brasileira de Medicina do Esporte*, 22, 31-34, 2016.
7. Mota MM, **Mesquita TRR***, Silva TL, Fontes MT, Santos SL, Capettini, LSA, Jesus ICG, Junior, LJQ, Angelis, KA, Wichi RB, Santos MR. Endothelium adjustments to acute resistance exercise are intensity-dependent in healthy animals. *Life Sciences* (1973), 142, 86-91, 2015.

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