

ORIGINAL ARTICLE



T- and L-Type Calcium Channels Maintain Calcium Oscillations in the Murine Zona Glomerulosa

Hoang An Dinh^{ID}, Marina Volkert, Ali Kerim Secener^{ID}; Ute I. Scholl^{ID}, Gabriel Stölting^{ID}

BACKGROUND: The zona glomerulosa of the adrenal gland is responsible for the synthesis and release of the mineralocorticoid aldosterone. This steroid hormone regulates salt reabsorption in the kidney and blood pressure. The most important stimuli of aldosterone synthesis are the serum concentrations of angiotensin II and potassium. In response to these stimuli, voltage and intracellular calcium levels in the zona glomerulosa oscillate, providing the signal for aldosterone synthesis. It was proposed that the voltage-gated T-type calcium channel $Ca_v3.2$ is necessary for the generation of these oscillations. However, *Cacna1h* knock-out mice have normal plasma aldosterone levels, suggesting additional calcium entry pathways.

METHODS: We used a combination of calcium imaging, patch clamp, and RNA sequencing to investigate calcium influx pathways in the murine zona glomerulosa.

RESULTS: *Cacna1h*^{-/-} glomerulosa cells still showed calcium oscillations with similar concentrations as wild-type mice. No calcium channels or transporters were upregulated to compensate for the loss of $Ca_v3.2$. The calcium oscillations observed were instead dependent on L-type voltage-gated calcium channels. Furthermore, we found that L-type channels can also partially compensate for an acute inhibition of $Ca_v3.2$ in wild-type mice. Only inhibition of both T- and L-type calcium channels abolished the increase of intracellular calcium caused by angiotensin II in wild-type.

CONCLUSIONS: Our study demonstrates that T-type calcium channels are not strictly required to maintain glomerulosa calcium oscillations and aldosterone production. Pharmacological inhibition of T-type channels alone will likely not significantly impact aldosterone production in the long term. (*Hypertension*. 2024;81:811–822. DOI: 10.1161/HYPERTENSIONAHA.123.21798.) • **Supplement Material.**

Key Words: aldosterone ■ calcium channel blockers ■ calcium ■ calcium signaling ■ zona glomerulosa

The adrenal glands are a pair of endocrine organs located above the kidneys. Within the adrenal cortex, steroid hormones are synthesized from the precursor cholesterol. The outermost zona glomerulosa (ZG) produces aldosterone, the zona fasciculata either cortisol in humans or corticosterone in mice and the innermost zona reticularis (in humans) androgens. Aldosterone is responsible for the maintenance of blood salt levels and volume via the regulation of various ion transporters in the kidney and intestine. Its synthesis is tightly controlled by stimuli linked to these targets,

primarily the serum concentrations of potassium and Ang II (angiotensin II).¹

ZG cells have a highly negative resting membrane potential at rest. Binding of Ang II to its cognate receptor leads to the closure of background potassium channels.² This results in oscillatory depolarizations of ZG cells,³ causing similarly oscillatory influx of calcium via voltage-gated calcium channels (VGCCs).^{2,4–9}

Calcium itself is required for several key functions in ZG cells, such as the regulation of transcription factors upstream of the aldosterone synthase *Cyp11b2*.^{10,11}

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NOVELTY AND RELEVANCE

What Is New?

Calcium signaling in the adrenal zona glomerulosa can be maintained in the absence of T-type calcium channels.

L-type calcium channels constitute the calcium influx pathway in the absence of T-type calcium channels.

What Is Relevant?

Aldosterone is a major component in maintaining salt and blood pressure homeostasis, primarily in response to angiotensin II or serum potassium.

Although $Ca_v3.2$ was thought to be essential for the regulation of aldosterone production in the zona glomerulosa, knock-out mice exhibit normal aldosterone levels due to their functional replacement by L-type calcium channels.

Clinical/Pathophysiological Implications?

Our results have implications for pharmacological strategies to lower aldosterone production. Only simultaneous blockade of T- and L-type calcium channels may inhibit calcium influx into the zona glomerulosa and thus lower aldosterone synthesis.

Nonstandard Abbreviations and Acronyms

Ang II	angiotensin II
KO	knock out
VGCC	voltage-gated calcium channel
WT	wild type
ZG	zona glomerulosa

and the transport of cholesterol to the inner membrane leaflet of mitochondria for conversion into aldosterone.¹² Tight control over $[Ca^{2+}]_{int}$ is therefore key to the control of aldosterone synthesis.

The original publication identifying murine ZG cells as voltage oscillators demonstrated that voltage fluctuations critically depend on the function of the T-type VGCC $Ca_v3.2$ (Gene: *Cacna1h*) and specific inhibition of this channel abolished all voltage oscillations.³ Even before, the specific inhibition of T-type VGCCs has been proposed as a potential strategy to pharmacologically suppress aldosterone production.¹³

However, despite its critical role in the electric excitability of the ZG, $Ca_v3.2$ knock-out mice did not show altered systemic aldosterone or renin levels.^{8,14} The molecular mechanisms that sustain aldosterone production in $Ca_v3.2$ knock-out mice currently remain unclear. We here set out to investigate the regulation of $[Ca^{2+}]_{int}$ in the ZG of mice lacking $Ca_v3.2$ and the implications of the results on wild-type (WT) ZG function and pharmacology.

METHODS

Data Availability

All data and materials, except raw calcium imaging videos, have been made publicly available on GitHub and can be accessed at <https://github.com/dhoangan/Cacna1hKO>. RNA-seq data have been made publicly available on ArrayExpress and can be accessed at <https://www.ebi.ac.uk/biostudies/arrayexpress/>

studies/E-MTAB-12999. Videos can be only be provided upon reasonable request due to their file sizes.

Detailed methods and the major resources table are available in the [Supplemental Material](#).

Mice and Organ Harvest

WT and *Cacna1h* KO mice⁸ were kept in IVF cages with water and food ad libitum according to local regulations. For extraction of adrenal glands, mice were anesthetized using isoflurane and euthanized by cervical dislocation.

Calcium Imaging

Calbryte 520 AM- or Fura-2 AM-stained acute slices (120- μ m thickness) were constantly perfused with bicarbonate-buffered, oxygenated solution. K^+ , Ang II, and inhibitors were added in indicated concentrations.

Electrophysiological Recordings of Dissociated Adrenal Cortical Cells

The adrenal cortex was manually dissected and cells isolated by enzyme- and shear-based dissociation. Cells were used for whole-cell patch-clamp recordings the next day.

Bulk RNA-Seq

RNA was extracted from dissected whole adrenal cortices (6 WT and 6 KO mice; 3 males, 3 females each). RNA was processed and sequenced in the Genomics core facility of the Berlin Institute of Health and analyzed as described.^{15–20}

Single Nucleus RNA-Seq

Adrenal glands (2 males and 2 females) were processed to obtain a nuclei suspension. Sequencing-ready libraries were generated from these and sequencing was performed on a HiSeq 4000 device.

Statistics

All *P* values (except for the differential expression analysis) are results from a likelihood ratio test of linear mixed models and are indicated as follows: ns, $P \geq 0.05$; * < 0.05 ; ** $P < 0.01$;

*** $P < 0.001$. All error bars and bands show the 95% CI of the mean value.

For differential expression analysis, P values were adjusted by Benjamini-Hochberg for multiple comparisons.

RESULTS

ZG Cells From *Cacna1h*^{-/-} Mice Still Exhibit Intracellular Calcium Oscillations

To study $[Ca^{2+}]_{int}$ in murine ZG cells, we stained acutely prepared slices from mouse adrenal glands with the fluorescent calcium indicators Calbryte 520 AM (high temporal resolution but no absolute quantification) or Fura-2 AM (allows for $[Ca^{2+}]_{int}$ quantification). We used WT as well as *Cacna1h*^{-/-} (KO) mouse lines that we established previously.⁸

When stimulated with 500 pmol/L Ang II and 4 mmol/L K^+ , ZG cells in slices from WT mice responded with pronounced Ca^{2+} oscillations. As observed previously,^{7,8} individual transients (spikes) were homogeneous in appearance. Spikes were clustered in bursts, separated

by pauses at a mostly constant baseline $[Ca^{2+}]_{int}$ (Figure 1A). Although lacking the $Ca_v3.2$ channel, $[Ca^{2+}]_{int}$ in ZG cells from KO mice exhibited similar behavior: Homogeneous spikes, clustered into bursts (Figure 1B).

The most prominent difference of the spiking in KO mice was the significantly increased overall number of spikes per second (activity; Figure 1C; Table S1). This was caused by an increased frequency of spikes within bursts and shorter gaps between bursts rather than longer bursts (Figure S1A through S1C).

ZG Stimulation Results in Similar Changes of Intracellular Calcium Levels in KO and WT Mice

We also investigated mean $[Ca^{2+}]_{int}$ over a wide range of concentrations of potassium (Figure 1D) and Ang II (Figure 1E). With increasing stimulation, $[Ca^{2+}]_{int}$ similarly increased in ZG cells of both WT and KO. Mean $[Ca^{2+}]_{int}$ were not significantly different between genotypes (Tables S2 and S3), suggesting that the higher activity and intraburst frequency of calcium spikes in KO

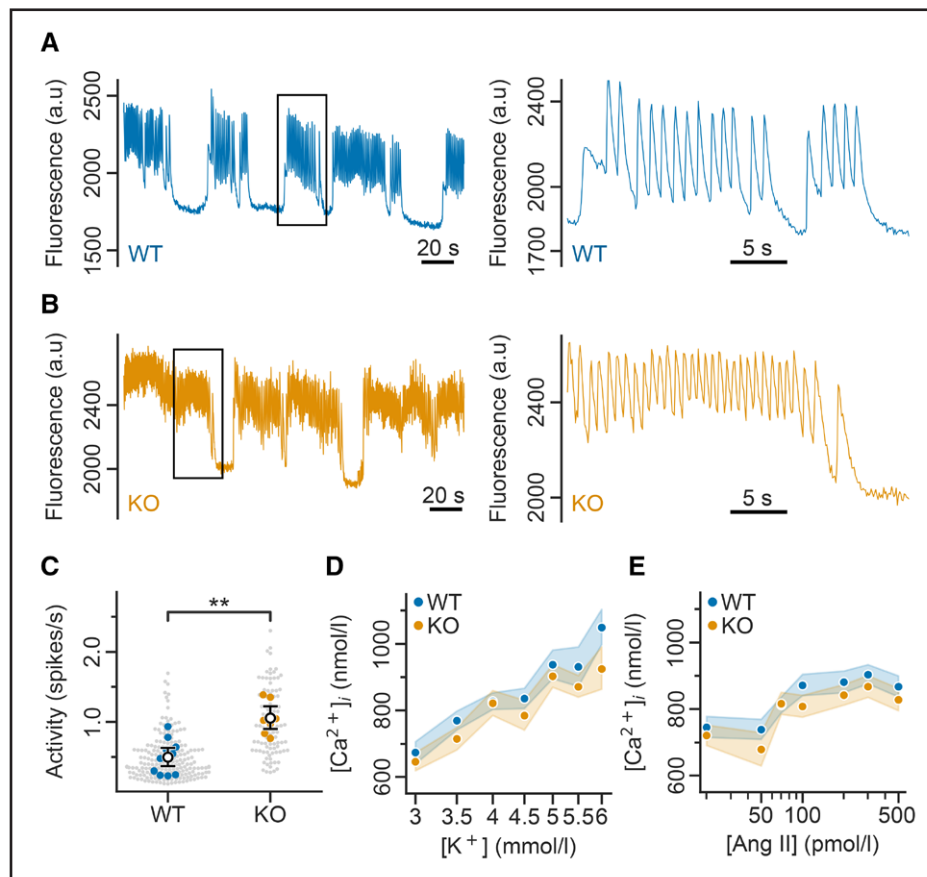


Figure 1. Calcium oscillations persist in *Cacna1h*^{-/-} mice.

A and **B**, Fluorescence signals recorded from one representative zona glomerulosa (ZG) cell each of a wild-type (WT; **A**) or knock out (**B**) Calbryte 520 AM-stained adrenal slice and stimulated with 4 mmol/L K^+ and 500 pmol/L Ang II (angiotensin II). A 30 s magnification (indicated by the black rectangle) is shown on the right. **C**, Calcium spike activity recorded in ZG cells from Calbryte 520 AM-stained adrenal slices over 7.5 minutes is lower in WT than in knock out (KO) ($n_{\text{animals,WT,male}}=7$; $n_{\text{animals,WT,female}}=0$; $n_{\text{animals,KO,male}}=3$; $n_{\text{animals,KO,female}}=2$; **D** and **E**) Mean $[Ca^{2+}]_{int}$ in ZG cells of Fura-2 AM-stained adrenal slices. The Ang II concentration was kept constant at 100 pmol/L in (**D**) while potassium was fixed at 4 mmol/L in (**E**; see Tables S2 and S3 for the sexes of the used mice).

cells does not result in higher calcium influx over time but rather maintains the sensitivity to physiological stimuli of aldosterone production.

Bulk RNA-Seq of Adrenal Cortices From KO Mice Revealed no Upregulation of Calcium Transport Genes

To identify differentially expressed genes between genotypes and to quantify the expression of VGCCs in WT, we performed bulk RNA sequencing of adrenal cortices from 6 WT and 5 KO mice. We prepared adrenal cortices by mechanical removal of the adrenal medulla. The resulting preparation thus includes both ZG and ZF.

In the WT adrenal cortex, *Cacna1h* ($Ca_v3.2$), *Cacna1c* ($Ca_v1.2$), and *Cacna1d* ($Ca_v1.3$) were the most strongly expressed VGCC α 1-subunit genes (Figure 2A; Tables S4 through S6). The most prominent accessory subunits were β 2 (*Cacnb2*) and α 2 δ 1 (*Cacna2d1*; Tables S4 through S6).

Apart from a downregulation of *Cacna1h* mRNA, likely due to nonsense-mediated decay, we did not observe any significant changes in the expression of known calcium channel genes in KO compared with WT mice (Table S4 through S6). In total, we observed 12 differentially expressed genes with at least a 2-fold change between genotypes (Figure 2B and 2C; Tables S7 to S9). Of these, besides *Cacna1h*, only *Creb5* has previously been associated with regulating aldosterone synthesis.²¹

Looking at other known genes involved in aldosterone synthesis (according to the KEGG database) without cutoff for the change in expression, we found 3 significant changes in KO (*Agtr1a*: 1.35-fold, $p_{\text{adj}}=0.048$; *Star*: 1.27-fold, $p_{\text{adj}}=0.007$; *Gna11*: 0.87-fold, $p_{\text{adj}}=0.023$; Tables S10 through S12). However, these changes were small and are therefore unlikely to carry a large functional impact on their own. *Star* may also play a role in the zona fasciculata and $Ca_v3.2$ was reported to regulate glucocorticoid production,²² so we assessed ACTH and corticosterone levels in WT and KO mice. No significant difference was observed (Figure S2).

GO term²³ and KEGG pathway²⁴ overrepresentation analyses for the set of differentially expressed genes did not yield any significant results.

Adrenocortical Cells From KO Mice Do Not Exhibit T-Type Calcium Currents

To investigate functional changes of existing calcium channels and to confirm the loss of $Ca_v3.2$ in our KO mice, we performed whole-cell patch-clamp recordings from isolated adrenocortical cells.⁵ Currents (Figure S3) were recorded in response to 2 separate voltage clamp protocols²⁵: First from a holding potential of -80 mV, then from a holding potential of -40 mV. At -80 mV, VGCCs reside in the closed but activatable state leading to currents from all different types (Figure S3, left).

With the second protocol (from -40 mV), the low-voltage activated T-type channels are already inactivated before the recording. Only non-T-type VGCCs (including the L-type channels $Ca_v1.2$ and $Ca_v1.3$) remain available (Figure S3, middle). The subtraction of the current from the holding potential of -40 mV from the one from -80 mV revealed the T-type channel component (Figure S3 right). In WT cells, we observed both T- and non-T-type currents in all recorded cells (7 cells from 3 animals, Figure 2D and 2E). In KO cells, we did not observe T-type currents in any of the recorded KO cells (9 cells from 4 animals; Figure 2D).

Non-T-type currents observed in KO cells were not different in their maximum amplitude or voltage dependence compared with WT cells (Figure 2E; Table S13). This excludes large posttranslational modifications to increase L-type currents in response to the loss of $Ca_v3.2$.

$Ca_v3.2$ is Not Required for Calcium Oscillations in WT Mice

RNA-seq and patch clamp suggested $Ca_v3.2$ as the only relevant T-type calcium channel in adrenocortical cells. To confirm its importance for calcium oscillations in the ZG, we studied the effect of the T-type calcium channel inhibitor 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2)²⁶ in adrenal slice preparations using calcium imaging. At the chosen concentration of $15 \mu\text{mol/L}$, virtually all T- but $<10\%$ of L-type VGCCs²⁷ are expected to be inhibited.

Calcium oscillations in ZG cells from KO mice were unaffected by the presence of TTA-P2 (Figure 3A), confirming the functional absence of other T-type channel isoforms. Treatment of WT slices led to a heterogeneous response, with some cells exhibiting continued calcium spiking (Figure 3B) and others turning completely silent (Figure 3C). Overall, $\approx 34\%$ of the WT cells and almost all KO cells remained active (WT: 34.4% [95% CI, 22.2%–47.3%]; KO: 90.3% [95% CI, 84.3%–95.1%]; Figure 3D). This resulted in a reduction of the mean activity in WT by about 45% when compared with control recordings (Figure 3E and 3F; Table S8 and S9; absolute data in Figure S4A and Tables S16 and S17).

The intraburst frequency in cells from WT slices was increased during TTA-P2 application while it remained unchanged in KO (Figure 3G; Tables S16 through S18; absolute data in Figure S4B and S4C). We also investigated the spatial arrangement of cells before and during TTA-P2 perfusion (Figure S5). The thickness of the ZG was similar in both genotypes before TTA-P2 (Figure S5A and S5B). This spatial distribution also remained similar during TTA-P2 perfusion (Figure S5C) indicating that cells in WT slices with remaining activity do not have a specific position within the ZG.

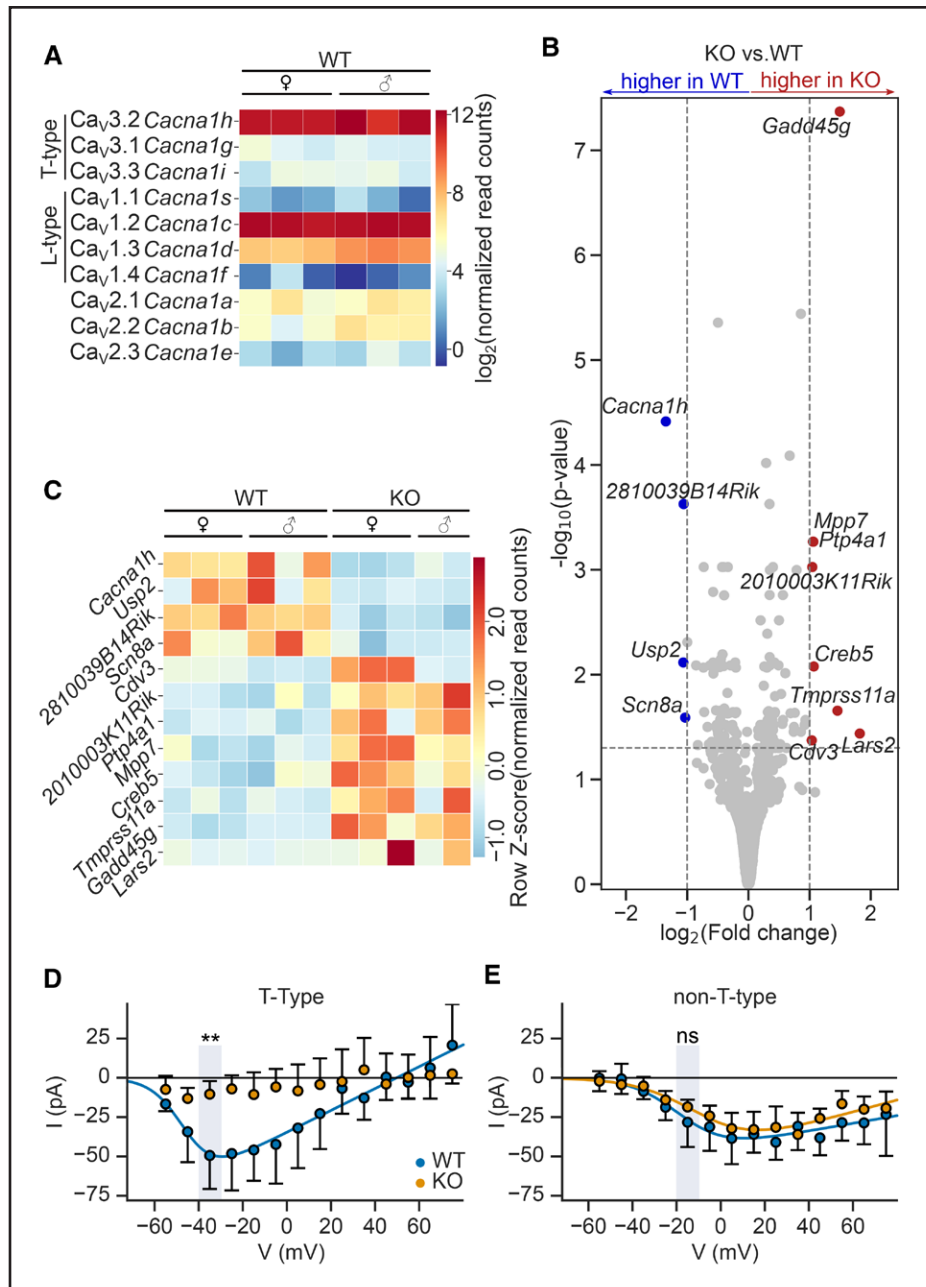


Figure 2. RNA-seq reveals only few differentially expressed genes (DEGs).

A, Heat map illustrating the normalized \log_2 transformed read counts of voltage-gated calcium channel (VGCC) transcripts detected in wild-type (WT) samples (columns, $n_{\text{animals,WT}}=6$). **B**, Volcano plots with the \log_2 -fold change (LFC, cutoff=|1|) in gene expression in knock out (KO) vs WT samples ($n_{\text{animals,WT,male}}=3$, $n_{\text{animals,WT,female}}=3$, $n_{\text{animals,KO,male}}=2$, $n_{\text{animals,KO,female}}=3$) and the \log_{10} transformed statistical significance (P value, cutoff=0.05). The 12 DEGs are highlighted as up- (red) or down- (blue) regulated. **C**, Heatmap illustrating the row Z score of the normalized read counts of DEGs sorted by their LFC. **D** and **E**, Isolated adrenocortical cells from KO mice lack T-type currents as shown by whole-cell patch-clamp recordings. Voltage dependence of the T-type (**D**) and non-T-type (**E**) peak current amplitudes. Solid lines represent fits of Equation (1) to our experimental data (see Supplemental Material). Circles represent mean values per cell. Only one half of the CI is displayed ($n_{\text{animals,WT,male}}=1$, $n_{\text{animals,WT,female}}=2$, $n_{\text{animals,KO,male}}=3$, $n_{\text{animals,KO,female}}=1$).

These findings support an important role of $\text{Ca}_v3.2$ in the generation of calcium spikes in WT mice. Still, at least in a subset of cells, other calcium influx pathways exist that can maintain calcium oscillations when T-type calcium channels are inhibited.

L-Type Calcium Channels Mediate Calcium Oscillations in KO Mice

Our RNA-seq data and previous publications also demonstrated the expression of L-type^{28–30} VGCCs in the

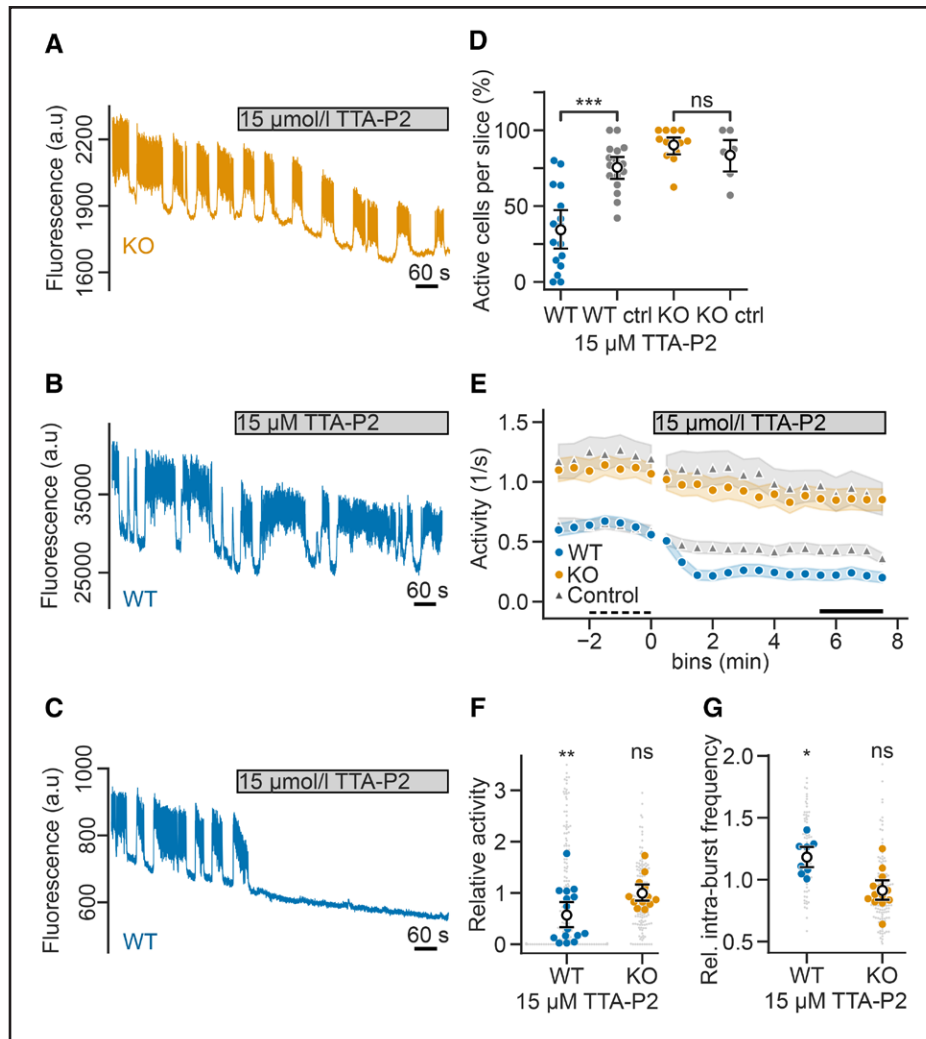


Figure 3. TTA-P2 only partially inhibits calcium oscillations in zona glomerulosa (ZG) cells of wild-type (WT) mice.

A through **C**, Variations in the fluorescence recorded in one representative knock out (KO; **A**) and 2 WT (**B** through **C**) ZG cells. Application of TTA-P2 inhibits calcium spikes in a fraction of the WT cells. **D**, Fraction of cells in a slice that are still active under TTA-P2 (determined at time span 5.5–7.5 minutes after addition of TTA-P2, indicated by the black line in **E**) compared with the number of active cells before application of TTA-P2 (–2 to 0 minutes, ie, baseline, indicated by the black dashed line in **E**). **E**, In WT but not KO ZG cells, calcium spike activity was lowered by TTA-P2 when compared with controls (no TTA-P2, triangles). Relative activity (**F**) and relative intraburst frequency (**G**) between 5.5 and 7.5 minutes after the start of the perfusion was significantly changed by TTA-P2 in WT but not KO. Data per genotype in **F** and **G** was calculated relative to and tested against control recordings. Cells were from adrenal slices stained with Calbryte 520 AM and perfused with 4 mmol/L K^+ and 500 pmol/L Ang II (angiotensin II) ($n_{\text{animals,WT,male}}=10$, $n_{\text{animals,WT,female}}=0$, $n_{\text{animals,WT ctrl,male}}=9$, $n_{\text{animals,WT ctrl,female}}=6$, $n_{\text{animals,KO,male}}=4$, $n_{\text{animals,KO,female}}=3$, $n_{\text{animals,KO ctrl,male}}=3$, $n_{\text{animals,KO ctrl,female}}=4$). TTA-P2 indicates 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoropiperidin-4-ylmethyl]-benzamide.

adrenal cortex. We perfused adrenal slices with the specific L-type channel inhibitor isradipine to isolate their contribution to calcium signals in the ZG.

The concentration of isradipine (0.05 $\mu\text{mol/L}$) was chosen to be specific for L-type over other VGCCs.^{31–34} The onset of inhibition was slow but resulted in an almost complete termination of calcium oscillations in KO cells. On the contrary, isradipine had nearly no effect on WT cells (Figure 4A through 4C; Figure S6A; Tables S19 through S21). The intraburst frequency remained unaffected by the inhibition of L-type channels in both genotypes (Figure S6B and S6C; Tables S20 through S22).

Overall, these results indicate that L-type calcium channels are essential to generate calcium spiking activity in mice chronically lacking $Ca_v3.2$. However, acute inhibition of L-type channels alone did not significantly change the calcium spiking in WT ZG cells.

Both T- and L-Type Calcium Channels Are Needed for Ang II-Dependent Calcium Signaling

To test whether L-type VGCCs underlie the remaining activity in WT cells subjected to TTA-P2 or other (non-T- nor L-type) VGCCs contribute to the generation of

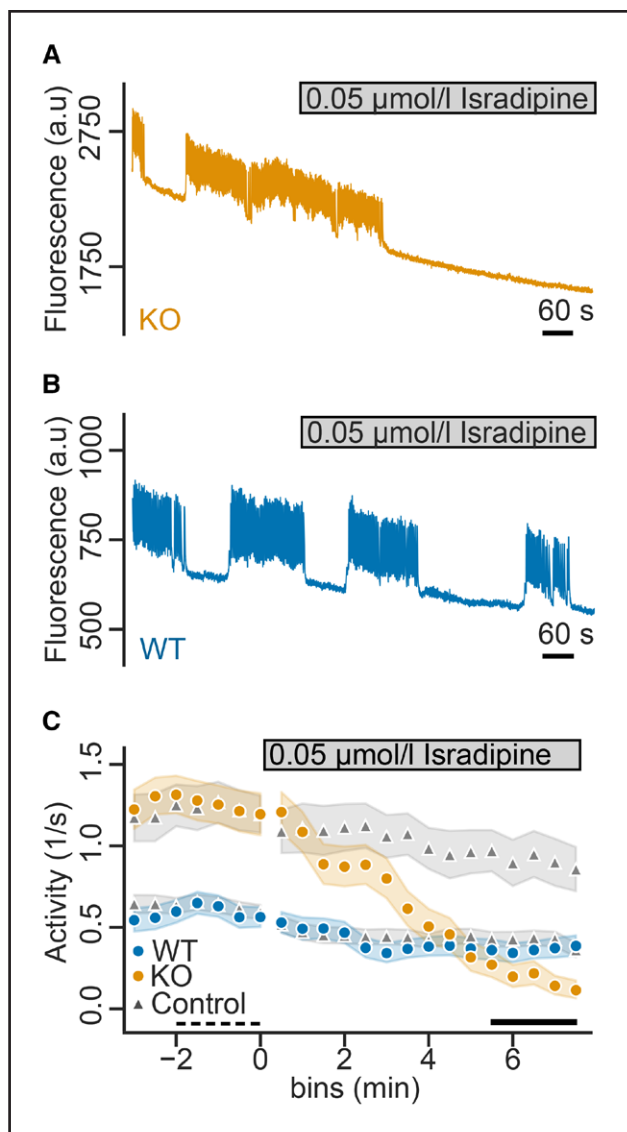


Figure 4. The L-type inhibitor isradipine inhibits calcium oscillations in knock out (KO) but not wild-type (WT) cells.

A and **B** Variations in the fluorescence recorded in one representative KO (**A**) and WT (**B**) zona glomerulosa (ZG) cell. Application of isradipine inhibits calcium spikes in almost all KO but not WT cells. **C**, Calcium spike activity. Cells were from adrenal slices stained with Calbryte 520 AM and perfused with 4 mmol/L K^+ and 500 pmol/L Ang II (angiotensin II) ($n_{\text{animals,WT, male}}=6$, $n_{\text{animals,WT, female}}=3$, $n_{\text{animals,WT ctrl, male}}=9$, $n_{\text{animals,WT ctrl, female}}=6$, $n_{\text{animals,KO, male}}=3$, $n_{\text{animals,KO, female}}=3$, $n_{\text{animals,KO ctrl, male}}=3$, $n_{\text{animals,KO ctrl, female}}=4$).

$[Ca^{2+}]_{\text{int}}$ oscillations, we studied the situation in ZG cells upon inhibition using both TTA-P2 and isradipine. The protocol consisted of a 10-minute period in which the slice was perfused with the T-type VGCC inhibitor TTA-P2 alone, followed by another 10 minutes during which TTA-P2 was supplemented with isradipine to additionally block L-type VGCCs. To maximize the specificity of the inhibition of T- over L-type channels, we lowered the concentration of TTA-P2 to 5 $\mu\text{mol/L}$. Furthermore, we increased the isradipine concentration to 300 nmol/L

to fully inhibit $Ca_v1.3$.³¹ A potential unspecific inhibition of T-type channels was considered less important in this context given the parallel incubation with TTA-P2. (Figure 5A and B).

As observed for the higher concentration (Figure 3D through 3G), TTA-P2 alone only incompletely reduced oscillatory activity (Figure 5B and 5C; Table S23 and S24; absolute data in Figure S7A and Tables S25 and S26) and the number of active cells (Figure S7B) but increased the intraburst frequency (Figure S7C and S7D; Tables S25 and S27). Addition of isradipine led to a cessation of all signals, demonstrating that the combination of both T- and L-type VGCCs, is necessary for calcium oscillations and cannot be substituted for by other calcium channels.

This does not only extend to the oscillations of calcium but to calcium levels in general. Recording mean $[Ca^{2+}]_{\text{int}}$ levels using Fura-2 AM revealed that, upon inhibition of T- and L-type channels, calcium levels were undistinguishable from levels before stimulation with Ang II (Figure 5D and 5E; Tables S28 and S29) but significantly lower than in controls (Figure S8A and S8B; Tables S30 and S31). We also incubated adrenal glands from WT mice with either control solution or solution containing TTA-P2 and isradipine. Inhibition of calcium channels significantly reduced aldosterone synthesis (Figure S9).

L-Type Calcium Channels Are Expressed in all ZG Cells

The heterogeneous nature of the effects of TTA-P2 and isradipine led us to investigate whether differences in the expression of the main L- and T-type VGCCs exist across ZG cells. For this, we obtained access to a single-nuclear RNA-seq data set of mouse adrenal glands (unpublished data by AKS and UIS). Selection of ZG cells by expression levels of *Cyp11b2* alone, while being largely continuous in mice³⁵ unlike in humans, is complicated by their low levels (Figure S10). We, therefore, selected ZG cells based on the expression of *Dab2*, *Cacnb2*, *Agtr1b*, and *Agtr1a* (see Supplemental Material). A histogram of the normalized counts of transcripts for the 3 main VGCCs in the ZG (*Cacna1c*, *Cacna1d*, and *Cacna1h*) revealed expression of $Ca_v1.2/Cacna1c$ in virtually all cells. About 20% of the cells, however, lacked expression of *Cacna1d* or *Cacna1h* (Figure 6A). Plotting the normalized, natural-log transformed counts of transcripts for the T-type channel $Ca_v3.2/Cacna1h$ versus the L-type channel $Ca_v1.3/Cacna1d$ reveals 4 subsets of cells (Figure 6B). The first consists of cells without detected RNA transcripts for either channel (red 1: 2252/6589 cells; 34%). There are also subsets of cells without detected *Cacna1d* but with *Cacna1h* transcripts (red 2: 1353 cells; 21%) and cells expressing *Cacna1d*

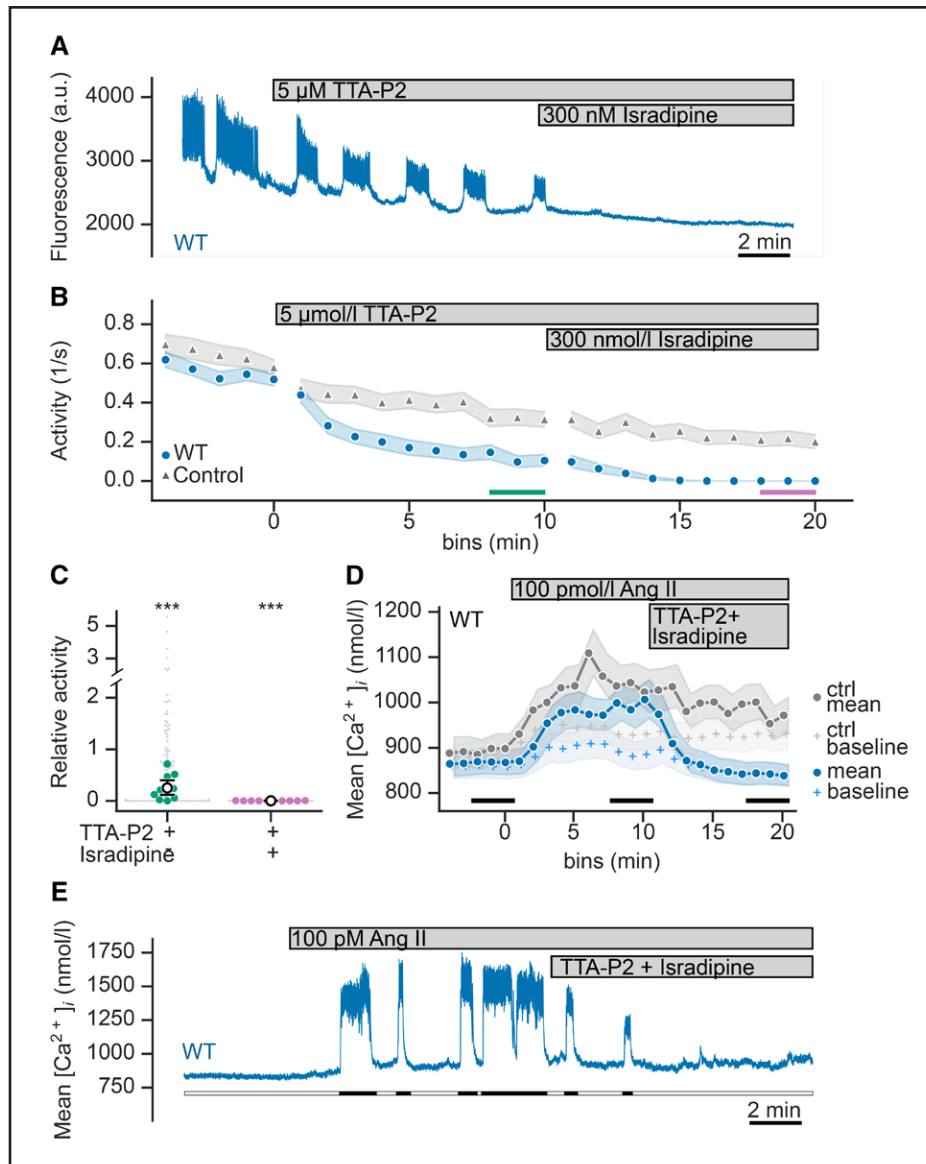


Figure 5. Simultaneous inhibition of L- and T-type calcium channels fully abolishes calcium signaling.

A, Fluorescence intensity recorded from one representative wild-type (WT) zona glomerulosa (ZG) cell. **B**, Calcium spike activity of WT ZG cells was lowered by TTA-P2 (3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide) and isradipine compared with control (no blockers, grey triangle). The last 2 minutes of each condition of the recording are highlighted with color-coded lines ($n_{\text{animals,WT, male}}=4$, $n_{\text{animals,WT, female}}=2$, $n_{\text{animals,WT ctrl, male}}=4$, $n_{\text{animals,WT ctrl, female}}=5$). **C**, Calcium activity in ZG cells from WT during the steady-state phases as highlighted in **B** relative to and tested against controls. **D**, Mean and baseline $[\text{Ca}^{2+}]_i$ in Fura-2 AM-stained ZG cells. Ang II-stimulated increase in $[\text{Ca}^{2+}]_i$ was abolished by inhibitors (grey: control, no inhibitors; $n_{\text{animals,WT, male}}=2$, $n_{\text{animals,WT, female}}=3$, $n_{\text{animals,WT ctrl, male}}=1$, $n_{\text{animals,WT ctrl, female}}=3$). **E**, Representative trace of $[\text{Ca}^{2+}]_i$ of a ZG WT cell (unfilled bars: baseline). Cells in **A** through **C** were from adrenal slices stained with Calbryte 520 AM and perfused with 4 mmol/L K^+ and 500 pmol/L Ang II. Cells in **D** through **E** were from Fura-2 AM-stained slices and perfused with 4 mmol/L K^+ .

without detected *Cacna1h* (red 3: 1729 cells; 26%). A similar number of cells expressed both *Cacna1d* and *Cacna1h* (red 4: 1255 cells; 19%).

DISCUSSION

The ZG is a complex system with several stimuli regulating the synthesis of aldosterone.² It has been postulated that most of these factors, including serum potassium

and Ang II, act by mediating oscillatory calcium influx into ZG cells elicited through depolarization of the cell membrane² requiring $\text{Ca}_v3.2$ ³ and potentially also through release of calcium from intracellular stores.^{36,37} In this article, we demonstrate that Ang II and potassium dependent $[\text{Ca}^{2+}]_{\text{int}}$ oscillations still occur in $\text{Ca}_v3.2$ knock-out mice and become instead dependent on L-type calcium channels. Furthermore, acute inhibition of T-type calcium channels does not abolish Ang II-dependent calcium influx

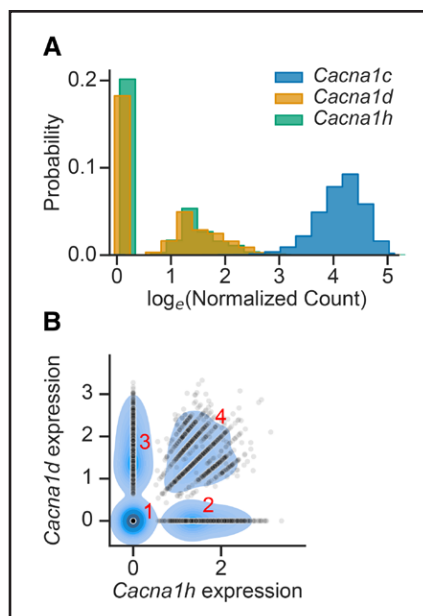


Figure 6. All zona glomerulosa (ZG) cells have transcripts for *Cacna1d* ($\text{Ca}_v1.2$) but not necessarily $\text{Ca}_v1.3$ or $\text{Ca}_v3.2$.

A, Histogram of the natural-log transformed normalized counts of indicated voltage-gated calcium channel (VGCC) transcripts found in the ZG ($n_{\text{animals,WT, male}}=2$, $n_{\text{animals,WT, female}}=2$). Almost all cells exhibit expression of *Cacna1c* but not *Cacna1d* or *Cacna1h*. Bars are slightly shifted for each isoform to improve visibility. **B**, Plot of the normalized, natural-log transformed counts of *Cacna1d* ($\text{Ca}_v1.3$) vs *Cacna1h* ($\text{Ca}_v3.2$) as individual points per cell. A kernel density plot of the data are plotted below in blue with darker color indicating higher density of values underneath. Red numbers indicate expression clusters explained in the text. The striped appearance in cluster 4 is due to the low value of *Cacna1h* and *Cacna1d* counts as seen in **A**.

into the wild-type ZG. Rather, a subset of cells still exhibits oscillatory calcium influx via L-type calcium channels.

Hu et al³ demonstrated that murine ZG cells constitute electric oscillators with action potential-like depolarizations. Maintenance of these fluctuations was critically dependent on the voltage-gated T-type calcium channel $\text{Ca}_v3.2$. Although not proven so far, it is likely that these voltage oscillations are the basis of ZG calcium oscillations as they exhibit similar frequencies and stimulus dependence (Figure 1).^{7–9} However, it is not the change in voltage but ultimately the influx of calcium that is required for the physiological function of the ZG. Contrary to this model, $\text{Ca}_v3.2$ knock-out mice did not show reduced aldosterone levels.^{8,14} However, these studies investigated KO mice on a systemic level, and secondary mechanisms (such as activation of the renin–angiotensin system or upregulation of other calcium influx pathways in the ZG) may have compensated for the loss. We, therefore, investigated the molecular mechanisms regulating calcium influx in the ZG of mice lacking the $\text{Ca}_v3.2$ channel in comparison to WT mice in acute slice preparations and isolated adrenocortical cells.

We found that calcium signaling was largely unaffected by the loss of $\text{Ca}_v3.2$ in KO mice (Figure 1).

Despite a slight increase in the spiking activity, mean levels of $[\text{Ca}^{2+}]_{\text{int}}$ in the ZG were indistinguishable from WT, explaining why aldosterone levels were unchanged (Figure 1D and 1E).

It was suggested that other T-type channels may also be expressed in the adrenal cortex.¹⁴ However, our data from RNA-seq (Figure 2A) did not suggest relevant expression of T-type channels other than *Cacna1h*. Adrenocortical cells from KO mice lacked T-type currents (Figure 2D and 2E). Furthermore, perfusion of slices from adrenals of KO mice with the T-type inhibitor TTA-P2 did not result in changes to calcium signaling (Figure 3), supporting that $\text{Ca}_v3.2$ is indeed the only relevant T-type VGCC in the murine ZG.

On the other hand, perfusion with the L-type calcium channel inhibitor isradipine almost completely suppressed $[\text{Ca}^{2+}]_{\text{int}}$ oscillations in KO mice (Figure 4), demonstrating that these signals instead depend on L-type calcium channels. Based on previous knowledge^{3,28,29,38} and our bulk RNA-seq analysis (Figure 2A), the L-type calcium channels most prominently expressed in the murine and human adrenal cortex are $\text{Ca}_v1.3$ and $\text{Ca}_v1.2$.^{3,28,29} We did not find evidence of compensatory upregulation of L-type or any other calcium channels or transporters in KO mice. Patch-clamp experiments also ruled out large posttranslational changes to VGCCs as current amplitudes and voltage dependencies were similar in WT and KO mice (Figure 2D–E). Also, the spatial distribution of cells exhibiting spiking dependent on L- and T-type calcium influx were similar in WT (Figure S5).

The importance of L-type channels had already been previously demonstrated by studies identifying somatic gain-of-function $\text{Ca}_v1.3$ mutations in patients with primary aldosteronism.^{28,30,38–40} Furthermore, there are previous reports that aldosterone synthesis in human ZG is dependent on both T- and L-type calcium channels.^{29,41} It is currently not known whether human ZG cells exhibit similar voltage and $[\text{Ca}^{2+}]_{\text{int}}$ oscillations as murine ones. However, species differences in the ion channels involved seem to be mainly in the composition of potassium channels,⁴² whereas the expression of calcium channels is rather similar.^{28,29,42,43}

Currently, we cannot explain why $[\text{Ca}^{2+}]_{\text{int}}$ oscillations were faster in KO than in WT mice. It had already been suggested that additional conductances must underlie the initial depolarization in WT,³ and this is confirmed by our results, as L-type channels ($\text{Ca}_v1.3$ and even more so 1.2) require stronger depolarization for activation than T-type VGCCs and are unlikely to drive depolarization from a potassium-defined resting membrane potential on their own. Clearly, the closure of TASK potassium channels is involved in permitting (Ang II-dependent) depolarization,^{5,6,44} but still other, currently unknown, conductances must mediate the initial depolarization itself.

Also, it is currently unclear how individual spikes and bursts are terminated. We previously observed slower

spiking in a gain-of-function *Cacna1h* knock-in model. Given that the knock out presented here should generally lead to the opposite effect, it is a tempting speculation that the frequency of calcium spiking is inversely linked to intracellular $[Ca^{2+}]_{int}$. In our knock-out model, this would serve to sustain physiological calcium levels (and hence aldosterone synthesis). Previous observations have suggested a role for calcium-dependent potassium channels^{45,46} in the ZG, which may control spiking, but further work is necessary to understand their functional importance. This would best combine in situ electrophysiological studies with simultaneous calcium imaging, which we were unfortunately unable to perform here.

Besides the situation of a chronic loss of $Ca_v3.2$ as in our KO model, our data also imply that physiological calcium signaling in the WT ZG is not solely dependent on $Ca_v3.2$. Acute inhibition of T-type VGCCs with the specific blocker TTA-P2 in WT mice only attenuated activity by $\approx 50\%$ to 80% (Figures 3 and 5). $[Ca^{2+}]_{int}$ oscillations could only be completely inhibited when simultaneously blocking L-type calcium channels using isradipine (Figure 5). Acute inhibition of L-type channels in isolation, however, did not alter calcium spiking (Figure 4). This suggests that the generation of oscillations in WT ZG cells is primarily dependent on $Ca_v3.2$. However, following the acute inhibition of T-type calcium channels, L-type channels can maintain calcium signaling in some of the WT cells (Figure 3). Cells with remaining activity after inhibition of $Ca_v3.2$ exhibited higher intraburst frequencies than before inhibition (Figure 3G; Figure S3B), similar to KO cells. This suggests that, while primarily dependent on $Ca_v3.2$, chronic loss or inhibition may upregulate calcium signaling via L-type VGCCs.

Our analysis of murine single-nuclear RNA-seq data suggests that $Ca_v1.2$ is expressed in all ZG cells while transcripts for $Ca_v1.3$ were not found in all ZG cells (Figure 6). The latter could be due to lower expression levels and low sensitivity of single-nuclear sequencing. It remains puzzling why no L-type currents were observed in ZG cells in the study by Hu et al⁹, even when stimulated with the L-type channel activator Bay K8644. In contrast, we observed L-type currents in all cells recorded using whole-cell patch clamp, even without further stimulation (Figure 2E). Although this discrepancy might be explained by the difference in cell preparation (in situ patch clamp³ versus dissociated adrenocortical cells in our study), our calcium imaging experiments and results from single-nuclear RNA-seq (Figure 6) also clearly support the relevance of L-type calcium channels in generating and maintaining calcium oscillations in the ZG of both genotypes, WT and KO, in situ.

Furthermore, we could demonstrate that Ang II mostly changes $[Ca^{2+}]_{int}$ through variations of the patterns of oscillations and not by altering baseline levels (Figure 5D and 5E). Simultaneous inhibition of T- and L-type calcium channels not only completely stopped Ang II-dependent

$[Ca^{2+}]_{int}$ oscillations but also abrogated any changes in baseline $[Ca^{2+}]_{int}$ and attenuated aldosterone synthesis (Figures S8 and S9) as previously also observed in human ZG in vitro.²⁹ This suggests that intracellular calcium stores may not play a large role in Ang II-dependent aldosterone synthesis but further studies are required.

PERSPECTIVES

Our findings also have implications in directing future pharmacological interventions. It has been suggested that the inhibition of T-type calcium channels might represent a promising target to lower aldosterone synthesis.¹³ Our results, however, imply that a long-term inhibition (as mimicked by our KO mice) would likely be countered via calcium influx through L-type calcium channels. This explains why mibefradil, a preferential T-type channel inhibitor, did not exert persistent effects on aldosterone levels or blood pressure in vivo.^{47,48}

Nevertheless, this may be different for the treatment of primary aldosteronism caused by gain-of-function mutations in either $Ca_v3.2$ ⁴⁹ or $Ca_v1.3$.^{28,38} We have recently investigated oral isradipine treatment of a mouse model carrying the $Ca_v1.3^{Ile772Met/+}$ mutation.³⁵ This L-type inhibitor was able to lower aldosterone serum levels in mice carrying the mutation but not in WT controls.

Interestingly, it has been observed in the H295R cell line that a blockade of both L- and T-type channels (eg, using the unspecific inhibitors benidipine⁵⁰ or efonidipine⁵¹) was efficient in reducing aldosterone production. Similarly, verapamil, which inhibits T- and L-type channels with micromolar affinity,⁵² may inhibit aldosterone synthesis in vitro⁵³ and in vivo.⁵⁴ In vivo, however, application of calcium channel antagonists modify the renin-angiotensin-aldosterone system on several levels, making clear molecular or cellular assignment of cause and effect difficult. Dual T- and L-type inhibition may be a promising strategy to lower aldosterone synthesis, but potential extra-adrenal side effects may limit their usefulness, and this approach, therefore, requires further investigation.

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Author Contributions

G. Stölting conceived the study; H.A. Dinh and M. Volkert performed calcium imaging; H.A. Dinh and G. Stölting analyzed calcium imaging data and bulk RNA-seq data; H.A. Dinh performed and analyzed patch-clamp experiments of adrenocortical cells; A.K. Secener and U.I. Scholl designed single nucleus RNA-seq experiments; A.K. Secener performed and analyzed single nucleus RNA-seq experiments; H.A. Dinh, U.I. Scholl, and G. Stölting wrote the manuscript with contributions from all authors.

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Disclosures

None.

REFERENCES

- Hattagady N, Olala L, Bollag WB, Rainey WE. Acute and chronic regulation of aldosterone production. *Mol Cell Endocrinol*. 2012;350:151–162. doi: 10.1016/j.mce.2011.07.034
- Spät A, Hunyady L. Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol Rev*. 2004;84:489–539. doi: 10.1152/physrev.00030.2003
- Hu C, Rusin CG, Tan Z, Guagliardo NA, Barrett PQ. Zona glomerulosa cells of the mouse adrenal cortex are intrinsic electrical oscillators. *J Clin Invest*. 2012;122:2046–2053. doi: 10.1172/JCI61996
- Capponi AM, Lew PD, Jornt L, Vallotton MB. Correlation between cytosolic free Ca²⁺ and aldosterone production in bovine adrenal glomerulosa cells. Evidence for a difference in the mode of action of angiotensin II and potassium. *J Biol Chem*. 1984;259:8863–8869. doi: 10.1016/S0021-9258(17)47233-4
- Penton D, Bandulik S, Schweda F, Haubs S, Tauber P, Reichold M, Cong LD, El Wakil A, Budde T, Lesage F, et al. Task3 potassium channel gene inactivation causes low renin and salt-sensitive arterial hypertension. *Endocrinology*. 2012;153:4740–4748. doi: 10.1210/en.2012-1527
- Heitzmann D, Derand R, Jungbauer S, Bandulik S, Sterner C, Schweda F, Wakil AE, Lalli E, Guy N, Mengual R, et al. Invalidation of TASK1 potassium channels disrupts adrenal gland zonation and mineralocorticoid homeostasis. *EMBO J*. 2008;27:179–187. doi: 10.1038/sj.emboj.7601934
- Schewe J, Seidel E, Forslund S, Marko L, Peters J, Muller DN, Fahlke C, Stölting G, Scholl U. Elevated aldosterone and blood pressure in a mouse model of familial hyperaldosteronism with CIC-2 mutation. *Nat Commun*. 2019;10:5155. doi: 10.1038/s41467-019-13033-4
- Seidel E, Schewe J, Zhang J, Dinh HA, Forslund SK, Markó L, Hellmig N, Peters J, Muller DN, Lifton RP, et al. Enhanced Ca²⁺ signaling, mild primary aldosteronism, and hypertension in a familial hyperaldosteronism mouse model (*Cacna1h*^{M1560V/+}). *Proc Natl Acad Sci USA*. 2021;118:e2014876118. doi: 10.1073/pnas.2014876118
- Guagliardo NA, Klein PM, Gancayco CA, Lu A, Leng S, Makarew RR, Cho C, Rusin CG, Breault DT, Barrett PQ, et al. Angiotensin II induces coordinated calcium bursts in aldosterone-producing adrenal rosettes. *Nat Commun*. 2020;11:1–15. doi: 10.1038/s41467-020-15408-4
- Clyne CD, White PC, Rainey WE. Calcium regulates human CYP11B2 transcription. *Endocr Res*. 1996;22:485–492. doi: 10.1080/07435809609043735
- Yagci A, Müller J. Induction of steroidogenic enzymes by potassium in cultured rat zona glomerulosa cells depends on calcium influx and intact protein synthesis. *Endocrinology*. 1996;137:4331–4338. doi: 10.1210/endo.137.10.8828493
- Kowluru R, Yamazaki T, McNamara BC, Jefcoate CR. Metabolism of exogenous cholesterol by rat adrenal mitochondria is stimulated equally by physiological levels of free Ca²⁺ and by GTP. *Mol Cell Endocrinol*. 1995;107:181–188. doi: 10.1016/0303-7207(94)03441-u
- Rossier MF, Ertel EA, Vallotton MB, Capponi AM. Inhibitory action of mibefradil on calcium signaling and aldosterone synthesis in bovine adrenal glomerulosa cells. *J Pharmacol Exp Ther*. 1998;287:824–831.
- Thuesen AD, Finsen SH, Rasmussen LL, Andersen DC, Jensen BL, Hansen PBL. Deficiency of T-type Ca²⁺ channels Ca_v 3.1 and Ca_v 3.2 has no effect on angiotensin II-induced hypertension but differential effect on plasma aldosterone in mice. *American Journal of Physiology-Renal Physiology*. 2019;317:F254–F263. doi: 10.1152/ajprenal.00121.2018
- Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods*. 2017;14:417–419. doi: 10.1038/nmeth.4197
- Ewels P, Magnusson M, Lundin S, Käller M. MultiQC. Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. 2016;32:3047–3048. doi: 10.1093/bioinformatics/btw354
- Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. 2018;34:i884–i890. doi: 10.1093/bioinformatics/bty560
- Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550. doi: 10.1186/s13059-014-0550-8
- Zhu A, Ibrahim JG, Love MI. Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics*. 2019;35:2084–2092. doi: 10.1093/bioinformatics/bty895
- Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, Fu X, Liu S, Bo X, Yu G. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innovation (Camb)*. 2021;2:100141. doi: 10.1016/j.xinn.2021.100141
- Nogueira EF, Rainey WE. Regulation of aldosterone synthase by activator transcription factor/cAMP response element-binding protein family members. *Endocrinology*. 2010;151:1060–1070. doi: 10.1210/en.2009-0977
- Enyeart JJ, Enyeart JA. Adrenal fasciculata cells express T-type and rapidly and slowly activating L-type Ca²⁺ channels that regulate cortisol secretion. *Am J Physiol Cell Physiol*. 2015;308:C899–C918. doi: 10.1152/ajpcell.00002.2015
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al. Gene ontology: tool for the unification of biology. *Nat Genet*. 2000;25:25–29. doi: 10.1038/75556
- Kanehisa M, Goto S. KEGG. Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. 2000;28:27–30. doi: 10.1093/nar/28.1.27
- Stölting G, Oliveira RC de, Guzman RE, Miranda-Laferte E, Conrad R, Jordan N, Schmidt S, Hendriks J, Gensch T, Hidalgo P. Direct interaction of Ca_vβ with actin up-regulates L-type calcium currents in HL-1 cardiomyocytes. *J Biol Chem*. 2015;290:4561–4572. doi: 10.1074/jbc.M114.573956
- Shipe WD, Barrow JC, Yang Z-Q, Lindsley CW, Yang FY, Schleslegel K-AS, Shu Y, Rittle KE, Bock MG, Hartman GD, et al. Design, synthesis, and evaluation of a novel 4-aminomethyl-4-fluoropiperidine as a T-type Ca²⁺ channel antagonist. *J Med Chem*. 2008;51:3692–3695. doi: 10.1021/jm800419w
- Choe W, Messinger RB, Leach E, Eckle V-S, Obradovic A, Salajegheh R, Jevtic-Todorovic V, Todorovic SM. TTA-P2 is a potent and selective blocker of T-type calcium channels in rat sensory neurons and a novel antinociceptive agent. *Mol Pharmacol*. 2011;80:900–910. doi: 10.1124/mol.111.073205
- Scholl UI, Goh G, Stölting G, de Oliveira RC, Choi M, Overton JD, Fonseca AL, Korah R, Starker LF, Kunstman JW, et al. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet*. 2013;45:1050–1054. doi: 10.1038/ng.2695
- Yang T, He M, Zhang H, Barrett PQ, Hu C. L- and T-type calcium channels control aldosterone production from human adrenals. *J Endocrinol*. 2020;244:237–247. doi: 10.1530/JOE-19-0259
- Nishimoto K, Rigsby CS, Wang T, Mukai K, Gomez-Sanchez CE, Rainey WE, Seki T. Transcriptome analysis reveals differentially expressed transcripts in rat adrenal zona glomerulosa and zona fasciculata. *Endocrinology*. 2012;153:1755–1763. doi: 10.1210/en.2011-1915
- Ortner NJ, Bock G, Dougalis A, Kharitonova M, Duda J, Hess S, Tuluc P, Pomberger T, Stefanova N, Pitterl F, et al. Lower affinity of isradipine for L-Type Ca²⁺ channels during substantia nigra dopamine neuron-like activity: implications for neuroprotection in parkinson's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2017;37:6761–6777. doi: 10.1523/JNEUROSCI.2946-16.2017
- Hockerman GH, Peterson BZ, Sharp E, Tanada TN, Schaefer T, Catterall WA. Construction of a high-affinity receptor site for dihydropyridine agonists and antagonists by single amino acid substitutions in a non-L-type Ca²⁺ channel. *Proc Natl Acad Sci USA*. 1997;94:14906–14911. doi: 10.1073/pnas.94.26.14906
- Lin M, Aladejebi O, Hockerman GH. Distinct properties of amlodipine and nifedipine block of the voltage-dependent Ca²⁺ channels Cav1.2 and Cav2.1 and the mutant channels Cav1.2/Dihydropyridine insensitive and Cav2.1/Dihydropyridine sensitive. *Eur J Pharmacol*. 2011;670:105–113. doi: 10.1016/j.ejphar.2011.08.005

34. Zamponi GW. Antagonist binding sites of voltage-dependent calcium channels. *Drug Dev Res.* 1997;42:131–143. doi: 10.1002/(sici)1098-2299(199711/12)42:3/4<131::aid-ddr4>3.0.co;2-r
35. Stölting G, Dinh HA, Volkert M, Hellmig N, Schewe J, Hennicke L, Seidel E, Oberacher H, Zhang J, Lifton RP, et al. Isradipine therapy in *Cacna1d*^{fl^o/772Met/+} mice ameliorates primary aldosteronism and neurologic abnormalities. *JCI Insight.* 2023;8:e162468. doi: 10.1172/jci.insight.162468
36. Hajnóczky G, Várnai P, Holló Z, Christensen SB, Balla T, Enyedi P, Spät A. Thapsigargin-induced increase in cytoplasmic Ca²⁺ concentration and aldosterone production in rat adrenal glomerulosa cells: interaction with potassium and angiotensin-II. *Endocrinology.* 1991;128:2639–2644. doi: 10.1210/endo-128-5-2639
37. Ely JA, Ambroz C, Baukal AJ, Christensen SB, Balla T, Catt KJ. Relationship between agonist- and thapsigargin-sensitive calcium pools in adrenal glomerulosa cells. Thapsigargin-induced Ca²⁺ mobilization and entry. *J Biol Chem.* 1991;266:18635–18641. doi: 10.1016/S0021-9258(18)55110-3
38. Azizan EAB, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, Maniero C, Garg S, Bochukova EG, Zhao W, et al. Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nat Genet.* 2013;45:1055–1060. doi: 10.1038/ng.2716
39. Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, Boulkroun S, Strom TM, Monticone S, Amar L, Meatchi T, et al. Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension.* 2014;64:354–361. doi: 10.1161/HYPERTENSIONAHA.114.03419
40. De Sousa K, Boulkroun S, Baron S, Nanba K, Wack M, Rainey WE, Rocha A, Giscos-Douriez I, Meatchi T, Amar L, et al. Genetic, cellular, and molecular heterogeneity in adrenals with aldosterone-producing adenoma. *Hypertension.* 2020;75:1034–1044. doi: 10.1161/HYPERTENSIONAHA.119.14177
41. Xie CB, Haris Shaikh L, Garg S, Tanriver G, Teo AED, Zhou J, Maniero C, Zhao W, Kang S, Silverman RB, et al. Regulation of aldosterone secretion by Ca_v1.3. *Sci Rep.* 2016;6:24697. doi: 10.1038/srep24697
42. Enyart JJ, Enyart JA. Human adrenal glomerulosa cells express K2P and GIRK potassium channels that are inhibited by ANG II and ACTH. *Am J Physiol Cell Physiol.* 2021;321:C158–C175. doi: 10.1152/ajpcell.00118.2021
43. Payet MD, Durroux T, Bilodeau L, Guillon G, Gallo-Payet N. Characterization of K⁺ and Ca²⁺ ionic currents in glomerulosa cells from human adrenal glands. *Endocrinology.* 1994;134:2589–2598. doi: 10.1210/endo.134.6.7515004
44. Gancayco CA, Gerding MR, Breault DT, Beenhakker MP, Barrett PQ, Guagliardo NA. Intrinsic adrenal TWIK-related acid-sensitive TASK channel dysfunction produces spontaneous calcium oscillations sufficient to drive AngII (Angiotensin II)-unresponsive hyperaldosteronism. *Hypertension.* 2022;79:2552–2564. doi: 10.1161/HYPERTENSIONAHA.122.19557
45. Barrett PQ, Guagliardo NA, Bayliss DA. Ion channel function and electrical excitability in the zona glomerulosa: a network perspective on aldosterone regulation. *Annu Rev Physiol.* 2021;83:451–475. doi: 10.1146/annurev-physiol-030220-113038
46. Yang T, Zhang H-L, Liang Q, Shi Y, Mei Y-A, Barrett PQ, Hu C. Small-conductance Ca²⁺-activated potassium channels negatively regulate aldosterone secretion in human adrenocortical cells. *Hypertension.* 2016;68:785. doi: 10.1161/HYPERTENSIONAHA.116.07094
47. Schmitt R, Kleinbloesem CH, Belz GG, Schroeter V, Feifel U, Pozenel H, Kirch W, Halabi A, Woittiez A-JJ, Welker HOA, et al. Hemodynamic and humoral effects of the novel calcium antagonist Ro 40-5967 in patients with hypertension. *Clin Pharmacol Ther.* 1992;52:314–323. doi: 10.1038/clpt.1992.147
48. Ragueneau I, Sao AB, Démolis J-L, Darné B, Funck-Brentano C, Jaillon P. Comparison of sympathetic modulation induced by single oral doses of mibefradil, amlodipine, and nifedipine in healthy volunteers. *Clin Pharmacol Ther.* 2001;69:122–129. doi: 10.1067/mcp.2001.113406
49. Scholl UI, Stölting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, Prasad ML, Goh G, Carling T, Juhlin CC, et al. Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. *eLife.* 2015;4:e06315. doi: 10.7554/eLife.06315
50. Akizuki O, Inayoshi A, Kitayama T, Yao K, Shirakura S, Sasaki K, Kusaka H, Matsubara M. Blockade of T-type voltage-dependent Ca²⁺ channels by benidipine, a dihydropyridine calcium channel blocker, inhibits aldosterone production in human adrenocortical cell line NCI-H295R. *Eur J Pharmacol.* 2008;584:424–434. doi: 10.1016/j.ejphar.2008.02.001
51. Imagawa K, Okayama S, Takaoka M, Kawata H, Naya N, Nakajima T, Horii M, Uemura S, Saito Y. Inhibitory effect of efonidipine on aldosterone synthesis and secretion in human adrenocarcinoma (H295R) cells. *J Cardiovasc Pharmacol.* 2006;47:133–138. doi: 10.1097/01.fjc.0000197539.12685.f5
52. Bergson P, Lipkind G, Lee SP, Duban M-E, Hanck DA. Verapamil block of T-type calcium channels. *Mol Pharmacol.* 2011;79:411–419. doi: 10.1124/mol.110.069492
53. Balla T, Hunyady L, spät A. Possible role of calcium uptake and calmodulin in adrenal glomerulosa cells: Effects of verapamil and trifluoperazine. *Biochem Pharmacol.* 1982;31:1267–1271. doi: 10.1016/0006-2952(82)90014-4
54. Guthrie GP Jr, Mcallister RG Jr, Kotchen T. Effects of intravenous and oral verapamil upon pressor and adrenal steroidogenic responses in normal man. *J Clin Endocrinol Metab.* 1983;57:339–343. doi: 10.1210/jcem-57-2-339